DESIGNING NANO-STRUCTURED HYDROGEL FOR CARTILAGE TISSUE ENGINEERING

A thesis submitted to the University of Manchester for the Degree of Doctor of Philosophy in the Faculty of Science and Engineering

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Illida Mohd Nawi
School of Materials
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<tbody>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>ART</td>
<td>Articular Cartilage</td>
</tr>
<tr>
<td>AC</td>
<td>Alternating current</td>
</tr>
<tr>
<td>ACI</td>
<td>Autologous chondrocyte implantation</td>
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<td>ACN</td>
<td>Acetonitrile</td>
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<tr>
<td>ADAMTS</td>
<td>A Disintegrin and Metalloproteinase with Thrombospondin motifs</td>
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<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>ADSC</td>
<td>Adipose stem cell</td>
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<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>BC</td>
<td>Bovine chondrocyte</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<td>BMSC</td>
<td>Bone marrow stem cell</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CGC</td>
<td>Critical gelation concentration</td>
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<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>DC</td>
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<td>dH₂O</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagles Medium</td>
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<td>DMMB</td>
<td>Dimethylmethylene blue</td>
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<td>Dimethyl Sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DPBS</td>
<td>Dulbecco’s phosphate buffer saline</td>
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<td>Focal adhesion kinase</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>FTIR</td>
<td>Fourier transform infrared</td>
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<td>G</td>
<td>Glycine</td>
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<td>Description</td>
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</tr>
<tr>
<td>G'</td>
<td>Elastic modulus</td>
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<td>G''</td>
<td>Viscous modulus</td>
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<td>GA</td>
<td>Guluronic acid</td>
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<td>Global domain 1</td>
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<td>Global domain 2</td>
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<td>GF</td>
<td>Growth Factor</td>
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<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
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<td>Hyaluronic Acid</td>
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<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
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<td>IGD</td>
<td>Inter-globular domain</td>
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<td>IgG</td>
<td>Immunoglobulin</td>
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<tr>
<td>ITS</td>
<td>Insulin Transferrin Selenium</td>
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<td>IVD</td>
<td>Intervertebral disc</td>
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<td>K</td>
<td>Lysine</td>
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<td>Leucine</td>
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<tr>
<td>LVR</td>
<td>Linear viscoelastic region</td>
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<td>MA</td>
<td>B-D-mannuronic acid</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>MPC</td>
<td>Mesenchymal Progenitor Cell</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
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<td>NaCl</td>
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<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drugs</td>
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</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
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<tr>
<td>OPF</td>
<td>Oligo(polyethylene glycol)fumarate</td>
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<td>P</td>
<td>Proline</td>
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<td>PA</td>
<td>Polyamide</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PCL</td>
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<td>PDGF</td>
<td>Platelet-derived Growth Factor</td>
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<td>Polyethylene</td>
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<td>Poly(ethylene)glycol diacrylate</td>
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<td>PEMF</td>
<td>Pulsed electromagnetic field</td>
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<td>Isoelectric point</td>
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<td>PRP</td>
<td>Platelet rich plasma</td>
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<td>PSF</td>
<td>Penicillin-streptomycin-fungizone</td>
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<td>PTFE</td>
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<td>PVA</td>
<td>Polyvinyl alcohol</td>
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<td>R</td>
<td>Arginine</td>
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<tr>
<td>R\text{a}</td>
<td>Mean of roughness</td>
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<td>RCCS</td>
<td>Rotary cell culture system</td>
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<td>RGD</td>
<td>Arginine-Glycine-Aspartate</td>
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<td>RLU</td>
<td>Relative light unit</td>
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<td>Ribonucleic acid</td>
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<td>Revolution per minute</td>
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<td>R\text{a}</td>
<td>Mean-square-root of roughness</td>
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<td>Description</td>
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</tr>
<tr>
<td>RWV</td>
<td>Rotating wall vessel</td>
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<td>SAPH</td>
<td>Self-assembled peptide hydrogel</td>
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<td>Scanning electron microscopy</td>
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<td>SLRP</td>
<td>Small-leucine rich PGs</td>
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<tr>
<td>TCP</td>
<td>Tissue culture plastic</td>
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<td>TE</td>
<td>Tissue Engineering</td>
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<td>Transmission electron microscopy</td>
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<td>Transforming Growth Factor</td>
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<td>TLR</td>
<td>Toll-like receptors</td>
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<td>Thrombospondin-1-repeat</td>
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<td>Ultraviolet</td>
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Abstract

As the natural turnover level of cartilage and its ability to repair itself are both relatively slow, minor injuries or lesions may lead to progressive damage of cartilage. Unlike other types of tissues that can heal intrinsically, cartilage, being avascular, has a confined supply of nutrients. For the past 25 years, researchers have turned to tissue engineering (TE) in treating cartilage injury. Cells, matrix and signalling molecules are three essential ingredients in TE. An ideal matrix such as biomaterial scaffold would support the cells at the primitive stage of tissue development, in terms of promoting desired phenotype and regeneration of neo-tissues. Physiologically, ~80% of the extracellular matrix of healthy cartilage comprises of water. This makes hydrogels, which are composed of >90% water, the best scaffold candidate to support the cells during cartilage regeneration.

Self-assembling peptide hydrogels (SAPH) are designed by embracing the nature of self-assembly in protein folding. SAPHs are seen to have great potential in TE applications; as the properties of peptide fibrils can be controlled by changing peptide sequences, concentration and pH. Other advantages of using SAPH in TE are their biocompatibility, biodegradability, tunability for desired mechanical properties, injectability and they can be functionalised. The primary objective of this thesis was to develop fully chemically and physically defined peptide-based hydrogels that can be used as three dimensional (3D) scaffolds for TE applications.

In the present study, we have shown that these peptide hydrogels have different storage moduli depending on the concentration and the pH of the hydrogels. This thesis also discusses the effects of charge in peptide hydrogels on cell viability, proliferation and production of GAGs. It was seen that bovine and human chondrocytes were viable for the duration of the study (21 days). In addition, these charged peptide hydrogels, when cultured without chondrogenic media, were observed to inhibit the production of GAG and type II collagen. In addition, cells were also found to respond differentially to the dynamic mechanical properties of the peptide hydrogels.

Cell - material interaction has been shown to play a vital role in cartilage engineering. Different biomaterial properties significantly influence the initial cell attachment to the substrate, which is crucial for chondrogenesis. The interaction of chondrocytes with the SAPH has been explored in this project. We show the effects of using different peptide hydrogels on the attachment, proliferation and spreading of immortalised human chondrocytes. Cell adhesion rate on SAPH was comparable to those on collagen hydrogels. Arginine was seen to induce cells’ elongated morphology.

This thesis shows that modifying the peptide sequence changes the gelation and degradation behaviour of the SAPH, which affects the cells bio-response due to cells’ mechanosensitivity. The work also contributes to the knowledge of substituting phenylalanine with arginine to the pioneering peptide sequence, FEFKFEFK. The study led an inaugural investigation on the interaction between the cells and SAPH.
Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.
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Throughout my PhD, it was almost every other day that I would doubt myself. Hitherto, I kept holding on to the slightest faith I have, thanks to the daily dosage of embraces from my husband, Ashraf and my two children Alif and Amily. To my husband, nothing can replace your sacrifice to let go of your permanent job and our steady life back at home just to be here with me. To Alif and Amily, I could put in a long list of requests from the two of you that I failed to fulfill throughout these four years, but I just chose not to. Instead, I would like to dedicate this PhD thesis to both of you and remember - Always keep your chin up and aim HIGH.
The Author

ACADEMIC QUALIFICATIONS

MSc (Tissue Engineering), Department of Biomedical Engineering, University of Malaya, Kuala Lumpur, Malaysia, 2007-2011

BSc in Biomedical Engineering, Department of Biomedical Engineering, Case Western Reserve University, Ohio, United States of America, 2002-2006

WORKING EXPERIENCE

Trainee Lecturer/ Academic Trainee, Department of Biomedical Engineering, University of Malaya, Malaysia, 2007-2010

Research Assistant, Tissue Engineering Laboratory, UKM Medical Centre, 2006-2007

JOURNAL PUBLICATIONS


CONFERENCE PUBLICATIONS


16th Annual Meeting of the Tissue and Cell Engineering Society (TCES), University College London, 4-6 July 2016, European Cells and Materials Vol 32. Supplement 4, 2016 (p76)

26th European Conference on Biomaterials (ESB 2014), Liverpool, United Kingdom, ISBN: 9781634399531; Publisher: European Society of Biomaterials.


Tissue Engineering and Regenerative Medicine International Society (TERMIS) 2010 Asia Pacific Meeting 2010: Sydney, Australia
# ACHIEVEMENTS

<table>
<thead>
<tr>
<th>Achievement</th>
<th>Year</th>
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<tr>
<td>Voted as runner-up for a flash talk at the ManBioMat network showcase</td>
<td>2017</td>
</tr>
<tr>
<td>Contributed to designing Bioreactor for Cartilaginous Tissue, led by Assoc.</td>
<td>2009</td>
</tr>
<tr>
<td>Dr Belinda Murphy, which is patented (PI 2009 5885)</td>
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# ACTIVITIES

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<td>Student service assistant – assisted with the registration week</td>
<td>2017</td>
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<tr>
<td>Involved in outreach program – running an interactive stand for MIB Open Day</td>
<td>2014-2017</td>
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<tr>
<td>Outreach program – running an interactive stand for Science Uncovered Manchester European Researchers Night at Manchester Museum</td>
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CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1. PROBLEM STATEMENT

1.1.1. Elastic cartilage

Microtia is a congenital anomaly of the ear that can be classified into four categories; Grade I being all the ear structures are present, but the auricle is malformed, to Grade IV with complete absence of the external part of the ear, also known as anotia (Baluch et al. 2014).

The prevalence of microtia was reported to be 1 in 7,000 live births every year worldwide, and in the European Union (EU) itself, there are 2 cases of microtia in every 10,000 births (Luquetti et al. 2011). Even though this figure is considered relatively low compared to other pathological aspects, abnormal appearance of the ears has a negative effect on youths’ self-confidence, quality of life and physiological development (Luquetti et al. 2011, Steffen et al. 2012). Besides microtia, there are other needs of ear reconstructions which are caused by trauma, burns or cancer. These, on the other hand, occur in more than 1:500 of the whole population. Unlike microtia where treatment is given at the age of 10, in the latter cases, the treatment is more urgent.

Total ear reconstruction remains one of many great challenges to medical practice, due to the failure of mimicking native tissue in terms of its flexibility, elasticity and complex shape. The field was pioneered by Tanzer using four-stage reconstruction of costal cartilage (Tanzer 1978). However, treatment includes multiple surgeries; from harvesting costal cartilage from the thoracic cage, subcutaneous implantation of the hand-carved cartilage in the lower abdomen for a few weeks, extracting the cartilage and re-implantation of the cartilage at the ear site (Bichara et al. 2014). Nagata came up with a two-stage technique which requires fewer operations and is currently the standard treatment in the EU and the United States (US) (Luquetti et al. 2011). This technique, however, is only carried out when the child turns ten years of age and has a high occurrence of partial necrosis of the posterior flap (Firmin 1998, Nagata 2000).

