THE MECHANICS OF CELL-SEEDED COLLAGEN HYDROGELS

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Copyright Statement

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Contents

1. Abstract

Objective: This study aims to measure the mechanical properties, namely permeability and stiffness, of HepG2 cell-seeded collagen hydrogels. Confined compression testing in conjunction with biphasic theory modelling was utilised to provide measures of these mechanical parameters of interest. Different cell seeding densities and culture durations were tested to observe their effects on mechanical properties in order to understand how significantly the cells remodel the gel and alter mechanical properties with their physical presence.

Background: Physical and mechanical interactions between cells and ECM components have been shown to affect cellular characteristics and the mechanics of the overall construct. The appropriateness of the scaffold can therefore influence cellular properties and function.

Methodology: 0.3% collagen hydrogels were seeded with HepG2 cells at different seeding densities ('no cell' control, $1x10^4$, $2x10^4$, $5x10^4$, $10x10^4$ and $20x10^4$ cells/well) and cultured for durations of 1, 3 and 5 days. An MTT assay was used to determine cell viability before mechanical tests were undertaken. Confined compression in conjunction with biphasic modelling was used to determine measures for the Aggregate Modulus (H_A), Hydraulic Permeability (k_0), Nonlinear Permeability Coefficient (M), Correlation Coefficient (r^2), peak stress and equilibrium stress.

Results and conclusions: No statistically significant trends were determined for the mechanical properties between cell seeding densities, however differences in hydraulic permeability and peak stress were observed with culture duration. r^2 values obtained from this study suggest experimental data

analysed with the biphasic model has large confidence intervals and may not be entirely suitable.

2. Introduction

2.1 The Liver

2.1.1 Liver tissue engineering

The drive for tissue engineering the liver arises from multiple avenues: organ-scale models for therapeutic replacement in patients with liver failure; laboratory models for studying the efficacy and safety of drugs; and mechanistic models to study mechanical influences on cellular properties, such as differentiation (Griffith et al. 2014; Dash et al. 2009). Where a patient suffers liver failure, multi-systemic complications emerge resulting in multi-organ failure and death. Success in treating liver disease once it has reached this level of severity ultimately falls to liver transplantation. However, donor organs are limited in availability and patients often die on the waiting list (Palakkan et al. 2013).

To ease the clinical dependence on donor organ availability, substitute techniques are being actively pursued. Surgical techniques such as partial liver grafts, whereby live donor liver tissue is removed from a living donor and supplied to the patient, have not bypassed the requirement for donor tissue. Tissue engineered constructs offer a promising alternative for liver failure treatment that will circumvent the need for donor tissue should a suitable cell source be found. In theory, a hepatocyte cell source that maintains its differentiated state, function and proliferative capabilities can be combined with a biocompatible scaffold to mimic liver structure in order to produce a construct capable of functioning as a replacement liver. Research is applying liver tissue engineering techniques to produce a range of clinical treatments. These may be for use in vitro, such as in the case of extracorporeal support

devices, or in vivo, in regards to implantable liver constructs or implantation of cells capable of regenerating the subjects own liver (Palakkan et al. 2013).

Better models are also required for drug assessment, discovery and development (Cattley & Cullen 2013). Drugs in development are often shelved in the later stages where drug-induced liver injury becomes apparent. In order to detect hepatotoxicity at an earlier stage (saving time and money) hepatic models are utilised (Gerets et al. 2012). These models provide hepatic cell sources that can undergo toxicity testing in order to determine the potential for liver injury. Where these current models need to be improved is in their attainment of a more in vivo like cell source and in the inclusion of in vivo mechanisms such as immune responses (Dash et al. 2009). Current drug assessment models may lack expression levels of key enzymes, such as cytochrome P450, which may deem drugs that become toxic as a result of mechanisms mediated by such enzymes as innocuous (Dash et al. 2009; Tuschl & Mueller 2006).

2.1.2 Liver cell types and their role in disease

The liver is composed of parenchymal hepatocytes (which make up almost 80% of the liver volume) and a range of non-parenchymal cells including hepatic stellate cells, Kupffer cells and sinusoidal endothelial cells (Roberts et al. 2007; Kmiec 2001). The non-parenchymal cells provide much of the biochemical and physical structure to the organ whereas hepatocytes make up the functional component of the liver and are the most metabolically active cells present (Roberts et al. 2007).

The sinusoidal endothelial cells play a structural role in dividing the functional compartments of the liver and providing a barrier to the sinusoidal space (Roberts et al. 2007). In addition to this structural role, liver endothelial cells

have also been shown to be a driving factor in liver regeneration through their upregulation of hepatocyte growth factor (HGF) expression in response to liver injury (Stutchfield & Forbes 2013; DeLeve 2013).

Kupffer cells are the resident liver macrophages and therefore have an essential role in immune homeostasis as well as a small role in the acute and chronic response to toxic agents (Roberts et al. 2007). They are located within the sinusoidal lumen although they are able to transverse the sinusoidal endothelial barrier to carry out their role in immunity, which may result in the release of cytokines and other signaling molecules (Roberts et al. 2007; Bilzer et al. 2006). Due to the 'strategic' location of the liver, any microbe or microbial product (i.e. bacterial endotoxins) entering the body via the gastrointestinal tract must first come into contact with macrophages via the Kupffer cell population in the liver (Bilzer et al. 2006). This primary contact results in the release of a vast array of growth factors, reactive oxygen species (ROS) and inflammatory mediators to inhibit infection (Roberts et al. 2007). Their role in governing the immunity of the liver has been shown to be part of mechanisms that result in acute hepatocyte injury and chronic diseases such as liver cancer (Roberts et al. 2007).

Hepatic stellate cells account for 5-8% of the resident cells in the liver and have vital roles in scar formation and liver regeneration (Yin & Evason 2013). Located in the Space of Disse, stellate cells are also known to have a critical influence upon the proliferation, morphogenesis and even differentiation of the other cell types found in liver during phases of development and regeneration (Yin & Evason 2013). In a normal healthy liver, stellate cells display a quiescent phenotype which can be identified due to their expression of neural markers and adipogenic factors (Tsukamoto 2005; Chen et al. 2015). In this quiescent state, stellate cells act as a large reservoir for retinoid (vitamin A and its substrates), which is stored in lipid droplets in the

cytoplasm of the cells (Blaner et al. 2009; Yin & Evason 2013). Upon insult to the liver, due to toxin introduction or viral infection, stellate cells respond to signals from the hepatocytes and immune cells in the form of pro-fibrotic mediators including transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) (Chen et al. 2015; Yin & Evason 2013). In response to such mediators, the stellate cells undergo activation in a process where the retinoid storage is lost, and the cells transdifferentiate into proliferative myofibroblast-like cells (Blaner et al. 2009; Chen et al. 2015). This heterogeneous population of hepatic myofibroblasts accumulate at the injured site where they generate scar tissue in order to protect any further damage to the liver (Chen et al. 2015; Yin & Evason 2013). They also promote regeneration of the epithelial cells by the production of cytokines and growth factors at the injury site. Where this can progress to chronic liver disease is in the prolonged activation of the scar-forming function of the activated stellate cells. Liver fibrosis is a reversible pathology characterised by widespread hepatic scarring as a result of impaired wound healing (Yin & Evason 2013). It manifests as a result of the excess production and deposition of ECM components (enriched in type I and III fibrillar collagens) put down by the activated stellate cells (Chen et al. 2015). Most chronic liver diseases result in liver fibrosis, which, if it persists, can go on to generate cirrhosis whereby the tissue scarring leads to disruption of the liver architecture and function. This can go on to develop into liver failure (Chen et al. 2015; Yin & Evason 2013).

Hepatocytes are the parenchymal cells of the liver and therefore perform most liver-specific functions. Where hepatic function is disturbed, the liver may become stressed, which is one way hepatocyte survival can be compromised. Under liver stress and hepatocyte injury, hepatocytes produce biomarkers such as serum aminotransferases. It is difficult to distinguish at what level of expression these biomarkers indicate an unhealthy liver as all humans have at

least a basal level of aminotransferase presence in their circulation (Malhi et al. 2010). A multitude of mechanisms, including toxic, metabolic and inflammatory insults, contribute to liver injury and can result in disease. These mechanisms typically feature the activation of hepatocyte necrosis and/or apoptosis. Hepatocytes in particular are susceptible targets to these mechanisms of cell death, in particular death receptor mediated apoptosis, due to their high expression levels of death receptors. Mitochondrial injury, more specifically mitochondrial permeabilisation and dysfunction, mediate the activation of this controlled cell death mechanism in accordance with mitochondrial-endoplasmic reticulum signaling as a result of excessive free fatty acids (Guicciardi 2013; Malhi et al. 2010). Altogether, liver injury is mediated by a multitude of mechanisms involving all of the cell types present in the liver to different capacities. Research into cellular functions and dysfunctions will help to augment understanding of liver pathology and hopefully aid clinical practice in future.