Despite being the current approved standard treatment, this technique also has limitations such as donor site morbidity, calcification and different biomechanical properties of the fibrocartilage, loss of projection and definition and high technicality (Mori et al. 2002, Uppal et al. 2008a, Jessop et al. 2016).

In the motivation of having off-the-shelf options, reducing donor site morbidity and major life-threatening surgeries, there has been massive progress in tissue engineered auricular cartilage. Polymers such as polyethylene, silicon or polytetrafluoroethylene, that have been materials are stiff and not able to bend (Nimeskern et al. 2014). In other studies, the prosthesis evaluated
induced significant inflammation and some even extruded from the skin (Nayyer et al. 2012). The use of allogenic porous polyethylene (PE, Medpor®, Stryker, Kalmanzoo, MI, USA) has risen in popularity in the past few years. Research have been carried out to reduce the inflammation resulted by using Medpor® by covering the scaffold with fibrin gel or oxidising it to increase its surface hydrophilicity. However, this does not overcome its limitation of being a non-biodegradable, rigid implant and causing skin erosion (Lee et al. 2011, Ruszymah et al. 2011, Bichara et al. 2012). Even though these polymers are designed to entirely replace the auricular cartilage that serves as alternatives to costal cartilage, these polymers do not fulfil the criteria of a real ear. Having an ear with not only the right look but also the feel of a real ear is one of the characteristics envisioned by patients who suffer the loss of auricle (Jacobs 2013).

Also, high biocompatibility, long-term stability, immunocompatibility, ability to grow and interact with host tissue made the autologous option as the current gold standard. Regenerative medicine has been explored widely in the past few years and is seen to have high potential in treating ear deformation. In regenerative medicine, the range of cell sources is wide; auricular cartilage and stem cells derived from bone marrow and adipose tissue (Jessop et al. 2016). Recently, the efficacy of chondroprogenitors found in the perichondral layer of auricular cartilage was studied and gave promising results (Kobayashi et al. 2011, Yanaga et al. 2012). When stem cells or articular cartilage were used, chemical and physical stimulation could be utilised to direct the new extracellular matrix (ECM) production leading to the formation of cartilage tissue that matches the function and elasticity of intrinsic native auricular cartilage. Besides, a wide variety of injectable hydrogel can serve as a better option due to the complex three-dimensional (3D) anatomical shape of the ear. The application of a suitable biodegradable injectable hydrogel in combination with 3D graphics and bio-printing may be the answer to regenerating refined customised implants for ear cartilage.

1.1.2. Articular cartilage

Osteoarthritis (OA) is a critical condition that can affect any joint in the human body - wrist, finger joints, hips, knees and neck. However, for discussion purposes, knee joint degenerative disease will be referred to throughout the thesis. Besides, because the knees are the most weight-bearing joint, the cases reported for knees are higher than other joints.

According to a report by the World Health Organisation (WHO) in 2010, nearly 50% of adults aged more than 65 are affected by osteoarthritis. This figure will continuously increase and it is projected that by 2050, 130 million individuals in the whole worldwide population will be suffering from this disease. The statistic is growing due to the increasing age of the society and epidemic of obesity (Allen and Golightly 2015, Willis 2015).

Cartilage, as a tissue, presents some unique challenges for orthopaedic medicine. Even minor injuries or lesions lead to progressive damage of cartilage, due to biomechanical stresses and in some cases worsen by metabolic or genetic defects (Fellows et al. 2016). Cartilage contains mainly mature chondrocytes which have slow intrinsic ability to repair itself. Its low repair
capacity also results from the cartilage being avascular, thus it has limited access to nutrients. Cartilage tissue depends on the supply of nutrients that diffuse through the matrix (Sophia Fox et al. 2009).

Currently, there are a number of drugs and OA treatment clinically available. Non-steroidal anti-inflammatory drugs (NSAIDs) are used to reduce the inflammation at the affected joints. Corticosteroids are being used as a painkiller, however, a study has shown that a high dose of corticosteroids may accelerate OA (McAlindon et al. 2017). Some patients are offered viscosupplementation (hyaluronic acid (HA) injection) and autologous platelet-rich plasma (PRP) (Jevsevar et al. 2015, Huang et al. 2018a). PRP, which contains platelets, growth factors, proteases and cytokines helps to reduce inflammation in osteoarthritic joints (Huang et al. 2018a).

Some other cases require surgical procedures such as microfracture and mosaicplasty. Microfracture, such as abrasion or drilling, is purposely done to the affected cartilage to allow the access of nutrients from the underlying bone marrow. This treatment is rather temporary due to the newly produced cartilage tending to become fibrocartilage (Mithoefer et al. 2009). Mosaicplasty is another surgical method widely applied. However, it is inferior due to donor site morbidity (Torrie et al. 2015). In mosaicplasty, the defective area of cartilage is replaced with a cylindrical cartilaginous plug obtained from the non-weight bearing area.

In the past two decades, researchers have been looking at the potency of autologous chondrocyte implantation (ACI). Recently, the autologous expanded chondrocytes were injected at the defect area and covered with a biodegradable collagen membrane instead of the periosteal flap (Brittberg et al. 2018). The cost incurred for ACI is high, and cells tend to dedifferentiate during expansion and lead to fibrocartilage formation. Thus, researchers turned to tissue engineered cartilage utilising a 3D scaffold.

As reviewed by Duval et al. (2017), in comparison of two-dimensional (2D) and 3D, cells cultured in 3D method have the benefit of cell interaction mimicking the cell physiologically, in terms of their morphology, motion and migration (Liu et al. 2018b). This leads to correct phenotypic gene expression, production of ECM and mechanical response. Conventional 2D cell culture uses flat surfaces such as a petri dish to culture the cells. Later on, researchers have used pseudo-3D such as placing the cells in between two layers of hydrogel (sandwich), creating microtopography (micropatterning) and designing surfaces with specific substrate stiffness (Duval et al. 2017).

Three-dimensional cell culture started when Benya and Shaffer (1982) found that the morphology of dedifferentiated chondrocytes was spherical and the cells re-expressed cartilaginous markers when cultured in 3D agarose hydrogel. Besides agarose, chondrocytes redifferentiation is also carried out using the hanging drop culture method of alginate beads in calcium chloride (CaCl₂), and free-scaffold 3D culture pellet obtained through centrifugation and cultured on an uncoated well plate (Caron et al. 2012).
Three main components of TE are cells, matrix and signalling molecules (O'Brien 2011, Kock et al. 2012, Hassanzadeh et al. 2018). Cells are the biological sources of elements for the extracellular matrix (ECM) (Muir 1995). Tissue engineering approaches are initiated by using large numbers of cells from tissues. By far, chondrocytes are the best cellular candidates for cartilage TE. The potential availability of healthy autologous cells that can be implanted in the impaired regions is insufficient and thus forces researchers to turn to expanding the cells to obtain a sufficient number. Initial problems faced in getting adequate chondrocytes are solved by growing cells in vitro. However, chondrocytes prolonged expansion has been reported to lead to chondrocytes losing their phenotype (Lin et al. 2008, Mounts et al. 2012). Besides cell dedifferentiation, there are also concerns related to donor site morbidity and the limited lifespan of cells once extracted (Uppal et al. 2008b). These drawbacks have motivated research into the possibility of using mesenchymal stem cells (MSCs) or mesenchymal progenitor cells (MPCs) for such applications (Kobayashi et al. 2011, Shi et al. 2017). Human bone marrow stem cells (hBMSC) is one of the first stem cells being studied. It was found that with expansion, its differentiation capacity reduced, and upon culturing, hBMSC led to calcified cartilage (Narcisi et al. 2015). Unlike BMSCs whose extraction requires painful procedures and leads to donor site morbidity, adipose-derived stem cells (ADSC) are abundantly available, can be easily isolated and produce chondrogenic markers (Mellor et al. 2015, Bielli et al. 2016). Mesenchymal stem cells can also be sourced from dental pulp and synovium (Fellows et al. 2016).

Signalling molecules have a role in regulating cell functioning, growth and remodelling (Kock et al. 2012, Green et al. 2015). Studies have shown that mechanical stimuli are capable of initiating biochemical signalling (Shiu 2007, Abbott and Kaplan 2015, Anderson and Johnstone 2017). Signalling cascades to stimulate chondrogenesis within auricular/articular engineering can also be triggered by growth factors (GFs). Insulin-like growth factors (IGFs), fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) are some of the growth factors used in tissue engineering (Huang et al. 2016, Yang et al. 2017b). Initially, GFs were injected directly into the tissues. However, this method is unfavourable since GFs have very short half-lives in vivo (Farach-Carson et al. 2007). Encapsulation of GFs into scaffolds results in stabilisation of GFs in the matrix, and controlled release of GFs into the cell microenvironment is possible. Encapsulation can either be done via physical mixing of the GFs in the hydrogels, covalent binding of the GFs to the hydrogels or microspheres (Yang et al. 2017b).

Besides cells and signalling molecules, a scaffold that works as a temporary matrix is also essential. Cell-seeded scaffolds act as structural supports for cells at the primitive stage of tissue development, both in terms of promoting desired phenotype and regeneration of cartilage. Cells attach to the matrix of the scaffold temporarily, and the matrix eventually degrades to provide more space for the newly synthesised ECM.
For the purpose of this project, a study to assess the possibility of using peptide hydrogel was carried out. A hydrogel is a hydrophilic polymer with the majority (> 90%) of the hydrogel volume contents being water (Li et al. 2014b). Having high water content, hydrogels have been shown to have high potential in biomedical applications due to their similarity to native ECM. Being microporous, hydrogels are capable of containing growth factors and drug molecules, making them very promising in the drug delivery field (Li et al. 2014b). Hydrogels have risen in popularity for use in tissue engineering and regenerative medicine, due to hydrogels being biodegradable, biocompatible and injectable in the liquid phase (Raghunath et al. 2007, Bidarra et al. 2014, Guan et al. 2017).

1.2. STRUCTURE-FUNCTION RELATIONSHIP IN ELASTIC CARTILAGE

The properties of elastic cartilage (EC) are a result of high concentrations of the elastin fibres, which are intertwined by the collagen type II fibres (Porth 2007, Nabzdyk et al. 2009). Besides elastin and collagen fibres, the matrix in EC also contains lipids, proteoglycans (PGs) and mainly water (Mahmoud Abdalla 2018). Histology staining shows chondrocytes are embedded in between elastin fibres. The scanning electron microscopy (SEM) images show that the chondrocytes fill small lacunae spaces within the matrix (Mahmoud Abdalla 2018). Chondrocytes in EC are denser than in articular cartilage, in similar polygonal morphology and evenly distributed throughout the ECM (see Figure 1-1) (Nabzdyk et al. 2009, Griffin et al. 2016).

![Schematic figure of elastic cartilage](image)

**Figure 1-1 Schematic figure of elastic cartilage.**
The ECM is surrounded by perichondrium, with vertical rows of immature chondroblast. Single mature chondrocytes can be seen between elastin fibres. Single chondrocyte can be seen in each lacuna.

The EC matrix is surrounded by the perichondrium. Perichondrium is a source of nutrient for the chondrocytes. Being avascular, EC depends on the nutrient which is delivered through diffusion. Within the perichondrium, lie vertical rows of chondroblast. Chondroblast is an immature version of chondrocytes and are relatively small and flat in morphology. Chondroblast is also known as perichondrial cell or mesenchymal progenitor cell (Mahmoud Abdalla 2018).

Elastic cartilage can be found at the ear pinna (auricle). The ear pinna plays a major role in hearing physiology due to the biomechanical properties of the cartilage. The ridges and concave enable the ear to localise the direction of the sound (Mahmoud Abdalla 2018). The
concave and ridges contribute the complexity of the shape of the ear cartilage, the thickness of the ear cartilage is significantly thinner at the helix region compared to other regions. The Young’s elastic modulus for all areas in the EC was insignificantly different, except for those measured at the concha region, which is significantly higher than the helix region (Griffin et al. 2016).

1.3. STRUCTURE-FUNCTION RELATIONSHIP IN ARTICULAR CARTILAGE

Articular cartilage (ART) can be found at the articulating joints of the hip, elbow, knee, shoulder, finger joint and wrist. Besides water, which accounted for 90% of the total wet weight of the ART matrix, proteoglycans and collagens are the main components in the matrix (Sophia Fox et al. 2009). These components were structured in an organised and sophisticated manner, of which the interaction between these components contributes toward the mechanical properties of the tissue. At the joints, ART works like a sponge that absorbs the compressive loading and distributes the weight throughout the matrix. Another function of ART can also be viewed as a protector to the gliding joint by reducing its frictional coefficient (Armiento et al. 2018).

Anisotropic ART is structured as four zones; superficial, middle, deep and calcified zones. As seen in Figure 1-2, at the top of the ART, closest to the synovial fluid, is the superficial zone. The superficial zone constitutes 10-20% of the full thickness. In this zone, the chondrocytes are condensed and flattened. The collagen fibres aligned parallel to the articular surface, which contributes to the ART resistance to the sheer and tensile forces experienced on the surface. The superficial zone contains the least amount of proteoglycans (PGs) compared to other zones (Malda et al. 2012).

The middle zone is also known as the transitional zone as the collagen fibres are arranged in oblique organisation. The collagen fibres are slightly thicker, and the spherical chondrocytes are sporadically distributed within this zone. The middle zone takes up 40-60% of the total thickness, while the deep zone is usually 30% of the thickness. The deep zone contains the highest concentration of PGs. Load bearing capability of ART is mainly due to the vertical arrangement of the thick collagen fibres in this zone. Chondrocytes within the deep zone are relatively larger and arranged in a columnar orientation in between the collagen fibres. Deep zone and calcified zone are attached via the collagen fibres. In between deep and calcified zone lies the tidemark. Tidemark is a thin basophilic layer that functions as a boundary between the mineralised and non-mineralised zones (Sophia Fox et al. 2009, Armiento et al. 2018).
1.4. **EXTRACELLULAR MATRIX COMPONENTS**

Extracellular matrix (ECM) is composed of collagen (primarily collagen type II) and elastin fibres, proteoglycans and adhesive glycoproteins such as fibronectin and laminin. ECM’s components work synergistically to provide mechanical support and regulate intercellular communication which is essential for cells’ normal function - migration, proliferation and phenotypic expression.

1.4.1. Collagen

Type II collagen constitutes 90-95% of the total collagen in cartilage ECM. Besides type II, there are also collagens type I, V, VI, IX, X, and XI in cartilage ECM (Sophia Fox *et al.* 2009, Mienaltowski and Birk 2014). Collagen is the most abundant structural protein component in cartilage ECM. Collagen makes up one-third of the total weight of human cartilage (Mankin *et al.* 1994). The mechanical property of the cartilage is highly dependent on the ratio of types of collagen (Pappa *et al.* 2014).

Collagens can be categorised according to their higher order structures. There are fibrillar-forming collagens (Type I, II, V and XI), sheet-forming collagens (Type IV, VIII and X) and anchoring collagens (Type VI, VII, XII and XIV) (Pollard *et al.* 2017). The fibril-forming collagens are secreted as procollagens. These procollagens contain C-terminal and N-terminal propeptide. The basic structural unit of collagen is a triple helix (see Figure 1-3). Three left-handed coiled precursor polypeptide α-chains, consequent to the huge quantities of proline and glycine, twist together to form a triple helix that coils right-handedly. The triple helix is stabilised by many hydrogen bonds. The amino acid sequences of the triple helix collagen are generally Gly-X-Pro and Gly-X-Hyp. The third residue of the triple helices is glycine. The steric hindrance requires that glycine be the third residue. The interior axis of the helix can only fit the single functional group of glycine; the hydrogen atom (Mankin *et al.* 1994).
The triple helix of α-chains undergoes modification before being secreted from the endoplasmic reticulum (ER). Selected proline and lysine amino acids are subjected to hydroxylation followed by glycosylation. Hydroxyproline is essential for constituting a helical structure, while the process of adding galactose and glucose to the hydroxyllysine determines the size of the collagen fibrils (Stockwell 1979).

Post-translational modification then takes place extracellularly, where crosslinking of enzymes initiates collagen molecules to self-assemble into D-staggered-arrays with unique patterns of bands repeated every 67 nm (see Figure 1-3) (Buckwalter et al. 1987).

![Figure 1-3](image)

**Figure 1-3** The synthesis of triple helix tropocollagen and collagen type I fibril. The precursor α chain coils in a left-handed manner forming a triple helix. These propeptides later cleaved together to form collagen fibrils. The distance between the “head” (↑) and the “tail” (•) of the next collagen molecule is constantly about 67nm. This constant value is important for the collagen fibrils to produce a structured formation called “staggered-array”. The interactions between parallel groups are moderated by covalent bonds. (Adapted from Lodish et al. (2000)).

1.4.2. Elastin

Elastin, which can be found in arteries, lung, skin, vocal chords and elastic cartilage, is composed of elastic fibres (90%) and microfibrils (Mithieux and Weiss 2005). The structure of elastin varies depending on the host tissue as the structure serves its functions. In elastic cartilage, elastic fibres are organised into a large 3D honeycomb configuration intercalating with collagen network (Mithieux and Weiss 2005, Nimeskern et al. 2016). In cartilage, the function of elastin is towards its elasticity and elastin fibres also work together with collagen fibres to withstand minimal load (He et al. 2013). The primary role of elastin in EC, however, is more into the flexibility of the EC, to retain the shape of EC and to hasten vestigial musculature connecting the ear to the head (Nimeskern et al. 2016). The diameter of elastin fibres (~μm) was also reported to be significantly larger than collagen fibrils (~nm) (Nimeskern et al. 2016).

Microfibrils can be found parallel to the long axis of the developing elastin fibre, next to the cells (Mithieux and Weiss 2005). Microfibrils are found to bind to versican, biglycan and decorin (Mithieux and Weiss 2005). Tropoelastin is the structural unit of elastin which aggregates together and binds through lysine-mediated crosslinking (Mithieux and Weiss 2005). Tropoelastin is composed of mainly non-polar amino acids such as glycine (G), valine (V),
proline (P) and leucine (L). These amino acids are arranged in an alternating hydrophobic and hydrophilic manner, where 82% of the amino acids are hydrophobic and provide the flexibility of elastin (elastin can extend up to eight times its resting length) (Wise et al. 2014). Proline offers rigidity, while glycine provides flexibility. When hydrated, these hydrophobic amino acids self-assemble into β-sheet/β-turn secondary structures (Wise et al. 2014). Elastin, however, does not have a stable, distinct tertiary structure. In an aqueous environment, elastin favours random coil due to its hydrophobic region. Upon stretching, elastin fibres tend to be long, straight and organised (Green et al. 2014). When not in stretched condition, the elastin fibres will be in high entropy state with low energy, turning into the disordered structure (Pollard et al. 2017).

Even though Yu and Urban (2010) reported that elastin fibres have a specific arrangement, concentration and fibre thickness throughout the depth of ART, a more recent study by Nimeskern et al. (2016) has shown that histological assessment shows no elastin is present in ART. Green et al. (2014) reported that researchers always overlooked the elastin fibres when imaging using light microscopy. In their study, an image of ART using two-photon excitation microscopy revealed the mesh network of elastin fibres in articular cartilage of the equine metacarpal-palmar-langeal joint. Pappa et al. (2014) found that microtic EC contains a reduced amount of elastin and Nimeskern et al. (2016) carried out a study to selectively deplete elastin and found that the compressive modulus of EC significantly reduced in result to the lack of elastin expression.

Besides accommodating the flexibility of the tissue, elastin receptors bind to αvβ3 integrins via the C-terminal of tropoelastin and interact with elastin binding proteins (EBP) via a hydrophobic sequence of VGVAPG (Yeo et al. 2015).

1.4.3. Proteoglycans

Proteoglycans (PGs) consist of one or more glycosaminoglycan chains which are covalently attached to the protein core (see Figure 1-4) (Bertrand and Held 2017). In healthy cartilage, PGs make up 25-35% of the cartilage dry weight (Buckwalter et al. 2005). PGs’ role in cartilage is not limited only to bind water; they are also found to be capable of binding to cytokines, chemokines, growth factors and morphogens (Bertrand and Held 2017). PGs are observed to modulate signalling pathways. Recently, studies have found that transmembrane PGs function as receptors that facilitate cell adhesion, cell-cell interactions and cell motility (Bertrand and Held 2017). PGs can be categorised according to their size; large-sized PGs (e.g., aggrecan, versican, agrin and perlecain) and small leucine-rich PGs (SLRPs, e.g., decorin, biglycan, asporin, fibromodulin and lumican). In large-sized PGs, each side chain can have more than 100 monosaccharides.
Aggrecan is the largest proteoglycan in size measuring 200kDa (Vynios 2014). The large molecular size of aggrecan is contributed by the number of GAGs attached to the core protein (Zhang et al. 2010). Aggrecan has three disulphide-bonded globular domains named as GD1, GD2 and GD3. The aggregation of aggrecans has become possible due to the GD1 domain of the core protein affixed to hyaluronan (HA) via link protein (see Figure 1-5) (Buckwalter et al. 1987, Sophia Fox et al. 2009). The region between GD1 and GD2 is known as the interglobulin domain (IGD) and this region is susceptible to proteases.

The space between GD2 and GD3 is filled by highly sulphated, linear glycosaminoglycans; keratan sulphate (KS) and chondroitin sulphate (CS). The building blocks of KS are repeating disaccharides of galactose and N-acetyl-glucosamine, and glucuronic acid and N-acetyl-galactosamine for CS (Zhang et al. 2010, Caterson 2012). As shown in Figure 1-5, the protein core in the middle of the PG attracted the GAG chains forming a bottlebrush-like structure and surrounded by the collagen network (Chandran and Horkay 2012).
Being highly sulphated, aggrecans are full of negative charges at pH 7 (Chandran and Horkay 2012). Negative charges create repulsion between aggrecans which provide osmotic force. This massive structure and charges trap water molecules and the PG-water structure contributes toward load-bearing properties of cartilage (Knudson and Knudson 2001, Roughley and Mort 2014). The interaction between the side chains forms a semi-rigid assembly that is strong enough to induce high hydrodynamic friction during compressive loading, and is permeable for nutrients to diffuse through (Chandran and Horkay 2012).