2.1.3 Hepatic Cell Sources

Human primary hepatocytes provide the closest in-vitro model to human liver cellular function and are therefore a powerful research tool (Gómez-Lechón 2003). Although hepatocytes are highly proliferative in vivo, they rarely complete a full cell cycle once in vitro (Vacanti & Kulig 2014), making them difficult cells to work with. Cryopreservation can prolong the availability of these cells once isolated, however, these cryopreserved hepatocytes are even less robust than their freshly isolated counterparts (Palakkan et al. 2013). Immortalised cell lines may therefore offer an advantage to certain studies as these cells retain their proliferative characteristics.

Once seeded, human primary hepatocytes have been shown to dedifferentiate in addition to losing some of their metabolic characteristics. A scaffold

containing ECM components is often required to maintain primary hepatocytes in culture. Differentiated functions of the cells have been shown to be expressed where hepatocytes are maintained on ECM components such as fibronectin, laminin, collagen and reconstituted basement membrane (Santhosh & Sudhakaran 1994). Therefore additional considerations must be made when culturing human primary hepatocytes to ensure the functions or characteristics that are being investigated are preserved.

Oncogene immortalised cell lines and hepatoma derived cell lines are widely used alternatives to primary hepatocytes. The main advantage they offer is the ease in which large quantities can be cultivated (Xu et al. 2004). These cells are limited however in that they are widely accepted to have lower levels of differentiated hepatocyte functions in comparison to primary hepatocytes (Khetani et al. 2015; Vacanti & Kulig 2014). An example of this is the SV40 large T antigen-immortalized Fa2N-4 cell line. These have been found to express lower levels of certain enzymes and uptake transporters, affecting metabolism of the cell. Additionally, these cells lack a nuclear receptor, which limits their ability to predict drug interactions in the liver (Khetani et al. 2015). Oncogene immortalised cell lines also have the potential to be metastatic and would not be able to be used for in vivo applications (Vacanti & Kulig 2014). Therefore considerations must be taken into the suitability of the immortalised cell line utilised for a specific study.

Human hepatoma HepG2 cells are a cell line of human liver carcinoma and are widely used as a model for liver studies. The cell line was derived from a well-differentiated hepatocellular carcinoma of a fifteen-year-old Caucasian American male in 1979. The cells themselves have an epithelial morphology and a model chromosome number of 55 (Costantini et al. 2013). Expression studies for HepG2 cells have found that they exhibit many of the genotypic features of parenchymal cells and are reasonably differentiated, display

hepatocyte morphology and they retain numerous functions and enzyme expression levels (Sassa et al. 1987; Valentin-Severin et al. 2003). For instance, HepG2 cells have been shown to synthesise bile acids from cholesterol; an example of function preservation (Javitt 1990). However, there are differences in expression between parenchymal cells and HepG2 cells which must be taken into consideration. Additionally, although derived from a cancer, HepG2 cells do not cause tumours in immunosuppressed mice but do in semi-solid medium and so long-term safety considerations must be addressed before clinical use (ICG Standards; Zhao et al. 2011; Fiegel et al. 2008).

HepG2 cells have been used for genotoxicity (mutagen screening) studies (Valentin-Severin et al. 2003), metabolic studies (Javitt 1990) and liver toxicity studies (Khetani et al. 2015). The differences in their expression profile when compared to in-vivo hepatocyte expression mean that, for each type of study, considerations must be made. For example, for toxicity studies, their sensitivity to accurate detection must be considered. The proposed sensitivities of this cell line to accurately detect toxicity are conflicting in the literature (Khetani et al. 2015). Where one study reports high sensitivity and low specificity (Khetani et al. 2015), another reports the opposite (Gerets et al. 2012). It is therefore only appropriate to use HepG2 cells for determining the toxicity of a parent drug; they are typically less suited for metabolite toxicity tests (Gerets et al. 2012). HepG2 cells are therefore limited in their usefulness by their differences in metabolic capacities and expression profiles when compared to in vivo hepatocytes (Xu et al. 2004). A subclone of HepG2 cells, HepC3a, have been shown to have higher activity levels and so may be a better alternative for certain studies (Vacanti & Kulig 2014).

Research has also moved forward in determining protocols for the directed differentiation of a variety of stem cells types (iPS, MSC etc.) to hepatocytes.

Where these cells would offer an advantage over primary cell cultures and immortailsed lines is that they are highly proliferative in vitro whilst maintaining in vivo like characteristics.

2.2 The extracellular matrix (ECM) and its influence on cellular properties

The in vivo environment cannot be perfectly replicated in vitro. Environmental factors such as the ECM, nutrient delivery and intercellular signalling can all impact cellular properties. The differentiation status, morphology and viability of the cells are all dependent on their environment. This ultimately culminates in cellular function changes. Advancements in cell culturing may focus on producing a more in vivo like environment for the cells in order to retain function. One of the better-studied methods for this is the introduction of ECM components to cellular scaffolds.

The ECM is the non-cellular component of a tissue. It is essential for the structural support of the cellular constituents through cell-matrix interactions but also for biomechanical and biochemical cues that can mediate signalling cascades and ultimately impact cellular properties (Frantz et al. 2010). The ECM is composed of two main macromolecule classes: glucosaminoglycans (GAGs), often covalently linked to proteoglycans; and fibrous proteins, the main being elastins, laminins, fibronectins and collagens (Alberts et al. 2002). Proteoglycans mediate cell-matrix/cell-cell interactions and therefore cell adhesion and inside-out and outside-in signalling (Schlie-Wolter et al. 2013). Examples of proteoglycans include integrins, syndecans and discoidin domain receptors (Frantz et al. 2010).

The ECM is composed of these fundamental components throughout the body, however, the composition and topology of these elements is unique to each tissue, and within this, each tissues ECM is distinctly heterologous. These

components may also be subjected to post-translational modifications and enzymatic/non-enzymatic remodelling and so there are a vast array of configurations the ECM could potentially take. Whilst the differences in composition, arrangement and post-translational modifications can affect cellular properties, they are also essential in the generation of the biochemical and mechanical properties of each tissue and therefore of each organ; properties such as the compressive/tensile strength and elasticity of an organ (Frantz et al. 2010). As each tissue has an unique ECM, it follows that the ECM composition may be a factor in tissue specific cellular properties (Schlie-Wolter et al. 2013).

In vitro studies have demonstrated the affect of the ECM on differentiation and cellular properties. Studies have shown stem cells can be directed down a certain lineage by the shape of the ECM they are adhered to. Additionally, migratory cells cultured on ECM arranged into a teardrop shapes have been shown to polarise and induce the formation of lamellepodia (Santhosh & Sudhakaran 1994; Liu and Chen, 2007). These studies act as a proof of concept for the impact the ECM can have on cellular properties, differentiation and behaviour.

2.3 Mechanical considerations

2.3.1 Mechanical mechanisms and properties of cells, ECM and liver tissues

The ECM, cells and external forces acting on a tissue all influence the mechanical properties of one another. The significance of mechanical forces at a cellular level have become increasingly accepted as important factors in overall tissue mechanics. Cellular level forces such as stretch, compression, static mechanical forces and shear stress from fluid flow have been shown to induce signalling mechanisms that can cause changes to cellular functions

downstream (Lam et al. 2012; Wells 2008). Altered cellular properties can include motility, viability, apoptosis initiation, adhesion, contractility and the differentiation state of the cell (Wells 2008). Studies into cellular mechanics have significantly contributed to our understanding of human diseases, including cancer, sickle cell anaemia and malaria in addition to liver pathologies such as non-alchoholic fatty liver disease and fibrosis (Lam et al. 2012). The aforementioned liver conditions have been shown to cause mechanical changes to the liver in terms of the organ itself, the ECM and the cells. Not only will continued research into cellular mechanics expand comprehension of pathologies as a whole, but it may also provide insight into diagnosis, prognosis and treatment further down the line (Lam et al. 2012). Although these in vitro studies of cellular mechanics have given such insight so far, many have yet to be validated in vivo.

Cells can be anchorage dependent, where adhesion is required for cell survival, or anchorage independent, such as in the case of blood and cancer cells. Most cells found in the body are anchorage dependent and are therefore unable to survive when dissociated and in suspension. Even with the addition of soluble proteins, such as RGD-binding integrins used to activate cell adhesion signalling pathways, the cells do not remain viable (Discher et al. 2005). Anchorage dependent cells adhere to the ECM through large macromolecular assemblies known as focal adhesions. Many components make up these complexes; including GTPases, scaffolding molecules and a range of enzymes (such as kinases, lipases, phosphatases and proteases). Modifications to focal adhesions are made by the cells in response to molecular and structural changes as well as the mechanical forces applied to the cell by the ECM (Wozniak et al. 2004).