In the disease state, the length of the aggrecans is truncated, reducing the negative charge and ability to retain water. Reports have shown that the GD3 region is absent in aggrecans upon being attacked by the metalloproteinase matrix proteins (MMPs) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) (Troebberg and Nagase 2012). Besides proteases and hyaluronidase, Cloos and Christgau (2002) found that non-enzymatic glycation modified the lysine residues at GD1 region which not only dissociate aggregation but also prevent aggregation.

Besides aggrecan, versican is another large-sized PGs found in cartilage. Versican can be found in a number of structures, but the principal structure of versican has G1 domain at the N-terminal and G3 domain at the C-terminal. G1 domain can attach to hyaluronan and has a role in promoting cell proliferation, migration and adhesion (Wight et al. 2014). Wu et al. (2002) found that the C-terminal of versican promotes cell adhesion via β1 integrin, tenascin and fibronectin. The middle region has CS that binds to the core protein through CD44 (Bertrand and Held 2017).

The building units of both SLRP decorin and biglycan are repeating disaccharides of dermatan sulphate and chondroitin sulphate (Roughley and Mort 2014). The repeating disaccharide units of dermatan sulphate (DS) consist of a hexosamine N-acetylated glucosamine and iduronic acid (Caterson 2012). Biglycan interacted with collagen fibril and elastin and was found to trigger pro-inflammatory signalling (Nastase et al. 2012). Both biglycan and decorin can bind to TGF-β and BMPs and cleaved by MMP-2, 3 and 13 (Nastase et al. 2012, Neill et al. 2012).

1.4.4. Adhesive glycoproteins

Glycoproteins in cartilage ECM function as a ‘glue’ in cell-cell and cell-matrix interactions. These glycoproteins are fibronectin and laminin. In cell communication complex, cell surface adhesion receptors such as integrins, CD-44, annexin V and intercellular adhesion molecule-1, ‘complete’ the signalling circuit and activated crucial pathways in the cells’ metabolism process.

1.4.4.1. Fibronectin

The word fibronectin comes from a combination of Latin words fibra (fibre) and nectare (to bind) (Xu and Mosher 2011). As suggested by its name, upon activation through cell surface interaction, fibronectin organised into fibrillar network adjacent to the cell surface (Xu and Mosher 2011, Almonte-Becerril et al. 2018). A work by Hynes and colleagues (1993) showed
that inactivation of the fibronectin gene resulted in lethal abnormalities in the neural and vascular development of mouse embryo. In each fibronectin subunit, there are three distinct functional domains, type I, II and III, each of which contains repeating homologous peptide sequences (Xu and Mosher 2011, Almonte-Becerril et al. 2018). Two of these subunits form a dimeric protein linked at the C-terminal ends via a pair of disulphide bonds (Bradshaw and Smith 2014). Type I and II domains have disulphide bonds.

Fibronectin has binding domains that recognise collagen and heparan sulphate proteoglycans. Each fibronectin can interact with multiple receptors through its variant binding domains (Grigoriou et al. 2017). It also contains binding sites to syndecans and arginine-glycine-aspartate (RGD) motifs that bind to α5β1 integrins (Almonte-Becerril et al. 2018). Upon interacting with integrin, the fibronectin-integrin complex will recruit more integrins thus attract more fibronectin, even through the fibronectin-fibronectin binding which later forms fibrillar fibronectin. These fibril formations are also found to be directional depending on the location of the focal contacts and to which protein the cytoplasmic integrin binds with (Singh et al. 2010). Fibronectin has the primary role in modulating cell adhesion, migration, growth and differentiation. Besides these, fibronectin is also found to function as a mechano-sensing mechanism (Carraher and Schwarzbauer 2013, Seong et al. 2013).

1.4.4.2. Laminin

Laminin is viewed as T-shape with two or three short arms which have N-termini, and one long arm which constitutes portions of all arms. Laminin is made of isoform consisting of three chains α, β and γ (see Figure 1-6) (Halper and Kjaer 2014). There are five α, three β and three γ chains, combinations of which could make up to 15 heterotrimeric laminin isoforms (Kaur and Reinhardt 2015). This relatively large protein (400-900kDa) has multiple genes on each subunit, offering binding sites to integrins at the N-terminal globular domains of α chains, and to collagen/heparan sulphate at the β and γ chains (Xu and Mosher 2011, Halper and Kjaer 2014).

A recent study by Foldager et al. (2016) has found depletion of laminin expression in degenerated articular cartilage, and Sun et al. (2017) reported that laminin is expressed in a spatiotemporal manner and can be detected mainly in the pericellular matrix region of adult hyaline cartilage.
Laminin is built by three domains $\alpha$, $\beta$ and $\gamma$. Each chain has different globular domains which provide multiple binding sites to integrins.

1.4.5. Integrin

Integrin works as an adhesive between the cells and the ECM. Integrin, a heterodimeric glycoprotein receptor, is made up of two subunits, $\alpha$ and $\beta$ (see Figure 1-7). After years of study, researchers have found there are 18 types of $\alpha$ and eight types of $\beta$ subunits in integrins (Hynes 2002, Srichai and Zent 2010, Aiyelabegan and Sadrooddiny 2017). These subunits make up to 24 distinct combinations of integrins (see Table 1-1 for some of the combinations that were detected in cartilage) (Srichai and Zent 2010, Loeser 2014). Loeser (2014) reported that some of these integrin dimers could only be detected in osteoarthritic cartilage.

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**Figure 1-6 Structure of laminin.**

Laminin is built by three domains $\alpha$, $\beta$ and $\gamma$. Each chain has different globular domains which provide multiple binding sites to integrins.

---

**Figure 1-7 Schematic drawing of integrin.**

Every $\alpha$ and $\beta$ subunit has a large extracellular domain and a short cytoplasmic tail, bridge by a transmembrane domain. 'Opened' and activated vinculin interacts with talin and actin and sends information to the intracellular part of the cell.
The head domain of the integrin heterodimer binds to the ECM protein, while the tail domain attached to other cytoskeletal domain such as paxillin and vinculin (Loeser 2014). At the intracellular level, the 116kDa vinculin was found to function as an adherence junction. The N-terminal of vinculin binds to talin, which then binds to the cytoplasmic tail of β integrin and its C-terminal binds to the actin and paxillin (Koshimizu et al. 2012). According to a report by Kuhne et al. (2010), there is no significant difference in the level of gene expression of β1 integrin in elastic and articular cartilage. The report also stated, however, vinculin expression is more pronounced in articular than in elastic cartilage. At the ECM side of the membrane, integrin binds to ECM proteins such as laminin, collagens and fibronectin (Srichai and Zent 2010).

<table>
<thead>
<tr>
<th>α subunit</th>
<th>β subunit</th>
<th>Receptors for</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>β1</td>
<td>Collagen</td>
</tr>
<tr>
<td>α2#</td>
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<td>α10</td>
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<tr>
<td>α3</td>
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<tr>
<td>α5</td>
<td></td>
<td>RGD</td>
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<tr>
<td>α8</td>
<td></td>
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<tr>
<td>α4#</td>
<td>β1, β3, β5</td>
<td></td>
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<tr>
<td>αV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α6#</td>
<td>β1</td>
<td>Laminin</td>
</tr>
</tbody>
</table>

Table 1-1 Combination of heterodimers integrin found in cartilage ECM.

Even though some integrins are quite specific, researchers have found that there are redundancies in the ligand which can be recognised by multiple integrin heterodimers. Integrins labelled with (#) were detected in OA cartilage. The table is generated based on reviews by Srichai and Zent (2010), Loeser (2014) and Halper and Kjaer (2014).

As stated earlier in Section 1.4.4.1, fibronectin has arginine-glycine-aspartate (RGD) sequence at the FNIII10 domain which is recognised by integrins (mainly α5β1 integrin) (Aiyelabegan and Sadroddiny 2017, Almonte-Becerril et al. 2018). The chronology of integrin-fibronectin interaction starts with the recognition of the RGD sequence by the integrin. Upon activation, the cytoplasmic tail of integrin subunits are separated from each other when the tail of β integrin binds to talin, vinculin and focal adhesion kinase (FAK) (Liu et al. 2015). The interaction between actin and talin/vinculin will initiate the arrangement of actin, which is believed to be indirectly responsible for the cell morphology and phenotypic expression (Kaur and Reinhardt 2015). The affinity of the binding domain is enhanced upon activation of the integrin, resulting in the recruitment of multiple integrin receptors. This clustering of integrins induces assembly of multiple fibronectins, which later forms fibrillar structures (Singh et al. 2010). The molecular assemblies involving a physical connection between ECM components and actin cytoskeleton via integrin receptors is termed as focal adhesion (FA) (Song and Park 2014). The anchoring and stabilisation of cells to the ECM protein is made possible by this focal adhesion.
Integrin also has a role in cell migration. Integrins from the rear of the cell are recycled to the front of the cell (Aiyelabegan and Sadroddiny 2017). Work by Seong et al. (2013) and Carraher and Schwarzbauer (2013) has shown that the activation of FA is rigidity-dependent. This suggests that integrin is a part of the cells’ mechano-sensing mechanism. Besides having a role in cell adhesion, migration and mechanotransduction, integrin is also pivotal in cartilage’s metabolic process as it mediates cell signalling mechanisms such as growth factors and cytokine production in the cartilage. In a report by Du et al. (2016), the integrin involved in the focal adhesion kinase which later activates the β-catenin/Wnt pathway. Pathologically, integrins are also capable of binding to the RGD domain at the fragmented fibronectin (FN-f). This binding is found to increase the production of pro-inflammation cytokines which leads to the production of catabolic proteins such as MMPs and ADAMTS (Ding et al. 2008, Loeser 2014).

1.4.6. MMPs and ADAMTS

Cartilage undergoes continuous remodelling which involves anabolic and catabolic activities. In healthy cartilage, these anabolic and catabolic processes are discretely balanced to support tissue turnovers (Li and Xu 2015). Matrix metalloproteinases (MMP), a disintegrin and metalloproteinases (ADAM) and ADAM with thrombospondin motifs (ADAMTS) are the proteinases found in cartilage ECM. Matrix metalloproteinase (MMP) is a zinc-dependent endopeptidase that is responsible for degrading ECM components. In terms of its structure, MMP consists of several domains, namely predomain, propeptide domain, N-terminal catalytic domain and C-terminal domain with hemopexin-like repeats (Jabłońska-Trypuć et al. 2016). Catalytic and hemopexin domains are connected via a connector known as a hinge. The propeptide domain contains a cysteine switch in the PRCGXPD sequence (Shiomi et al. 2010). Catalysis of the propeptide domain will activate these enzymes. There are 23 known types of MMP in the human body (Shiomi et al. 2010). MMPs can be classified into a sub-category of either collagenases, stromelysins, gelatinases, matrilysins and other, based on their domain organisation, sequence similarities and specificity to the substrate (Jabłońska-Trypuć et al. 2016).