As the cell engages its focal adhesions to adhere to ECM components, or a substrate provided in the scaffold, it pulls on the anchored substrate. This

cellular ability to transmit a contractile force to the substrate is dependent upon the interactions of myosin and actin filaments. The anchored substrate provides a resistance to the applied force, which in turn causes alterations to the cytoskeletal and focal adhesion organisation of the cells. Where an adherent cell is cultured on a soft scaffold such as a film or gel, these cellular contractions can sometimes be observed. The mechanical forces provided by the cells can cause the scaffold to shrink or wrinkle as the cells pull on the substrate. There is a general trend between substrate stiffness and cell structural arrangement in that the stiffer the substrate is, the more stable and organised the cytoskeleton and focal adhesions are. Studies have also shown that collagen fibril stiffness can be reproducibly altered to give different resistances to cellular mechanical forces. Variation in substrate stiffness was shown to have a significant impact on cellular features (Discher et al. 2005; Levental et al 2007; Pelham et al 1997; Wakatsuki et al. 2007).

The mechanical properties of the ECM are unique to each tissue. The composition and arrangement of ECM components determine the stiffness of the matrix and in so doing influence cellular behaviour. Where a matrix is stiffer it offers more resistance to the contractile forces of the anchored cells. Cells were first shown to detect and respond to variations in ECM stiffness by seeding epithelial cells and fibroblasts on ligand-coated gels (Discher et al. 2005). One of the factors since been discovered that can be predicted from matrix stiffness is cell motility. The cellular mechanism, durotaxis, describes the tendency for cells to migrate from areas of low matrix stiffness to areas of high matrix stiffness (Lo et al. 2000). Additionally, NIH 3T3 cells have been shown to slow their cell cycle and initiate apoptosis more frequently on softer matrices (Wang et al. 2000). These studies offer evidence for the influence of the ECM on overall cellular behaviour. The ECM also has influence at a molecular level and can direct post translational modifications; focal adhesion sites have been shown to undergo tyrosine phosphorylation where the ECM is

stiff (Pelham 1998). Commonly, to initiate a certain cellular behaviour through matrix stiffness in culture, it is best to replicate the Young's modulus of the in vivo tissue. For instance, it has been shown that Hepatic Stellate cells undergo differentiation to myofibroblasts when cultured on scaffolds with similar stiffness to cirrhotic liver whereas on matrices imitating normal liver properties, the cells remain quiescent (Wells 2008).

Cell-directed organisation of the ECM is also significant in influencing mechanical properties of a tissue. Collagen hydrogels are often used as scaffolds for in vitro tissue engineering studies as they allow for investigation into cell-matrix interactions (Busby et al. 2013). Hepatic cells have been shown to actuate post-translational modifications to collagen in response to liver injury. Lysyl oxidases undergo upregulation as a part of the response mechanism to liver injury. These enzymes, in combination with transglutaminase and lysyl hydroxylases, cause collagen crosslinking. This has been shown to significantly increase collagen stiffness in collagen gels in vitro and has been shown to likely affect similar changes in vivo (Elbjeirami et al. 2003). Liver fibrosis is also known to upregulate proteoglycan expression. These glycosylated proteins have a role in regulating the hydration status of the ECM. Increasing their expression makes the matrix more resistant to compression thus increasing the stiffness of the liver (Wakatsuki et al. 2000).

The Young's modulus of a normal liver ranges from 300 to 600 Pa. This can reach beyond 20 KPa in a fibrotic or cirrhotic liver (Georges et al. 2007). Hepatocytes are often cultured in monolayers on plastic (José et al. 2009), the Young's modulus of which is far greater than any physiological environment (Yeung et al. 2005). Studies have shown that hepatocytes adopt a dedifferentiated phenotype and are highly proliferative on stiff scaffolds and a growth arrested, differentiated phenotype on soft scaffolds. This was nicely demonstrated by Semler et al. 2000 where matrigel and collagen gels were

progressively crosslinked with glutaraldehyde to increase scaffold stiffness. As the stiffness of the gel increased, hepatocytes underwent dedifferentiation and increased proliferation accordingly (Hansen et al. 2006). It is therefore possible that studies investigating cellular properties where cells have been cultured on scaffolds as stiff as plastic, may in fact be drawing conclusions wholly unrelated to in vivo applications. Such in vitro mechanical studies have however successfully predicted cellular behaviour in animal studies of the central nervous system (Georges & Janmey 2005). Glia are the primary cells involved in scar formation following injury. Implanting hydrogel to the injury site was shown to inhibit scar formation as the glia were unable to survive on the soft matrices and neurones were able to grow as would be predicted by in vitro studies (Georges & Janmey 2005). Further research is required to determine if the liver has similar in vivo to in vitro correlations for mechanical studies yet progress has been made in predicting in vivo liver toxicity from in vitro analysis (Deng et al. 2010). The use of collagen hydrogels in culture may act to nullify this problem as we work to provide gels with stiffness's, stiffness gradients and cell-matrix interactions typical of physiological conditions, including liver and liver fibrosis (Tse & Engler 2011).

2.3.2 Mechanical Testing

Human tissues can be considered viscoelastic, consisting of an elastic solid and viscous liquid phase (Wells 2008; Discher et al. 2005). Experiments observing the short term elasticity and long term viscous behaviour of collagen gels have indicated they behave like a Maxwell fluid in their linear viscoelastic range (<15% strain) (Chandran & Barocas 2004). Numerous methods of mechanical testing and modelling have been found in the literature for soft hydrated tissues and different gel types (agarose, collagen etc.) mainly centring on indentation, shear, uniaxial tension and confined or unconfined compression. These mechanical testing methods are often undertaken in

conjunction with viscoelastic, elastic, biphasic or triphasic models (Périé et al. 2005).

Uniaxial tension and shear tests examine the properties of only the matrix network. This is because although gels are highly hydrated, the interstitial flow resistance is negligible as the network and fluid phases essentially deform together (Chandran & Barocas 2004; Knapp 1997). Compression studies allow analysis of both the matrix network and the interstitial fluid (Chandran & Barocas 2004). Under compression, the network stiffness (i.e. collagen fibrils) is much less as the fibrils are more resistant to tensile forces than they are to compressive (Li et al. 2005). The contribution of the interstitial fluid may therefore not be negligible and must also be considered. As compressive tests consider both phases of a material, they are often utilised in analysing biphasic materials.

2.3.3 Confined and unconfined compression

Unconfined compression is where a sample is compressed, with a uniaxial force, between two impermeable, smooth surfaces without any lateral containment (Fortin et al. 2000). The sample therefore undergoes deformation both radially and axially (Riches 2015). Although unconfined compression has been utilised for studies into the biological responses to applied load, it is rarely used for the mechanical analysis of biphasic materials as the biphasic model does not offer a good description of the materials response (Fortin et al. 2000). This is because certain assumptions are required which cannot be met by unconfined compression (Riches 2015). More complex analysis, including the incorporation into the biphasic model of anisotropy or composite solid phases composed of fibrillar and nonfibrillar components, has been shown to provide a good bridge between theory and experimentation (Fortin et al.

2000). However, confined compression testing can avoid this added complexity.

Confined compression analyses the response of a sample to an applied load whereby the load bearing and viscous properties of both the solid and fluid phases are relevant. It differs from unconfined compression in that a porous platen is used to compress a laterally confined sample. As the sample is compressed, the interstitial fluid flows through the interconnected pores of the sample (an assumption of the model) and evacuates the gel via the porous platen in order to conserve the volume. Once equilibrium is reached, the solid phase takes the load (Chandran & Barocas 2004).

For confined compression, stress relaxation and creep experiments can be conducted. These can be performed as either cyclic or static tests (Périé et al. 2005). A creep test applies a stress and records the deformation whereas a relaxation test applies a defined deformation and evaluates the stress required to perform that constant applied deformation. For stress relaxation, the material viscosity damps the response to strain in the ramp phase and relaxes the stress in the hold phase (Chandran & Barocas 2004). Experimental protocols differ between studies in their tare load (between 0.03-0.05 and 0.1-0.26N), ramp rate (between 0.1 to 1μms^{-1}) and ramp amplitude (between 2% and 10%) (Périé et al. 2005). Therefore the experimental design can take many forms and an understanding of the test protocols is imperative for producing conclusions relevant to the literature.

2.3.4 Characterising the collagen gel

As previously stated, collagen gels are made up of two component phases: a highly interwoven yet sparse collagen fibril network (usually making up only 0.1-0.5% of the gel) and an excess of interstitial fluid (typically 99.5-99.9% of

the gel) (Knapp 1997). The collagen fibrils have effective shear and tensile strength and thus most of the load is supported by the fibril network in response to shear and tensile forces. However, under compression the collagen fibril network provides little resistance. Collagen gels are able to endure compressive loads due to the resistance provided by the interstitial fluid. Compression facilitates high solution pressures of the interstitial fluid which, in combination with the fluids incompressibility, impedes the collapse of the network and thus offers resistance to compression (Knapp 1997). It has been shown that under compression, collagen gel mechanics arise from interstitial fluid flow and collagen fibrils bending at cross-linkage sites (Chandran & Barocas 2004).The rate at which the fibril network transfers the load to the fluid phase is determined by the stiffness and permeability of matrix (Busby et al. 2013; Knapp 1997). As both phases contribute to the compressive behaviour of collagen gels, the damping or stress relaxation behaviour may be governed by the gels resistance to permeation by the fluid or the solid phases intrinsic viscosity (Chandran & Barocas 2004).

2.3.5 Biphasic modelling of mechanical behaviour

There have been several forms of empirical equations proposed to quantify the properties of permeability and stiffness that can be applied to collagen hydrogels (Gu et al. 2003). In a study by Knapp et al. (1997), confined compression of collagen hydrogels was analysed using a biphasic theory originally described by Barocas and Tranquillo in 1997, yet this theory should only be used in accordance with unconfined compression tests and rheological data (Busby et al. 2013). For confined compression tests, the linear biphasic theory proposed by Mow et al. (1980) considers the stretch ratio (λ) and resultant stress response to determine an estimate of the aggregate modulus ($H_{A)}$ and hydraulic permeability (k) (Busby et al. 2013). The linear model of Périé et al. (2005) has since been applied to 2% agarose gels to successfully

determine the properties of deformation-dependent permeability (Busby et al. 2013; Gu et al. 2003). Busby et al. (2013) has gone on to apply this linear biphasic model to 0.2% and 0.4% collagen gels in order to determine if the model is adequately sensitive to establish differences in the permeability and stiffness of such gels. This study showed that the aggregate modulus, hydraulic permeability and both peak and equilibrium stresses correlated to the content of collagen, even at such small percentages. It could therefore be hypothesised that this linear biphasic model could be sensitive enough to recognise the result of matrix remodelling (Busby et al. 2013).

Poroviscoelastic models, originally developed from soil mechanics, may also be suited in determining mechanical differences in collagen hydrogels. The biphasic poroviscoelastic (BPVE) models developed by Suh and DiSilvestro (1999) have been applied to cartilage, a soft biological tissue whose mechanical properties are largely dependent upon its fibrillar collagen and hydrophilic proteoglycan network, in order to determine the mechanical repercussions of collagen crosslinking (McGann et al. 2014). Therefore, further development of this theory may provide an equally or better model for determining changes in mechanical properties in response to small scaffold alterations attributed to matrix remodeling by cells or the addition of enzymes etc. (Busby et al. 2013).

The linear biphasic theory relies on three assumptions: The material is homogenous; the liquid phase is an incompressible viscous fluid and the solid phase behaves elastically; and there is an interconnected pore structure made up of infinitesimally small pores to allow fluid flow relative to the solid phase (Riches 2015). Darcy's law is used to model fluid flow through the gel:

$$v = -\frac{\kappa}{\mu} \nabla P \qquad (1)$$

Where v is the fluid velocity, P is the pressure (▽P being the pressure gradient), $\varkappa$ is the Darcy permeability and μ is the viscosity coefficient (Johnson & Deen 1996). The Hydraulic permeability (k) is related to Darcy's permeability by:

$$k = \frac{\kappa}{\mu} \qquad (2)$$

The hydraulic permeability governs the interstitial fluid flow as it acts to resist it. It is a proportionality constant relating the pressure (P) to the fluid velocity (v) (Johnson & Deen 1996; Gu et al. 2003). k is also a function of the stress ratio (λ) where $k=k[\lambda(z,t)]$ in the z direction at time t.

For the linear biphasic model, Mow et al. (1980) allows the aggregate modulus (H_A) and the hydraulic permeability (k) to be estimated from confined compression tests by fitting equation (3) (Busby et al. 2013):

$$\frac{\delta^2 u}{\delta z^2} = \frac{1}{H_A k} \frac{\delta u}{\delta t} \qquad (3)$$

Here u=u(z,t) where u is the displacement of the samples solid phase in the z direction at time t.

The aggregate modulus can be considered a measure of stiffness as it is related to the young's modulus (E) and Poisson's ratio (ν) of the sample by (Busby et al. 2013):

$$H_A = \frac{E(1-\nu)}{[(1+\nu)(1-2\nu)]} \qquad (4)$$

2.4 Research question and aims

The stiffness and permeability of a collagen gel and how they change in response to different cellular densities and culture durations is a question with implications in both tissue engineering and in vitro toxicity testing. Understanding to what extent these parameters vary may help to gain a better understanding of what the results of these studies represent as well as helping to design better experiments where mechanical considerations can be applied. Toxicity testing relies on the mass transfer of a toxin through a construct and so knowledge of how permeability may be affected over time should be an essential part of these studies. For tissue-engineered constructs, the changes in mechanical parameters are critical to its success as an implant. Implanted constructs will be subjected to cellular ingrowth, matrix remodelling and diffusion of signalling molecules and growth factors. Researchers would want to control the rate of these processes in order to elicit a desired response. One important consideration to achieve this is through a design where the mechanics of the construct over its lifetime can be predicted and this will come through a better understanding of the mechanics of in vitro cell seeded constructs.

The mechanical properties of HepG2 cell-seeded collagen hydrogels in a mixture configuration are to be evaluated in this project. This is to determine if there are any significant differences in the mechanical properties of:

- collagen gels seeded with different HepG2 cell densities.
- cell seeded collagen gels over different culture times.
- collagen gels in monolayer and mixture configurations.

3.0 Materials and Methods

3.1 Storage and maintenance of HepG2 cell cultures

HepG2 cells used for this study were derived from a cryogenically preserved stock in liquid nitrogen from the University of Strathclyde. Cultures were then stored in 1x Dulbecco's Modified Eagle Medium, DMEM, (Cambrex) which was supplemented per litre with 50ml sterile FCS (foetal calf serum), 5ml PEST (penicillin and streptomycin) and 5ml of 1% NEAA (non-essential amino acids). Cultures were stored in 25cm^3 T-flasks and incubated in 5% carbon dioxide/air humidified 37°C incubator.

In order to maintain a viable culture for the extent of the study, HepG2 cells were passaged every 3-4 days. Hep G2 cells are an adherent cell line and once 90% confluence was approximately reached (usually every 3-4 days), the medium was decanted and any traces of serum removed by washing the T-flask with 5ml of versene, an EDTA solution. 1ml of the protease solution, 1 (w/v) trypsin, was then added to the cells and left for a few minutes to detach the adhered cells from the T-flask surface. To break up any remaining cell aggregations and surface attachments, the T-flasks were gently tapped. Their separation from the T-flask and each other was confirmed by observation under a microscope. 5ml of DMEM was then added to dilute and stop the action of the trypsin, achieved as a result of inhibition by the serum present in the DMEM. The culture was then split by an appropriate ratio (1:6 was routinely used for confluent growth) and added to a fresh T-flask with enough DMEM to make the volume up to 10ml. For cell culture, sterile conditions must always be maintained.

3.2 Collagen gel preparation and cell seeding

The type I collagen stock used for collagen gel preparation was obtained from rat tail tendons in a method described by Rajan et al. (2007). Additional sterile stock solutions of 1M Sodium Hydroxide (NaOH), DMEM:NaOH (2:1 mixture) and 1/1000 v/v acetic acid were also required. The volume of each solution required was dependent upon the objective concentration of the collagen gel to be made (0.3%) and the concentration of the type I collagen stock; this can vary each time it is isolated.

A stock of 5.55mg/ml of collagen solution was used throughout this study with a prescribed collagen gel concentration of 0.3%. Therefore for 10ml of collagen gel solution, the volumes required were as follows: 5.4ml collagen solution, 3.6ml 1/1000 v/v acetic acid and 1ml DMEM:NaOH (2:1 mixture).

Care needed to be taken to avoid introducing bubbles through the collagen solution; this would affect the results of mechanical testing later on. Bubbles tend to form when the last of the solution is evacuated from the pipette. Therefore, to resolve this, the technique of reverse pipetting was utilised with Gilson pipettes (where liquid is expelled only to the first stop and not the second) and a technique with the same principle was used for the motorised pipette fillers: an extra ml was taken up and left in the pipette. Additionally, the pipette opening was rested against the inside surface of the glass beaker to reduce the inclusion of bubbles.

To prepare the gels, all solutions were kept on ice as warmer temperatures can promote the setting of the collagen gel solution before plating can occur. The collagen solution and 1/1000 v/v acetic acid were added together first followed by the DMEM:NaOH (2:1 mixture). The pH of the resulting solution was then adjusted to 8.0-8.5 by the dropwise addition of the 1M NaOH, which

is required in order for the solution to gel. The change of pH was observed in the yellow to pink colour change as a result of the phenol red pH indicator present in the DMEM. At this point, cells could be seeded into the solution by their addition with a Gilson pipette. The solution was swirled to ensure the cells were evenly spread and then pipetted as 0.4ml per well in a 24-well plate using the reverse pipetting technique. Completed plates were then left to set in an incubator for an hour before the addition of 1ml of DMEM.