According to Rose and Kooyman (2016), in normal conditions, cartilage has MMP-1/2/13 and -14 at a low concentration. However, these enzymes, along with MMP-3, MMP-8 and MMP-9, are over-expressed in disease state resulting to loss of the balance of remodelling, thus leading to osteoarthritis and rheumatoid arthritis (Rose and Kooyman 2016). As stated by Yang et al. (2017a) and Haslauer et al. (2013), besides these MMPs, ADAMTS-4 and ADAMTS-5 were also upregulated in anterior cruciate ligament (ACL) injury. Obesity and joint injury causes excessive mechanical stress at the joints. In some cases, it would lead to osteoarthritis when other factors such as genetic, age, ethnic background, nutrient deficiency and gender weigh in (Findlay and Atkins 2014).
Each ADAMTS has a unique structure. However, they are built by similar domains namely propeptide domain, catalytic domain, disintegrin-like domain, thrombospondin type 1 repeat (TSR) and cysteine-rich and spacer domain (Shiomi et al. 2010). All ADAMTS and some of the types of MMPs have a consensus sequence (R/KX,R/K↓R) (where n = 0, 2, 4 or 6 and X is any amino acid, ↓ is the cleavage site), which is recognisable by furin (Kelwick et al. 2015). Once the protease convertase, furin cleave at this site, the enzyme is activated (Stanton et al. 2011).

1.5. BIOMATERIAL SCAFFOLDS

At the earliest stages of tissue development, cells attach to the matrix, and the matrix eventually degrades to provide more space for the newly synthesised ECM (Nayyer et al. 2012, Dhote et al. 2013).

In cartilage tissue engineering, cells cultured on 2D culture plates tend to change phenotype, and the cells have been shown to exhibit more fibroblastic characteristics (An et al. 2001, Vinatier et al. 2009). In 2D, cartilage cells tend to express collagen types I and III, instead of cartilage-specific collagen (collagen type II) (Goldring et al. 1986, Saadeh et al. 1999). Thus the requirements to use a three-dimensional scaffold arises.

1.5.1. Requirements for biomaterial scaffolds for cartilage regeneration

Generally, a biomaterial scaffold must be non-toxic and biocompatible. It should not induce inflammation or immunoreaction (dos Santos et al. 2017, Iulian et al. 2018). This includes the by-products of the chemical reaction and biodegradation, which must be non-toxic and able to exit the body without disturbing other metabolic processes of the body (O’Brien 2011). The scaffold also must facilitate the diffusion of nutrients and metabolites (Liu et al. 2017). This has been achieved by having a porous scaffold. Interconnected pores within scaffolds allow cellular penetration and provide a high surface-to-volume ratio (Dhandayuthapani et al. 2011). A high density of pores also contributes to diffusion of nutrients to cells and of waste products out of the scaffold. Dey et al. (2018) investigated the effect of increasing porosity. In their work, it was found osteoblast (SaOS-2) adhesion and proliferation significantly increased with increasing porosity of the scaffold. Having porosity, however, compromises the scaffold’s mechanical properties (Kim et al. 2011).

The most prominent challenge in articular cartilage TE is to engineer weight-bearing tissue with multiphasic cellular architecture. In ART regeneration, the scaffold must be capable of withstanding compressive load and strain from the cyclic load. In EC regeneration, even though the scaffold does not need to bear weight, it has to sustain the strain from the skin constriction (Otto et al. 2015). Ideally, the scaffolds should have mechanical properties that mimic the anatomical site (O’Brien 2011). Matching the elasticity of the matrix in the native tissue will assist the cell orientation and phenotypic expression (dos Santos et al. 2017, Guan et al. 2017).

Researchers recognised another challenge as the scaffold’s stiffness is considered as a dynamic property depending on its degradation kinetic. Degradation of the scaffold is required
for the cells to undergo spreading, migration, proliferation and differentiation (Khetan et al. 2013, Trappmann et al. 2017). In ART regeneration, for example, the scaffold should be able to support the loads, gradually degrading to provide space for the newly formed ECM matrix, and later transfer the load to the new tissue (Pascual-Garrido et al. 2018). Scaffold degradation can be hydrolytic, enzyme-mediated or dissolution. The presence of cells and other enzymes produced by encapsulated cells and native tissue will affect the degradation kinetics of the scaffold (Nicodemus and Bryant 2008). A superior scaffold not only degrades after in vitro and in vivo implantation, but its degradation rate must also match the newly produced tissue (Dhandayuthapani et al. 2011). Two separate studies (among others) by Sarem et al. (2018) and Feng et al. (2014) have shown that cells morphology, aggrecan and type II collagen expression of chondrocytes and MSCs were modulated by the degradation rate of the scaffold. Rapid degradation reduced the retention of ECM and would impede cell remodelling.

One of the essential characteristics of a scaffold is providing adherence sites for the cells and integrating with the native tissue. Studies have been carried out by researchers to increase cells integration with the scaffold through physical and chemical modifications. When designing the biomaterial scaffold, researchers have to keep in mind the scaffold’s hydrophobicity, charges, surface chemistry and surface roughness that determine protein adsorption and cellular affinity (dos Santos et al. 2017, Liu et al. 2018b).

As stated in the previous section, due to the requirement of dimensional aspect of the cell culture system, 3D culture is highly favoured. This is due to the advantages of 3D cell culture which provides cell-cell and cell-matrix interactions that mimic the physiological environment, thus helps to maintain the cell phenotype (Liu et al. 2018b). According to Duval et al. (2017), 3D scaffolds can be classified into i) scaffold-free 3D spheroids (i.e. microfluidics and hanging drop cultures), ii) biopolymer scaffolds such as hyaluronan, gelatine, collagen, alginate and chitosan, iii) prefabricated scaffolds utilising polymers in 3D printing and stereolithography, and iv) hydrogels, which can be sourced naturally and synthetically.

Ticking the boxes of the requirements listed above, hydrogel is considered as a suitable candidate for cartilage regeneration, and this is going to be the main focus of this thesis. As stated in Section 1.1.3, hydrogel mainly consists of water (> 90%) and is supported by networks of physically or chemically crosslinked polymer chains (Radhakrishnan et al. 2017). Hydrogel has been utilised as a delivery medium for cells and growth factors as cells can be mixed in hydrogel while it was in soluble state (Dobratz et al. 2009, Mark and Daniel 2013, Pleumeekers et al. 2014, Abbadessa et al. 2016). Besides providing the right cell interaction, encapsulating cells within hydrogels will bring forth protection to the cells, and prolonged retention of the cells at the injury site. Injecting the cell suspension on its own will expose the cells to the shear force, and cells would be washed out right after injection (Bidarra et al. 2014). Aguado et al. (2012) have observed that cell survival increased when cells were injected while encapsulated within alginate compared to being suspended in saline solution. In situ gelation of hydrogel is also possible, as hydrogel undergoes a phase transition from sol state to gel phase. This has also
given hydrogel another positive point, which is injectability. Injectable hydrogel would allow for minimally invasive localised administration of cells or drug (Bidarra et al. 2014), complete filling of irregular-shaped defects or complex shape such as auricle (Pascual-Garrido et al. 2018) and could be used as bio-ink (Radhakrishnan et al. 2017).

1.5.2. Natural Polymers

1.5.2.1. Hyaluronan

Natural polymers such as collagens and hyaluronan attract researchers as these same components can be found within ECM. Hyaluronan or hyaluronic acid (HA) is an anionic non-sulphated glycosaminoglycan in cartilaginous ECM (Fraser et al. 1997). It consists of 250 to 25000 repeating disaccharide units of glucuronic acid and N-acetylglycosamine (Avenoso et al. 2018). In native tissue, hyaluronan has many vital roles in cell adhesion and cell proliferation, organisation and production of ECM (Hansen et al. 2001). Cell surface receptors for HA are integrins, toll-like receptor (TLRs) and CD44; and HA-CD44 binding is critical for downstream signalling of cartilage metabolism (Takahashi et al. 2010, Avenoso et al. 2018). HA has been reported to take a role in both anti- and pro-inflammatory mechanisms depending on the molecular weight (MW) of the protein. Low-MW HA, known as HA fragments, induces the production of interleukin (IL-1β), MMP-13 and ADAMTS4 and ADAMTS5 (Campo et al. 2009, Ariyoshi et al. 2012).

Single-injection of HA (known as viscosupplementation) has been given to OA patients as one of the earlier treatments, even though the benefit of it has been reported to be irrelevant (Rutjes et al. 2012, Jevsevar et al. 2015). However, when hBMSCs were cultured in commercially available HA-based hydrogels (HYAFF-11), Jakobsen et al. (2010) found that the stem cells produced the same level of COL2A1 and SOX9 expressions as human chondrocytes. Recently, HA was used to encapsulate human umbilical cord blood-derived MSCs, and a favourable outcome was observed (Park et al. 2017c). HYAFF-11 was also used to replace the collagen membrane in the third generation of ACI (Brittberg 2014). A five-year follow up of implanting BMSCs using HA into cartilage defect has shown positive outcomes compared to the control group which received microfracture treatment alone (Gobbi and Whyte 2016).

Even though these natural polymers are beneficial in terms of biocompatibility, their mechanical integrity has always been seen as a disadvantage (Bernhard and Vunjak-Novakovic 2016). Thus, researchers attempt to reduce hydrolytic degradation of HA through its photo-polymerisation (Feng et al. 2014). However, an in vitro culture of swine articular and auricular chondrocytes by Chung et al. (2008) has shown that the level of collagen type I increased after 14 days of culture (no GF was used). It was also reported by Bian et al. (2013b), even though photo-polymerisation of HA increased its storage modulus, the diffusivity of methacrylated HA (meHA) was reduced. When MSCs were cultured in meHA, the GAG level decreased and led to hypertrophic gene production (Toh et al. 2012). Due to this, studies have been carried out by
this and other groups to modulate the degree of crosslinking by changing the monomer concentration and UV/ visible light exposure time (Bian et al. 2013b, Fenn and Oldinski 2016).

Low diffusivity of crosslinked HA was resolved by incorporating gelatin microspheres. The microspheres will provide microcavity once degraded (Fan and Wang 2015). Another group induced controlled and local proteolytic degradation of HA by tagging meHA with MMP-sensitive binding peptide (Feng et al. 2014). It was reported that cell-mediated degradation in this modified meHA has successfully reduced the hypertrophic behaviour of hMSCs. Since then, researchers have been modifying HA using other methods of crosslinking and functionalised HA for specific biomedical application. For example, the same group bound sulphate to the MMP bound-HA hydrogels, and found it impeded its degradation and slowed down the release of TGF-β1, thus promoting the chondrogenesis of the hMSCs encapsulated within the HA (Feng et al. 2017). Besides encapsulating cells, HA was also used to transport ADAMTS-5 inhibitor to treat osteoarthritis (Chen et al. 2014b).

1.5.2.2. Collagen

Collagen is found abundantly in native ECM and plays a big role in cell attachment (Bernhard and Vunjak-Novakovic 2016). The properties of collagen hydrogel differ to each other, depending on the collagen source and the extraction method. Collagen type I, for instance, can be extracted from rat tail tendon, porcine skin or bovine skin through acid extraction, pepsin digestion, acid-salt precipitation or a combination of these methods. The preparation of hydrogel from collagen stock solution can vary depending on the concentration of hydrogel, polymerisation temperature and pH (Antoine et al. 2014). The pH relies on the type of neutralisation agent (HEPES, NaOH or HCl), strength and type of buffer (PBS, DMEM or MEM) (Antoine et al. 2014). Researchers tend to use collagen type I hydrogel, even though there is more collagen type II in cartilage (Zhou et al. 2011, Reiffel et al. 2013, Cohen et al. 2016). This is due to the difficulty of forming type II collagen hydrogel that has specific mechanical properties (Nimeskern et al. 2016).

Type I collagen hydrogel was fabricated into 3D ear structures using computer-assisted drawing/manufacturing (CAD/CAM) technique, injected with ovine auricular chondrocyte suspension, and subcutaneously implanted in nude rats (Zhou et al. 2011). A separate study by Bonassar and colleagues (2013), using 3D printed collagen hydrogel was carried out encapsulating bovine auricular chondrocyte. After six months in vivo study, the newly formed cartilage had uniformly distributed and showed intense elastin fibre histological staining (Cohen et al. 2016). On the other hand, Ren et al. (2016) utilised collagen type II hydrogel to engineer zonal cartilage by seeding the hydrogel in gradient chondrocyte seeding density. This approach was reported to increase the production of COL2A1 and aggrecan expression, even though the cell density was observed to be uniform throughout the depth at the end of the study, which does not mimic the physiological cartilage. Collagen’s compressive modulus and injectability
were modulated by changing the ratio and mixing different type of collagen content in the hydrogel (Nimeskern et al. 2016).

Despite collagen being favoured over other natural polymers to be used as a matrix scaffold in auricular cartilage engineering, results have shown that collagen scaffolds tend to lose their three-dimensional shape. The deformation is likely due to cell motility within the scaffold. In an attempt to enhance this collagen scaffold, Cervantes et al. (2013) embedded titanium wire within the collagen scaffold and found that this increased the scaffolds’ shape fidelity and flexibility. The retention of ear shape of collagen-wire-strengthen encapsulated with sheep auricular chondrocytes was also reported by Zhou et al. (2011).

Other researchers have used different strategies to improve the mechanical properties of collagen hydrogels. Its properties were enhanced by glycation (glutaraldehyde or genipin), enzymatic- or photo-crosslinking (Thiele et al. 2014, Walters and Stegemann 2014). An interesting strategy was reported by Garvin et al. (2013) to control the microstructure of collagen fibres. As shown in Figure 1-3, collagen molecules assembled and bundled in a staggered manner to form long thin fibrils. This fibril assembly is controlled by the concentration of collagen, environmental temperature and pH, and ionic strength of buffer. Garvin et al. (2013) have also shown that exogenous mechanical forces such as ultrasound can be used to tune the microstructure of collagen fibril.

1.5.2.3. Alginate

Besides collagens and hyaluronan, alginate and agarose are also classified as natural polymers. Alginate is extracted from brown seaweed. Alginate is a hydrophilic, cationic unbranched polysaccharide built by repeating disaccharide of β-D-mannuronic acid (MA) and α- L-guluronic acid (GA) (Radhakrishnan et al. 2017). Naturally, the ratio of these two uronic acids depends on the species of seaweed, part of the seaweed used to extract the alginate and the harvest location and season. The ratio of these uronic acids determines the stiffness of the alginate hydrogel (Lee and Mooney 2012).

Alginate promotes rounded cell morphology which indicates chondrocyte phenotypic property (Tuan et al. 2003, Dashtdar et al. 2011, Zeng et al. 2014, Markstedt et al. 2015). Clinical-grade alginate forms hydrogel through i) ionic crosslinking, ii) external gelation, iii) internal gelation and iv) covalent crosslinking (Bidarra et al. 2014). Gelation happens with the exchange of sodium ions in GA with divalent cations such as Ca$^{2+}$, Sr$^{2+}$ and Ba$^{2+}$. The selection of cation determines alginate stability, permeability and stiffness. Due to alginate interaction with cations, its degradation, when used in culture media, is deliberated.

Alginate can also be made into microbeads which can be prepared by dropping alginate solution into soluble salt (CaCl$_2$). Because the drops solely depend on gravity, the crosslinking density and alginate concentration were inhomogeneous (Coates and Fisher 2012, Lee et al. 2013). Hence, electrostatic has been applied to control the alginate flow rate and the diameter
of the microbead (Koh et al. 2017). One of the major concerns with the application of alginate in cartilage tissue engineering is its inferiority in mechanical properties. To overcome this problem, alginate has been chemically modified (Desai et al. 2015). Alsb erg’s group photocrosslinked methacrylated alginates using ultraviolet (UV) light to increase the storage modulus of the modified hydrogel (Jeon et al. 2009). The ability of alginate to form a gel in situ upon exposure to UV light is beneficial as this hydrogel can be delivered by minimal invasive procedure.

Even though photocrosslinked alginate improves its mechanical properties, researchers found that this modified alginate has a slow degradation rate. As alginate is plant-based, no enzyme available in mammals can degrade this hydrogel in the human body. Thus, Jeon et al. (2012) incorporated 2-aminoethyl methacrylate (AEMA), a biodegradable crosslinker to oxidised photocrosslinked alginate to induce controllable degradation. The same group, later on, functionalised the modified alginate to enhance its bio-adhesiveness (Jeon and Alsberg 2013). Recently, there have been studies carried out to use photo-crosslinked alginate’s functionalisation ability to track the gel via imaging such as magnetic resonance imaging (MRI) (Koh et al. 2017). This new strategy is beneficial in avoiding the need for invasive biopsies as non-invasive assessment can be done, and tracking the delivery of the hydrogel and small molecules by-product or its removal from the body is now possible (Santoso and Yang 2016, Ngen and Artemov 2017).

In a study by Andrew et al. (2016), different types of hydrogel were tested for their efficacy as bio-inks, and it was reported that BMMS Cs encapsulated within 3D printed alginate supported hyaline-like cartilage phenotype. In another study, when human chondrocytes extracted from the ear, nasal and articular cartilage were cultured in alginate and implanted in vivo, only ear and nasal chondrocytes had increased production of GAG and retained its elastin fibres (Pleumeekers et al. 2014). Mannoor et al. (2013) utilised alginate’s printability to print chondrocyte-seeded alginate into an ear shape with silver nanoparticle coil that conducts electrical signal to electrodes that function as cochlear.

1.5.2.4. Agarose

Agarose is a biocompatible natural material that has a low degree of branch complexity (Steward et al. 2011). It is a polysaccharide consisting of alternating sequences of 1,3-linked β-D-galactose and 1,4-linked 3,6-anhydro-α-L-galactose (Yang et al. 2017b, Zarrintaj et al. 2018). Agarose forms a thermoreversible hydrogel when molten agarose solution is incubated at a temperature lower than 40°C (Yixue et al. 2013). Agarose has been shown to promote chondrogenesis (Hong and Reddi 2013, Mark and Daniel 2013, Nims et al. 2014, Nover et al. 2016). The capability of agarose to support cartilage regeneration may be contributed to its property which resists the invasion of blood vessel and thus mimics the low oxygen tension of avascular native cartilage (Ashley and Lakshmi 2012). Cigan et al. (2016) encapsulated human articular chondrocytes at high seeding density (60 million cells per ml) and produced new
cartilage with mechanical properties that matched the native cartilage. High production of collagen type II and GAG was observed when 20 million cells per ml of bovine chondrocytes were cultured in 2% (w/v) agarose for an extended period (>3 months) (Farrell et al. 2014). This same study, however, found negative results when BMMSCs were cultured for the same duration in vitro. In another study, Mark and Daniel (2013) reported that agarose cultured with chondrogenic progenitor cells produced significantly higher GAG and stiffness than those cultured in fibrin.

Low concentrations of low-melting-point agarose have been widely used in studies that investigate the effect of mechanical loading and electrical stimulation on chondrocytes (Bian et al. 2010a, Kaupp et al. 2012, Pingguan-Murphy and Nawi 2012, Zignego et al. 2014, McCutchen et al. 2017). Agarose was chosen for this application as it offers a uniform environment and does not contain any ionic charge (Kelly et al. 2006). In such studies, it is essential that the scaffolds used are fabricated from polymers that provide solely construction matrix that support chondrocytes while exposed to mechanical stimulation, without other additional factors (such as growth factor or anionic charge), as the objective of these studies is to observe the effect of mechanical loading.

Agarose viscoelastic properties can be controlled by its molar mass and solution concentration (Thiele et al. 2014, Jutila et al. 2015). The concentration of agarose was modulated to see its effect on mechano-sensitivity of chondrocytes encapsulated within the variable-stiffness agarose hydrogel. Studies by June and colleagues have found that when encapsulated in higher stiffness agarose hydrogel (4.5% w/v), chondrocytes experience more substantial deformation, even after a short exposure to compressive loading (Zignego et al. 2014, McCutchen et al. 2017).

Agarose is degradable by agarase, however, this enzyme is not available in the human body. Besides that, agarose also lacks bioactive properties (Thiele et al. 2014). Attempts to overcome this problem were carried out by others either by adding peptide binding ligand or through hybrid/composite hydrogels (Ingavle et al. 2014, García-Martínez et al. 2017). In another study, dedifferentiation of chondrocytes was augmented through the incorporation of ECM extracts to agarose. This, however, was not capable of guiding the MSCs towards a chondrogenic phenotype (Youngstrom et al. 2016).

These two polymers, alginate and agarose, have received a great deal of attention mainly because of their non-inflammatory and potential for injectable delivery (Ashley and Lakshmi 2012). An ideal situation in clinical tissue engineering is to have an injectable hydrogel, which may or may not contain cells, allowing costly invasive major surgery to be avoided. Besides that, as stated in Section 1.5.1 injectable hydrogel has the benefits of in situ gelation, is capable of filling irregularly shaped defects and applicable as bio-ink.

Being one of the most conventional hydrogels, agarose has been utilised in pioneer works in producing large cartilage tissue. To date, studies of scaffold development of hydrogel are
restricted to 2mm thickness. Otherwise, there would be a problem of cell necrosis in the middle of the scaffold due to the nutrient inaccessibility. Goldman and Barabino (2017) modified 5mm thickness agarose slab with microfluidic channels. Circulation of media flowing through the channel not only enhanced the transportation of nutrient, but it also provided hydrodynamic shear to the cells. It was, however, reported that this modification compromised the mechanical integrity of the large scaffold. Nover et al. (2016) used a different approach- producing agarose-chondrocyte hydrogel in the shape of puzzles. The puzzles then assembled through interlocking, and this aggregation was able to withstand large loading strains compared to a bulk square-shaped hydrogel.