Collagen gels were seeded with set concentrations of HepG2 cells: $1x10^4$, $2x10^4$, $5x10^4$, $10x10^4$ and $20x10^4$ cells/well in addition to collagen gels with no seeded HepG2 cells which acted as the control. Prior to seeding, HepG2 cell suspensions were prepared through the versene/trypsin passaging method previously stated (section 2.1). Cell numbers were then counted using a haemocytometer to allow for the HepG2 suspension concentration to be subsequently calculated.

3.3 Determination of cell viability

Viability of the HepG2 cells seeded into the collagen gels was determined by MTT reduction assay. A 10mM solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in pH 6.75 phosphate-buffered saline (PBS) was made up by dissolving 0.4143g of MTT in 100ml of pH 6.75 PBS. The solution was taken up into a syringe and sterilised through a 0.2μm filter. This solution could then be stored for 2 weeks at 4°C.

To assess viability, the medium bathing the gels was removed from the wells and 500μl of the MTT solution was added. The sample was incubated for a further hour at 37°C before the MTT solution was removed. Metabolically active cells reduce MTT to a purple formazan product whereas non-viable

cells are not stained (Riss et al. 2004). This colour change could be observed and photos taken to give evidence of cell viability.

3.4 Confined compression mechanical testing

To determine the mechanical properties of the samples, the confined compression and biphasic theory technique validated by Busby et al. 2013 was utilised.

The DMEM bathing the collagen hydrogel samples was firstly drained from the wells and the gel was detached from the well wall with a 200µl pipette tip. Care was taken to remove all the bathing fluid and any bubbles. The sample was then loaded onto the BOSE ElectroForce 3200 Test Instrument (BOSE, UK) with a 22N load cell (BOSE, UK). The porous platen ($k = 5.95x10^{-7}$ m^4/Ns) was lowered onto the gel surface until full contact was made and a preload of 0.01N (0.05KPa) was achieved. Prior to testing, the system was rested to allow the stress response to equilibrate. Samples underwent compression by displacement control at a rate of 25 µm/s (~1.1% strain/s) by 250 µm (~11% strain). The subsequent hold phase was maintained for 300s whilst stress response data was acquired using WinTest software.

The peak and equilibrium stress were determined from the load applied and the area ($1.91cm^2$) using Microsoft Excel. Additionally, data was converted using Microsoft Excel to fit requirements for analysis in MATLAB®. MATLAB®, in combination with the corresponding biphasic theory code provided by Dr. Phil Riches, was used to determine estimates of the aggregate modulus (H_A), hydraulic permeability (k_0), coefficient of permeability (M) and correlation coefficient (r^2).

3.5 Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 21.0 software. For each of the three culture durations and 6 cell seeding densities (no cells, $1x10^4$, $2x10^4$, $5x10^4$, $10x10^4$, and $20x10^4$ cells/well) varying numbers of repeats were taken. This gave a sample set with varying values for n in each group. A multivariate general linear model analysis was carried out, followed by a Dunnett's test. This allowed for inspection of the between-subjects effects and pairwise comparisons of the group means. Normal distribution was assumed in order to implement these tests. Confidence intervals were set to 95% with a p value of 0.05 indicating significance.

4. Results

There is no significant relationship between the concentrations of the cells seeded and the hydraulic permeability (k_0), aggregate modulus (H_A), r^2, non-linear permeability coefficient (M), peak stress or equilibrium stress. The results do not appear to show any established trends and so data suggests the parameters tested are independent of changes in concentration between zero and $20x10^4$ cells per well.

Culture time dependent effects are not seen for the aggregate modulus (H_A) or r^2, nor can any clear trends be identified. In cell-seeded samples, the hydraulic permeability (k_0) significantly decreases with culture time ($p=0.041$) as depicted in figure 2A. The non-linear permeability coefficient (M) decreases significantly with culture time also ($p=0.034$) although the only significant reduction is between day 3 and day 5 ($p=0.19$) (figure 2B). In contrast, control samples show relatively stable values throughout culture time for these parameters (figure 1A and 1B).

Control samples for the peak stress show no significant change over culture time (figure 1C). The peak stress however demonstrates a significant increase over culture time in cell-seeded samples ($p=0.010$) (figure 2C), however the equilibrium stress shows no significance nor can any trend be identified in the data.

Standard error of the mean error bars in the cell-seeded samples (figure 2) are reasonably small and therefore infer good repeatability in the results. Control data error bars (figure 1) are slightly less so but still infer a steady response over culture time.

Cell viability was determined via MTT assay, photographic evidence for which can be found in the appendix. This does not allow quantification of the proliferation of the HepG2 cells over culture duration but it can be observed that the cell numbers present reflect the seeding densities applied and therefore the method for seeding the gels is accurate enough.

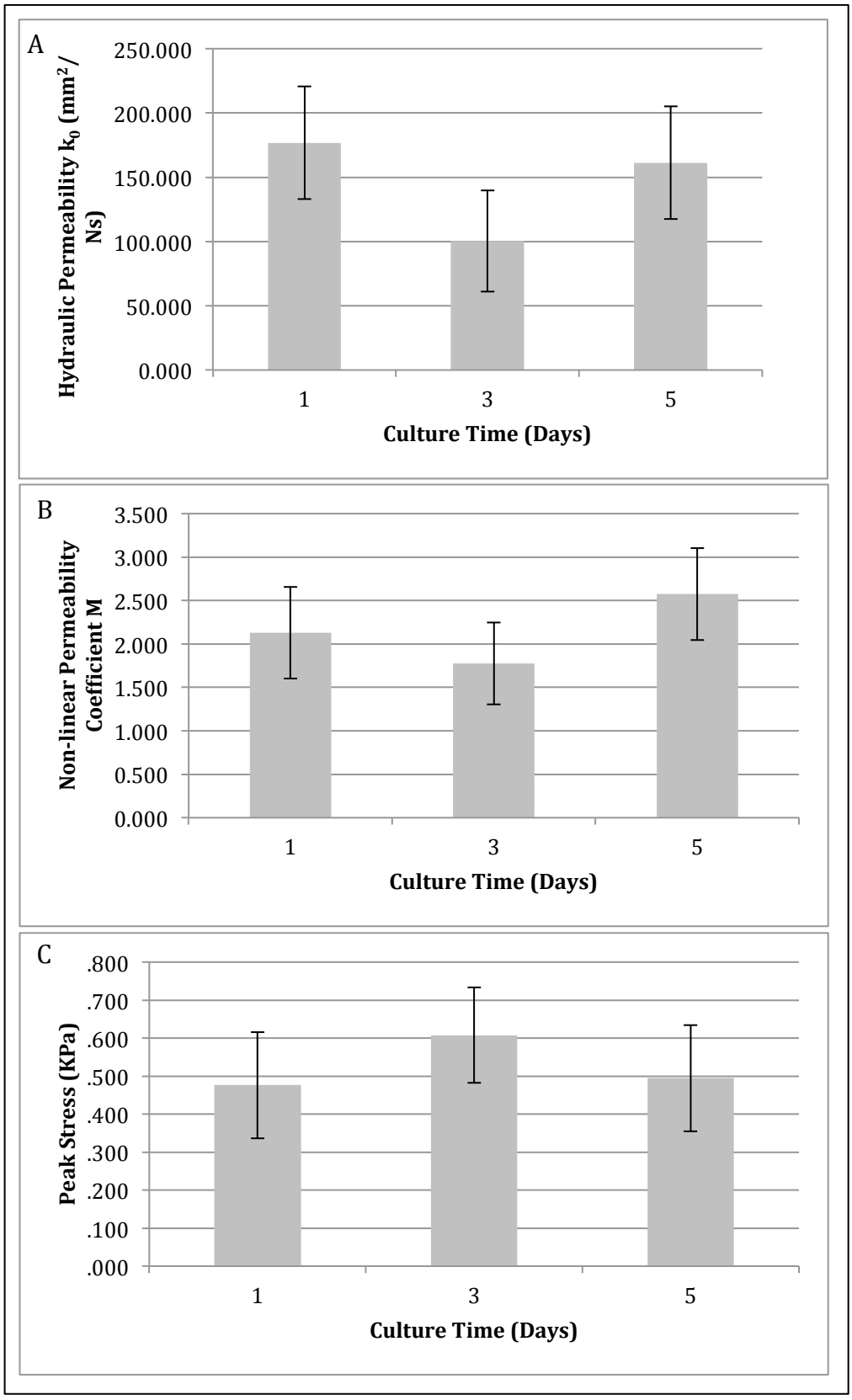

Figure 1. Pooled data from all non-cell seeding densities showing changes in hydraulic permeability (k_0), non-linear permeability coefficient (M) and peak stress over culture time. Error bars indicate standard errors of the mean.