1.5.2.5. Fibrin

Being malleable, fibrin gel has been a great candidate for injectable vehicle cell delivery and hydrogel ink-jet printing to produce 3D constructs (Noori et al. 2017). One of the most apparent advantages of using fibrin that can be prepared from autologous blood plasma is that it is unquestionably biocompatible. Commercially available fibrin glue is also accessible by mixing thrombin and fibrinogen, even though autologous fibrin is preferable due to the risk of viral transmission (Wysocka et al. 2010, de la Puente and Ludeña 2014, Noori et al. 2017). The fibrinogen molecule is comprised of three peptide chains, which are linked by disulphide bridges (Brown and Barker 2014). Physiologically, fibrinogen initiates the blood clotting process (Noori et al. 2017). A work by Gasparotto et al. (2014) has looked into the feasibility of extracting fibrin from snake venom and used as a scaffold to deliver rat MSCs.

Modulation of fibrin properties is governed by the thickness and density of the fibres, number of branch points, porosity and permeability of the gels (Noori et al. 2017). These characteristics are controlled by the concentration of gel precursors (thrombin/ fibrinogen), Ca\(^{2+}\), salt, pH, temperature and the presence of other plasma protein such as fibronectin and albumin (Brown and Barker 2014, Noori et al. 2017). Ruszymah et al. (2007) seeded paediatric auricular cartilage into fibrin scaffolds derived from human plasma to investigate its plausibility in ear reconstructive surgery. The same group also found it was feasible to subcutaneously implant fibrin hydrogel with microtic auricular chondrocytes, and resulted in an increase of collagen type II, aggrecan, SOX-9 and elastin production (Ishak et al. 2015). Even though it was not reported by the previous group, fibrin hydrogel tends to shrink, especially when implanted acellularly (Neumeister et al. 2006).

Due to its capability of forming gel \textit{in situ}, fibrin has also been used in articular cartilage regeneration. Wysocka et al. (2010) observed a promising result using autologous fibrin seeded with autologous chondrocytes from the non-load bearing area and implanted during ACI procedure. In another study, using an equine model, Frisbie et al. (2015) found that autologous chondroprogenitor cells with fibrin hydrogel produced significantly better tissue than the acellular fibrin, even though Kim and colleagues who used human MSCs in fibrin hydrogel reported that this system was not superior to the acellular ones (Kim et al. 2015b).
As stated in the previous section, agarose was found to be more chondro-inductive than fibrin (Mark and Daniel 2013). It was also reported by Almeida et al. (2016) that fibrin was found to be less superior than other established hydrogels, even though de Windt et al. (2015) reported that direct co-culture of ART:MSC within fibrin is better than within alginate, showing the importance of bioactive adhesion sites availability in fibrin. There are multiple cell binding sites readily available in fibrin. Fibrin interacts with fibronectin and HA mainly through integrin via tripeptide binding sequence RGD (de la Puente and Ludeña 2014). Due to this, researchers utilised fibrin, which is degradable through proteolysis, to sustain the supply of TGF-β for an extended period of time (Diederichs et al. 2012, Henning et al. 2014). Despite being biocompatible, a major hurdle with applying fibrin as a construct for cartilage TE is unfavourable mechanical properties and its shrinkage in vivo (Raghunath et al. 2007, Nayyer et al. 2012, Thiele et al. 2014, Tomaszewski et al. 2014, Noori et al. 2017).

1.5.3. Synthetic Polymers

On the other hand, synthetic polymers are more favourable than the natural ones as their porosity, mechanical properties and degradation rate can be tailored based on intended use (Dhandayuthapani et al. 2011). Hydrophilic polyethylene glycol (PEG) has been widely studied as a construct that supports chondrogenic re-differentiation (Cui et al. 2012, Skaalure et al. 2015). The feasibility of utilising PEG in cartilage regeneration has been explored due to the fact that it is possible to tune its physical properties by changing the crosslinking density of photocrosslinkable PEG (Skaalure et al. 2015). Increasing the crosslinking density would enhance PEG stiffness. However, the dense mesh would disrupt the diffusivity and degradability of PEG (Neumann et al. 2016). Thus, researchers have been working on techniques to modulate the degradation rate of PEG; either through the incorporation of hydrolysis labile or enzyme-sensitive peptide. Peng et al. (2018) controlled the degradation rate of block copolymer of PLA-PEG-PLA by changing the number of lactic acid oligomers. Others have incorporated a peptide sequence that is cleavable by an enzyme to facilitate cell-modulated degradation (Skaalure et al. 2015, Sridhar et al. 2015a). In addition, PEG does not have any cell or protein binding sites (Kharkar et al. 2013). Despite that, poly(ethylene glycol) dimethacrylate (PEGDMA) has been used to regenerate auricular chondrocytes, and in 3D bioprinting (Papadopoulos et al. 2011, Cui et al. 2012).

Flores et al. (2017) used high-resolution 3D images to print a 3D construct of polylactic acid (PLA) ear implant. PLA was also copolymerised with either PLA (producing PLLA) or polyglycolic acid (PGA), producing poly(lactic-co-glycolic) acid (PLGA). The ratio of PLA and PGA governs the properties of PLGA such as biocompatibility, gelation and degradation rate (Caminal et al. 2014). Auricular chondrocytes cultured in both PLLA and PLGA produced a high amount of collagen type II compared to PLA and polycaprolactone (PCL), according to a study by Tanaka et al. (2012), even though PLLA was found to cause an immunological response (Fujihara et al. 2010). A recent work by the same group, however, reported no inflammation
when PLLA was used to culture auricular chondrocytes with high proliferation rate and implanted in vivo (Ishibashi et al. 2017).

Safranin-O and collagen II staining showed microtic tissue had the same biological properties as normal auricular cartilage, even though the cells lost their phenotypes upon in vitro expansion (Zhang et al. 2014b). Thus, the group co-cultured microtic tissue with hBMSCs in PLA coated with PGA. Immersion of PLA into PGA was reported to increase the scaffold’s ability to withstand skin constriction and maintain its shape (Otto et al. 2015). Despite cell dedifferentiation, long-term (40 weeks) in vivo implantation of PLGA seeded with chondrocytes extracted from either type of cartilage, produced tissues that have a comparable amount of cartilaginous gene expression (Nakao et al. 2017). In a separate study, PLGA prepared as microspheres, was loaded with TGF-β1. Morille et al. (2016) found sustainable release of the growth factor for up to a month. Also, PLGA encapsulating articular chondrocytes was found to improve the joint repair when used in combination with mosaicplasty (Zuo et al. 2016). Another study, however, reported the failure of PLGA after a year of in vivo implantation, due to lack of integration with the host tissue (Caminal et al. 2014).

PLGA and PCL are two of many polymers that have US Food and Drug Administration (US FDA) approval. PCL is favoured due to its strong mechanical properties which mimic the viscoelasticity of articular cartilage (ART) (Karuppuswamy et al. 2015). The Mikos group used PCL to redifferentiate expanded ART (Meretoja et al. 2012, Dahlin et al. 2014c). The downside of PCL is due to its slow degradability. In a study by Adeola et al. (2016), the practicality of using PCL as a bio-ink depends on its molecular weight.

High-density polyethylene, Medpor® is a commercially available construct that has been widely studied to regenerate ear cartilage. According to Reinisch and Lewin (2009), Medpor® is a better solution than rib cartilage as there is less morbidity and no need to wait until the patient turns ten years old to wait for the rib cartilage to grow to a certain size. Even so, it is still not the best solution as the rate of failure is always high caused by extrusion and infection (Nayyer et al. 2014). Low elastic modulus of Medpor® led to skin perforation, necrosis and low cell adherence (Baluch et al. 2014, Nayyer et al. 2014, Storck et al. 2014). Atomic force microscopy (AFM) analysis of Medpor® surface was shown to be smooth at the micro level, which may have caused inadequate cellular adhesion (Nayyer et al. 2014). Even though reduction in failure has been reported when Medpor® was covered entirely under temporoparietal fascial flap, the rigid implant still caused a chronic inflammatory response (Nayyer et al. 2016, Kim et al. 2017).

1.5.4. Hybrid and Composite hydrogels

Photo-polymerised PEG-based hydrogel PEG diacrylate (PEGDA) was used to facilitate joint repair upon microfracture procedure. PEGDA was beneficial regarding its ability to support the mechanical load. However, it was not capable of adhering to the slippery surface of articular cartilage. Earlier work from Elisseeff's group found that covalent binding of HA to PEGDA
increased the adhesiveness of PEGDA to the surface (Wang et al. 2007). Following that, PEGDA-HA co-culturing BMSCs and chondrocytes filled the defects and enhanced the integration of the host and new tissue (Sharma et al. 2013). As stated earlier in Section 1.4.3, HA is a type of glycosaminoglycan that can be found abundantly in native cartilage, but the application of HA itself in cartilage was found to be inferior in terms of its mechanical properties.

On the other hand, PEG has excellent mechanical properties, but lacks cell adherence. Thus, combining PEG and HA will benefit both parties. Lee et al. (2015) found blending HA in PEGDA not only increased the cell interaction with the scaffold but also facilitate its biodegradability. In addition to HA and PEG, collagen type II hydrogel was added to mimic the native cartilage and Zhang et al. (2015a) took a step ahead incorporating magnetic nanoparticles to help deliver BMSCs to the defect site. In another study, the interpenetrating network (IPN) of PEGDA and chondrocytes-agarose hydrogel was fabricated, and its stiffness was higher than each hydrogel on its own (Rennerfeldt et al. 2013). However, since both have no bioactive binding site, Ingavle et al. (2014) had to incorporate cell binding peptide RGD to the construct.

Collagen I and HA mixed hydrogel was used to encapsulate ADSCs and chondrocytes and injected into 3D printed PCL (Morrison et al. 2018). HA was also added to alginate to facilitate cell adhesiveness of alginate (Park and Lee 2014, Reppel et al. 2015). Being injectable, alginate was studied for its feasibility as bio-ink and work was carried out by multiple groups to build 3D constructs of layer-by-layer deposition of PCL and alginate for both articular and elastic cartilage (Kundu et al. 2015, Park et al. 2017b). Besides that, due to their high biocompatibility, alginate and agarose were mixed and commercially available as Cartipatch (Huang et al. 2016). It was, however, found in a study by Yang et al. (2018) comparing alginate-agarose and alginate-collagen hydrogels, that the later has better cell adhesion and significantly reduced the dedifferentiation of chondrocytes.

As stated earlier in Section 1.5.2.5, fibrin has poor mechanical properties. Thus, researchers have been designing hybrid fibrin hydrogel with HA, alginate, agarose or collagen (Thiele et al. 2014, Garcia-Martinez et al. 2017). Fibrin hydrogel has always been reported to shrink hydrogel upon culturing, Ma et al. (2012) found this problem is solved by adding alginate to it. Reduced volume and shape deformation has been reported for fibrin/HA encapsulated with auricular chondrocytes (Gillette et al. 2008). Visscher et al. (2016) retains fibrin hydrogel shape and volume by mixing it with collagen hydrogel and injecting into PCL cage. Varying the ratio of fibrin:collagen and fibrin:alginate changes its microstructure and mechanical properties. Studies have found the mechanical properties of fibrin gel could be reinforced by incorporation of crosslinked HA, and cell viability increased in this hybrid system (Snyder et al. 2014, Zhang et al. 2016b). However, having higher compressive modulus does not affect hMSCs chondrogenicity, unless TGF-β was present (Snyder et al. 2014). Having said that, fibrin has always been a good candidate in TE hydrogel as it naturally contains cell binding sites. Thus, it was used to improve bioactivity of PCL and alloplastic Medpor® which were used to regenerate
auricular cartilage and was reported to have reduced inflammatory reaction when used with fibrin (Hwang et al. 2014).