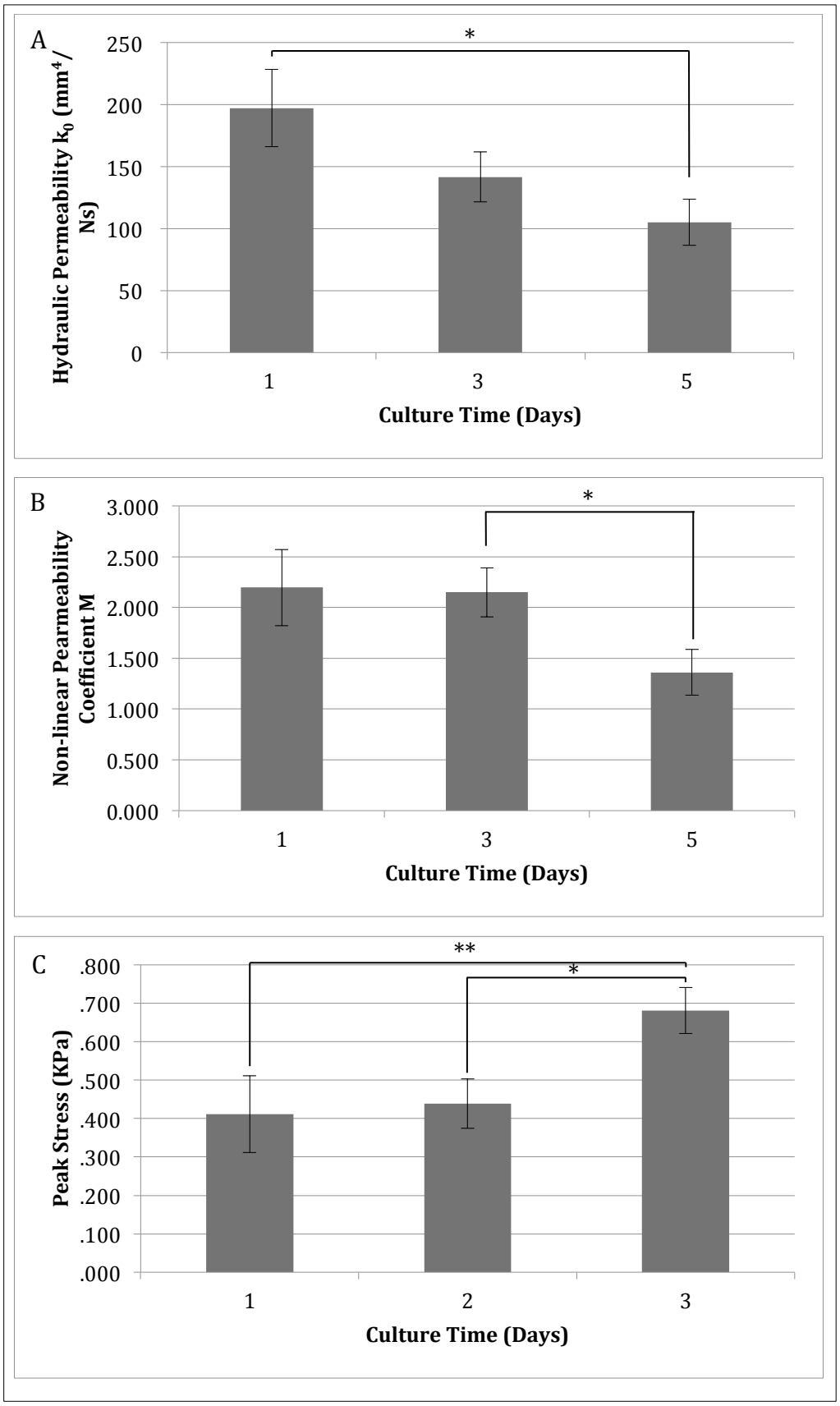

Figure 2. Pooled data from all of the seeding densities showing changes in hydraulic permeability (k_0), non-linear permeability coefficient (M) and peak stress over culture time. Error bars indicate standard errors of the mean. Significance is depicted by *$p<0.05$ and **$p<0.01$.

Table 1. Summary of the range of r^2 values found in the data.

Range of r^2 values	Number of r^2 values	Percentage of total r^2 values (%)	Percentage of r^2 values above the lowest range value (%)
$r^2>0.99$	0	0	0
$0.99>r^2>0.95$	13	11.7	11.7
$0.95>r^2>0.9$	43	27.0	38.7
$0.9>r^2>0.8$	83	36.0	74.8
$0.8>r^2>0.7$	102	17.1	91.9
$0.7>r^2>0.6$	108	5.41	97.3
$0.6>r^2>0.5$	110	1.80	99.1
$0.5>r^2>0.4$	110	0	99.1
$0.4>r^2>0.3$	110	0	99.1
$0.3>r^2>0.2$	110	0	99.1
$0.2>r^2>0.1$	110	0	99.1
$0.1>r^2>0.5$	111	0.901	100

r^2 depicts the percentage of variance explained by the model and can determine how well the biphasic model fits the experimental data. r^2 values range from 0.981 to 0.0676 (Table 1). The highest proportion of values (36.036%) of r^2 range between 0.8 and 0.9. Using standard deviations (1SD = 0.119) from the mean (0.844) to detect outliers, any value of r^2 less than 0.606 may be considered an outlier. Therefore 3 of our r^2 values, and resulting parameter values, may be considered outside of the normal goodness of fit for the biphasic model.

Figure 3A shows a good example of the experimental data fitting to the biphasic model. Figures 3B, 3C and 3D represent a number of different ways in which the model fails to appropriately fit to the data. In 3C the ramp stress lags behind the model and has a smaller value for equilibrium, figure 3D showing the opposite. Figure 3B shows two stress peaks, which is not expected from a normal stress response, and so the model fits the second peak to the curve, negating the first.

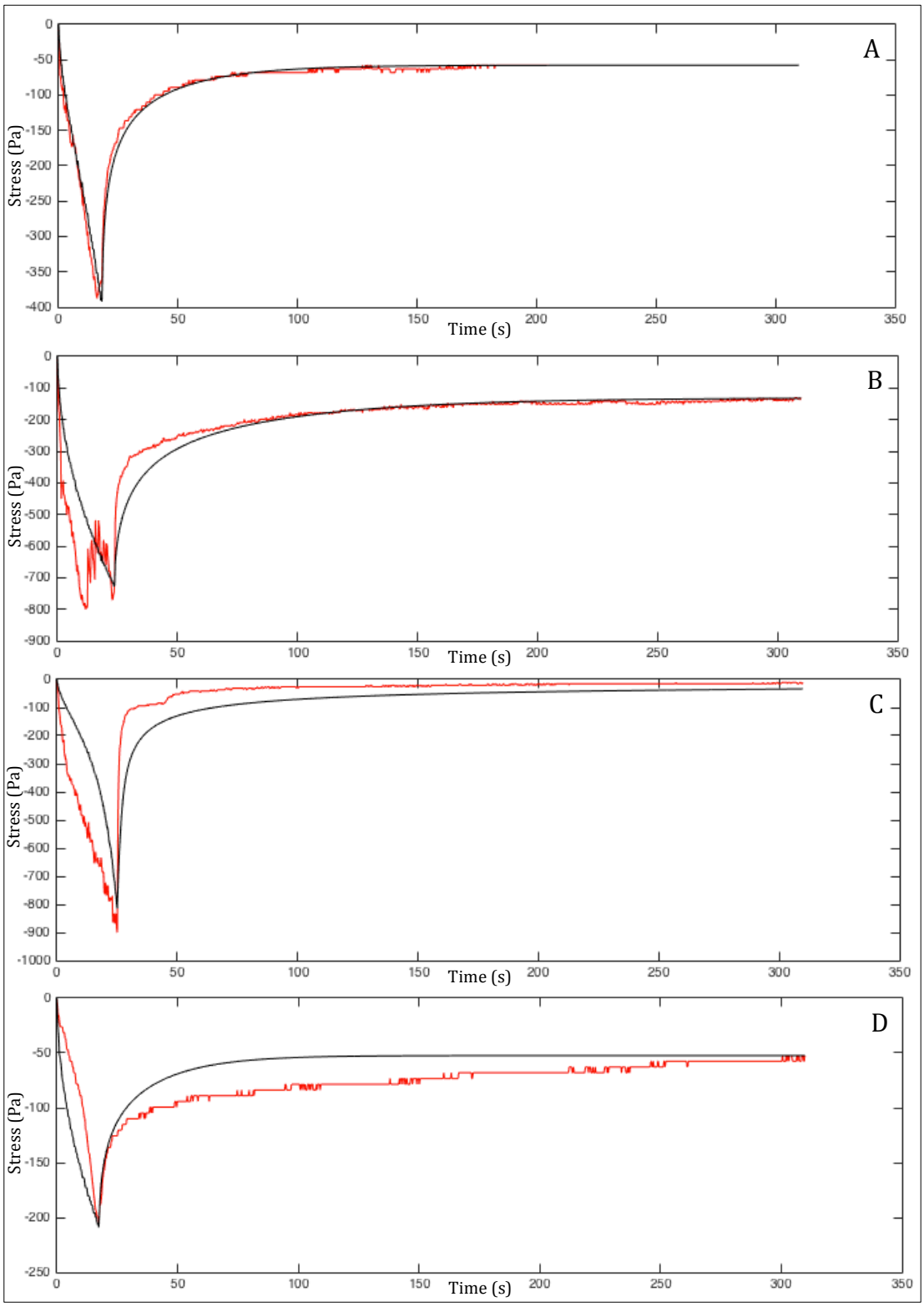

Figure 3. Matlab output figures depicting successes and inaccuracies in fitting of the experimental stress response to the biphasic model. A) Good fit, r^2 value of 0.981. B) Dual stress response peak, r^2 value of 0.676. C) Poor fit, r^2 value of 0.689. D) Poor fit, r^2 value of 0.0676.

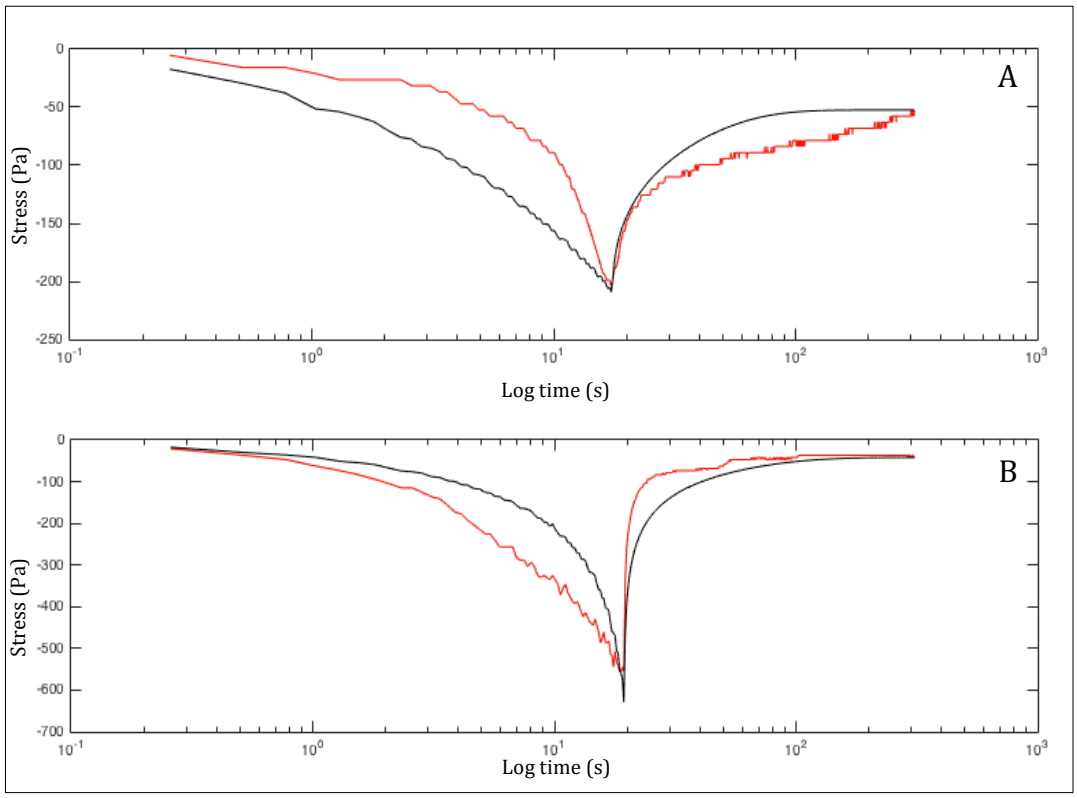

Figure 4. Semi-log Matlab output figures for stress response against time to determine if equilibrium has been reached in 300s. A) Example of response where equilibrium has not been reached. B) Example of response where equilibrium has been reached.

Taking the semi-log of the stress response against time allows for observation of the gradient (Figure 4). If equilibrium is reached there should be no gradient as the graph approaches 300s. Equilibrium is not always reached after 300s as seen in figure 4A, although, some data does reach equilibrium (Figure 4B).

5. Discussion

5.1. Summary

The purpose of this project was to measure the mechanical characteristics of HepG2 cell-seeded collagen hydrogels to determine if culture time and seeding density had any effect. Cells are able to remodel their matrix environment enzymatically and mechanically to alter the permeability and stiffness of the construct (Elbjeirami et al. 2003; Lam et al. 2012). In turn, the permeability and stiffness of a scaffold can influence cellular characteristics and behaviours such as proliferation, viability, motility and differentiation (Mehta et al. 2010). There are few studies investigating the mechanical changes of collagen gels due to the influence of hepatocytes. Analysing how the seeding densities and culture time affects the collagen gel mechanics would be useful for tissue engineering as it allows for a prediction of how a given construct will act during the course of a study. This would be relevant for studies looking to maintain certain cellular characteristics in culture (i.e. for toxicity testing and clinical tissue engineered constructs)(Du et al. 2008), for models looking to maintain mechanical properties (for instance to be similar to in vivo) and potentially for studies looking to induce stem cell fate through mechanical matrix interactions (Trappmann et al. 2012).

In this study, collagen gels were seeded with HepG2 cells in densities of $1x10^4$, $2x10^4$, $5x10^4$, $10x10^4$, and $20x10^4$ cells/well as well as a 'no cell' control to give results comparable to similar studies by Sprott 2014 and Pellizzoni 2014. The mechanical characteristics of collagen hydrogels seeded with HepG2 cells in a monolayer configuration have been previously investigated (Pellizzoni 2014). This study aimed to consider the mechanical properties of the collagen gel as a whole to observe the interactions of the gel and the cells. During confined compression however, the platen directly contacted the cells seeded on the surface of the gel, which may have influenced the results. In this study, HepG2

cells are seeded into collagen hydrogels in a mixture configuration in order to resolve this issue.

5.2. Mechanical properties

The data obtained has shown significant trends for the hydraulic permeability (k_0), peak stress and the non-linear permeability coefficient (m) over culture time but not for differing seeding densities.

The hydraulic permeability is a measure of the ability of a porous construct to allow fluid transfer under an applied pressure which drives the flow of the fluid (Serpooshan et al. 2010). Hydraulic permeability of a collagen gel therefore has an influence on mechanical properties such as stiffness as well as the mass transfer of the gel. This therefore allows for nutrient transport and cell-cell/cell-matrix communication and signalling transduction which may lead to alterations in function, morphology and differentiation state (Serpooshan et al. 2010). In addition, viscous shear forces brought about by localised fluid flux can cause cellular responses, such as activation of the MAPK signalling pathway which is important in mechanotransduction signalling and is activated in response to any mechanical stimuli (Glossop & Cartmell 2009; Busby et al. 2013). Therefore, hydraulic permeability can give insight into the amount of mass transfer and shear forces a gel is subjected to by fluid flux under compression.

For this study, the hydraulic permeability has been shown to decrease significantly with culture duration; however, there is no effect of seeding concentration. Where the 'no cell' culture is taken as a control, there is no significant change in hydraulic permeability. This suggests that the presence of cells are not influencing the gel permeability and therefore cell ingrowth is not sufficient to physically obstruct fluid flow significantly. The rate of HepG2 proliferation in collagen hydrogels has not yet been characterised, however, their utilisation in this study over primary hepatocytes is in part due to their

highly proliferative nature (Palakkan et al. 2013). It is therefore difficult to categorise the number of cells present in gels of different culture times. If these significantly surpass the range of seeding densities applied to the gels then these larger cellular densities may in fact be a significant physical obstruction to fluid flow but for this to be a factor you would expect to see a trend between the seeding concentrations. For quantifiable data on the changes to seeding densities, a full MTT assay could be performed or cells could be photographed with a microscope and counted over the same size field of view. Another factor to consider is changes in the collagen gel itself over time. Hydrogels can undergo swelling or shrinking until an equilibrium is reached which may contribute to changes in k_0 over culture duration (Patel & Mequanint 2007). The non-linear permeability coefficient (M) describes the loss of permeability with compression and is used to model the permeability of the construct (Riches 2011). It therefore follows that it should have a similar trend to the hydraulic permeability as this parameter is calculated from M.

HepG2 cells cultured on a monolayer showed no significant reduction in hydraulic permeability with culture time but did show a decreasing trend (Pellizzoni 2014). Monolayer studies did however show a significantly lower hydraulic permeability for cell-seeded gels compared to the 'no cell' collagen gel. This suggests the cells did offer an obstruction to fluid flow but this may be due to their direct contact between the porous platen and gel surface, restricting flow though the top of the gel. A study has shown that HepG2 cells have slower proliferation rates in 3D culture than monolayer culture and that HepG2 cells put down fibronectin in the 3D configuration but not whilst seeded in monolayers (Pruksakorn et al. 2010). This would suggest that monolayer cultures may be less permeable than 3D culture due to their lack of fibronectin synthesis regardless of their higher proliferation rate.

The aggregate modulus (H_A) is a measure of stiffness where fluid flow has ceased and the collagen gel is at equilibrium (Mansour & Ph 2009). Analysis of

this parameter is important to mechanical studies as the stiffness of a scaffold can have significant effects on cellular properties (Busby et al. 2013; Tse & Engler 2011; Helary et al. 2010). The stiffness of a cell seeded collagen gel can be influenced by cellular degradation of the matrix to reduce the aggregate modulus or the laying down of collagen by cells as well as the physical presence of the cells to increase the aggregate modulus (Saddiq et al. 2009). Evidence of enzymatic degradation of the collagen fibres has been shown by Saddiq et al. 2009 whereby gels are incubated with bacterial collagenase.

HepG2 cells seeded as monolayers on collagen gels have shown an increase in H_A with culture time (Pellizzoni 2014). In contrast, collagen gels and collagen gel cross-linked composites seeded with 3T3 mouse fibroblasts and human skin fibroblasts have shown an overall decrease in H_A (Saddiq et al. 2009). The difference in results here may be due to the cultured cell type. Fibroblasts are more actively involved in collagen synthesis and degradation than the HepG2 cells. Additionally, in the monolayer study, the increase in H_A may be more to do with the testing setup whereby the platen is introduced to a layer of cells on top of the gel and thus may be reading the stiffness of the cells more than the whole construct.

This study has established no trend or significance to the effects of cell concentration and culture duration on the aggregate modulus. To account for this, we could either be seeing a balance of these mechanisms of matrix degradation combined with collagen resynthesis and physical cellular interactions or these mechanisms may not be occurring to any significant extent at all. Collagen gels in growth medium have been shown to have a relatively stable stiffness when not seeded (Saddiq et al. 2009). This could therefore support our claim that the concentration of cells present in the 3D matrix mixture is not enough to affect any significant changes.

The results obtained for our aggregate modulus however are in the same order of magnitude as some in vivo measurements. Indentation tests and a novel

method for mechanical testing which utilises FE modelling has shown that a healthy liver has a stiffness corresponding to 0.62±0.09 kPa for the FE model and 0.59±0.1 kPa for indenter tests whereas a diseased liver has a range of 0.6 - 1.64 kPa for the FE model and 0.96 - 1.88 kPa for the indenter (Barnes et al. 2007). The values of H_A obtained from this study are between 0.266 - 0.387 KPa which is closer to a healthy liver phenotype that the fibrotic liver. This may in some way account for the lack of matrix remodeling as the HepG2 cells are cultured on a healthy liver-like scaffold and so do not initiate any fibrotic mechanisms but instead happily proliferate.

Although the aggregate modulus shows no significant changes in stiffness, the peak stress is shown to increase with culture time, although there are no concentration dependent effects. This suggests that the stiffness of the gel does in fact increase with culture time, however, as the equilibrium stress and H_A show no trend in agreement with this statement, the increase in peak stress is not conclusive to the state of the gel stiffness over time. During confined compression, a fluid leaving surface (FLS) cake can form. An FLS cake is a collagen dense region at the surface of the gel. This forms as the gel is compressed and the fluid leaves but collagen is retained. This may increase hydraulic resistance at the surface and therefore influence results (Serpooshan et al. 2010; Serpooshan et al. 2013; Chandran & Barocas 2004). Additionally, as the value for H_A is dependent upon biphasic modelling, it may be that issues (to be later discussed) with this model have produced inaccurate estimates of H_A. However, without a similar trend in equilibrium stress supporting the peak stress trend, a conclusion cannot be drawn.

5.3. Biphasic modelling

Determination of the aggregate modulus (H_A), non-linear permeability coefficient (M) and hydraulic permeability (k_0) is achieved using a Nelder–Mead scheme put forward by Riches 2011. In this, the biphasic theory is modelled

whereby there is an exponential decrease in permeability with strain as described here:

$$k(\lambda) = k_0 e^{M(\lambda-1)}$$

This model determines the H_A from the equilibrium stress. As shown in figure 3A, experimental data does not always reach equilibrium and thus an imprecise value for H_A may in fact be produced. Once estimated, the H_A is fixed, along with other parameters, to enable the permeability parameters to be determined in a time-dependent manner. Therefore the fitting of H_A accurately may also have an effect on permeability measures. Future studies should therefore ensure equilibrium is reached in the data to void any additional error this brings (Riches 2011).

To determine how well the experimental data fits the biphasic model, a coefficient was used to characterise the goodness of fit (r^2). A range of different combinations of the permeability parameters can give the same r^2 value as described by the manifolds presented in Riches 2010. The manifold surface also has multiple local maxima that may draw the conclusive parameter values away from more accurate measurements. The Nelder–Mead simplex method implemented in this study by Riches 2010 prevents convergence on these local maxima and instead focuses the measurements to the global maximum, giving a result closer to true. In spite of this, there are still a range of parameter values that can give the same r^2 value. Therefore the values determined from this study should be taken with a pitch of salt and large confidence intervals should be assumed. A parameter sensitivity study which looks at the 4-dimentional interactions of H_A, k_0, M and β (a parameter fixed at 0.256 to determine H_A) may help determine how confident we can be in this method (Riches 2011).

Our results show a wide range of r^2 values as represented in Table 1. As there are already large confidence intervals assumed in the parameter values from the biphasic modelling method, low r^2 values would suggest results may be far from the true value. Only 11.7% of the r^2 values produced from this study are

over 0.95 which acts as a good r^2 value. 74.7% of our data has an r^2 value above 0.8 which may be considered a reasonable value, however, at this point we are just decreasing the confidence we can have in our data. For future studies, a r^2 cut off value could be implemented in order to reduce some of the uncertainty associated with this method.

r^2 values were analysed using a general linear model in order to determine if culture time and concentration had any effect on how well the parameters were fitted to the model. This produced insignificant results as expected, however, the statistical analysis may not be appropriate to this study. r^2 values are skewed towards 1 and therefore normal distribution cannot be assumed although it is a requirement for general linear analysis.

5.4. Problems and limitations

Experimental limitations are evident in many features of this study. Confidence issues associated with the biphasic modeling have already been described however, there are additional concerns in other experimental techniques. Cell seeding concentrations required the accurate measurement of small volumes of liquid. This may not have mixed appropriately in the gel causing an uneven spread of cells and cells may have been lost in transfer. Additionally, there was always variation in cell count due to aggregations of cells which could not be broken up. For these reasons, the seeding densities for each sample may have seen large variations and may therefore account for the lack of significance in data between cell seeding concentrations. Another experimental problem may be in the loading of the gels. It is difficult to get an exact preload of 0.1N which may affect the stress response recorded for each sample.

5.5. Conclusion

The aim of this study was to determine the mechanical effects of seeding collagen gels with HepG2 cells in different densities and with varying culture

durations. Our study showed no concentration-dependent mechanical effects. Culture duration was shown to influence the permeability of the constructs, however, these results cannot be taken as conclusive due to the low r^2 values recorded and additional factors associated with the biphasic modelling method. The peak stress was shown to significantly increase with culture time, which may be due to the behaviour of the collagen gel itself over time, however this trend was not supported by the equilibrium stress or H_A. Future research into parameter sensitivity studies may enable for more confidence in results from biphasic modelling.

A decrease in permeability over culture time is a factor that will have implications on tissue engineering and in vitro toxicity studies. The changes observed in this parameter will reduce mass transfer through the sample and so for toxicity testing this could affect toxin uptake by the cell population. This should therefore be a factor taken into consideration when implementing a toxicity test. Additionally, an understanding of the mechanical characteristics of a tissue-engineered construct is imperative to controlling the effectiveness of an implant. Once implanted into the patient, the construct will experience cell ingrowth, matrix remodelling and signalling molecule permeation. The permeability of the construct will affect the rate of these processes and therefore the behaviour of the body response to the implant. As we have shown the permeability decreases over a comparably short culture time than would be required for in vivo tissue engineering it would be interesting to observe how mechanical parameters behave over a more clinically relevant culture duration.

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7. Appendix

Appendix shows MTT assay photographic data. Each photo represents a different cell-seeding density and culture duration. Colour changes from yellow to pink are due to the presence/absence of culture media.