1.5.5. Self-Assembling Peptide Hydrogels (SAPH)

Due to its robustness, tunability and functionality, peptides have gained attention from researchers over the last ten years (Liu et al. 2018a). Peptides have been utilised in biomedical and drug delivery application designed to i) mimic growth factors such as TGF-β, ii) assist cell penetration and adhesion and iii) self-assembled peptide hydrogels. Cytomodulin, which contains 4-6 amino acids with β-turn secondary structures under physiologic conditions, has the same function as TGF-β (Liu et al. 2018a). Zhang et al. (2015b) found cytomodulins induced chondrogenic differentiation of BMSCs when ligated to the surface of microspheres. Besides, proteins, small interfering RNAs, nanoparticles and oligonucleotides can also be enveloped in peptides that are capable of permeabilising cell membranes (Pan et al. 2015).

Peptides have also been used to modify and enhance the functionality of biomaterials, such as CD-44 affinity binding peptide, MSC affinity binding peptide (E7), HA binding peptide, RGD functional peptide, N-cadherin mimetic peptide and MMP sensitive peptide (Shao et al. 2012, Bian et al. 2013a, Roberts et al. 2014, Ugarte-Berzal et al. 2014, Kim et al. 2015a, Sridhar et al. 2015a, Ustun Yaylacı et al. 2016).

The field of self-assembly peptide hydrogel (SAPH) was pioneered by Zhang and co-workers through the discovery of Ac-(AEAEAKAK)₂-ConH₂ in yeast extract (Zhang et al. 1993), followed by designer SAPH, (RADA)₄ (Zhang and Altman 1999) and Ac-(KLDL)₃-ConH₂ (Kisiday et al. 2002a). Self-assembly peptides are characterised by amino acid sequences that self-assemble into nanoscopic or microscopic structures.

Self-assembly peptides are seen to have high potential in tissue engineering applications as the properties of peptide fibrils (e.g. functionality and geometry) can be controlled by changing peptide sequences, concentration and pH (Hogrebe et al. 2018). Koch et al. (2018) utilised SAPH designed by Aggeli and co-workers, P₁₁. Based on P₁₁ peptide library, a series of peptide sequences with the net charge of +2 or -2 (at pH 7) were studied. Koch et al. (2018) found that the hydrogel stiffness and critical gelation concentration varied as different ionic composition was used, according to the peptides’ charges.

Properties of biomaterials can be altered to suit the needs in tissue engineering applications; due to over 20 natural amino acids that each has different physical properties (polar, non-polar, acidic, basic and aromatic) (Miller and Saiani 2010). Each peptide chain interacts with another chain through non-covalent interactions such as ionic, hydrophobic and hydrogen bond and π-stacking (Ulijn and Smith 2008). These interactions are weak on their own. However, the combination of these interactions are responsible for the stability of self-assembly peptide (Koutsopoulos 2016).

1.5.5.1. SAPH nanostructures
Self-assembly peptides that are being used as a 3D scaffold in tissue engineering can be divided into three categories based on their structures; which are peptide amphiphiles (PAs), α-helix and β-sheet (Miller and Saiani 2010).

Amphiphiles are elongated rod micelles that subsequently entangle to form 3D networks (Miller and Saiani 2010). PAs are built up by four domains; hydrophobic alkyl tail domain, intermolecular hydrogen bonding, charged amino acids for pH and salt responsive nanostructures, and hydrophobic tail. The hydrophobic tail can be replaced with various lengths of chains to accommodate its functionality. Referring to Figure 1-8, the second domain is built by amino acids that have a high tendency to create β-sheet hydrogen bonding (Webber et al. 2013). Alteration to this second region is a way to control the shape of assembled nanostructures. For example, replacing the β-sheet peptide region with a bulky aromatic side chain will trigger the formation of twisted ribbon from helical ribbon structures using non-aromatic residues (Pashuck and Stupp 2010). The third region provides charge which controls the solubility of the peptide in water. This region is the switch that triggers gelation of PAs upon pH changes and the addition of salts. A signature design by the Stupp’s group is to add a fourth region to a traditional PAs design that enables binding of bioactive signals. This region can be used to bind RGD which will facilitate cell adhesion (Webber et al. 2013).

Upon self-assembly in water, the molecular energy within PAs distributed towards hydrophobic interactions of the alkyl tail, hydrogen bonding among middle amino acids in the PA and electrostatic repulsions between charged amino acids (Wang et al. 2014a).

![Figure 1-8 Molecular structure of peptide amphiphiles (PAs).](image)

**Figure 1-8 Molecular structure of peptide amphiphiles (PAs).**

Such PA is designed based on four domains; 1) alkyl tail, 2) hydrogen domain, 3) charged residues and 4) hydrophobic tail. Image obtained from Webber et al. (2013) with permission.

In cartilage engineering applications, Shah et al. (2010) benefits the fourth region of PAs by adapting the TGF-binding domain. The integration of controlled continuous growth factors while culturing cells is essential. TGF-β1 has been shown to induce chondrogenesis and inhibit matrix degradation (Farach-Carson et al. 2007). It was reported that PAs synthesised with TGF-β1 binding sequence increased cartilage-specific markers gene expression (Shah et al. 2010).

The design of α-helix self-assembly peptide has been inspired by nature, where most typical globular proteins are made up of α-helix domains. Peptides with α-helical structures self-
assemble through hydrophobic interactions (Saiani et al. 2009). This highly ordered structure can be designed with specific morphologies and different helical pitch (Saiani et al. 2009, Miller and Saiani 2010). α-helical structures can be arranged in parallel, antiparallel or mixed topologies. α-helices structures self-assemble by burying the hydrophobic surface residues (Saiani et al. 2009, Miller and Saiani 2010). α-helical structures can be arranged in parallel, antiparallel or mixed topologies. α-helical structures self-assemble by burying the hydrophobic surface residues (Fletcher et al. 2012). The self-assembly and gelation properties of the peptide can be modulated by manipulating the distribution of polar groups along the helices (Saiani et al. 2009). Mehrban et al. (2014) used α-helical self-assembling peptides complemented with Arg-Gly-Asp-Ser (RGDS) to enhance cell adhesion.

Both PAs and α-helix peptides have high fibre rigidity which leads to difficult hydrogel formation, as compared to β-sheet peptides (Miller and Saiani 2010). β-sheet self-assembly is triggered by the addition of salts (Jonker et al. 2012). Upon self-assembly into β-sheet fibrils, hydrophobic and charged amino acids superimposed with adjacent peptide chain through van der Waals and electrostatic interactions (Wang et al. 2014a).

Peptides having β-sheet structures can be further classified based on their morphology; short peptide, β-hairpin and ionic self-complementary peptides. The short peptide contains 2-3 amino acids with aromatic groups initiating self-assembly through π-stacking that leads into a cylindrical nanostructure (Miller and Saiani 2010).

β-hairpin structured hydrogels are usually homogeneous and form stable hydrogels. MAX1, which consists of 20 amino acids with alternating valine (V) and lysine (K), is one of the examples of β-hairpin. At physiological pH, the electrostatic repulsions between lysine residues are secluded, and hydrogen bonds will stabilise the self-assembly structure (Branco et al. 2009). Based on this design, the Schneider group came up with another peptide sequence MAX8, where lysine at position 15 on the hydrophilic chain is replaced with glutamic acid. With glutamic acid, this peptide sequence was seen to react faster in terms of its self-assembly kinetics. Another paper by the Schneider group reported their attempt to design a peptide hydrogel (HLT2) that had the same nanostructure with MAX8 but carried less charge (Sinthuvanich et al. 2012). At neutral pH, HLT2 carries +5 charges, while MAX8 carries +7 charges per monomer. These peptides were first dissolved in the aqueous buffer before adding cell culture media that was responsible for triggering β-hairpin peptide folding. Both peptides were designed to have similar morphology and storage moduli. It was found that chondrocytes prefered the less electropositive HLT2 (Sinthuvanich et al. 2012).

Established on the alternation of one charged and one non-charged amino acid, the ionic self-complementary peptide comprises of hydrophobic and hydrophilic on each side of its chain (Saiani et al. 2009). Ionic self-complementary peptide turns into hydrogel at above critical concentrations (Miller and Saiani 2010). Inside aqueous media, two β-sheet fibrils come together forming a single fibril to bury the hydrophobic side chains. If the individual peptides are arranged in the sheet with the N- and C-termini alternating then it is identified as anti-parallel β-sheet (Ulijn and Smith 2008). Being in β-sheet fibrils formation, it can be assumed that non-
polar amino acids control fibre strength, while polar amino acids manipulate lateral fibre-fibre interactions (Miller and Saiani 2010).

KLD-12 is another self-assembly peptide that self-assembled when the pH changed as the net charge of the peptide molecules approached zero. A study was carried out by Grodzinsky and co-workers (2002b) utilising KLD-12 hydrogel. The KLD-12 solution was solubilised in sucrose solution to reach physiological osmolarity before 15 million chondrocytes per ml were seeded in peptide hydrogel. It was reported in this study that chondrocytes with rounded morphology were observed to be distributed throughout the scaffold. $^{35}$S-sulphate incorporation was quantified to assess proteoglycan synthesis by cells. It was found that this value increased at day 5 and started to decrease at day 15. This same trend was also observed for chondrocytes seeded within agarose scaffold that served as control samples (Kisiday et al. 2002b).

1.5.5.2. SAPH in cartilage engineering

It is reported that β-sheet peptides promote cartilage regeneration (Kisiday et al. 2002b, Shah et al. 2010, Mujeeb et al. 2013). In fact, in comparison studies between SAPHs and other 3D hydrogels, SAPHs were observed to be superior in supporting chondrogenesis. Three-dimensional KLD-12 was better in terms of providing a more suitable environment than agarose and alginate (Kisiday et al. 2002b). More recent work from the same group not only found that cells cultured in RADA produced a higher level of sGAG than those in agarose hydrogel, but they also observed that adding dexamethasone to the system only worked for agarose, not for RADA (Florine et al. 2013). Recently, Ulijn and co-workers (2017) discovered that the level of SOX-9, collagen type II and aggrecan produced by adipose-derived pericytes cultured in Fmoc based diphenylalanine-serine SAPH were significantly higher than those in alginate, even in the absence of chondrogenic media.

Earlier work carried out within our group using chondrocyte-seeded FEFEFKFK showed high cell viability, with good cell proliferation and morphology (Mujeeb et al. 2013). In this study, the peptide hydrogel was prepared at physiological pH and was found to obtain pore size of 15-30nm. Wang et al. (2014a) discussed the rational design of peptide nanofibre scaffolds for cartilage tissue engineering application. Being hydrogel, ionic self-complementary nanofibre contains more than 99% water, with a fibre diameter of 10-20nm and 5-200nm pore size; these properties are in good agreement with natural ECM (Wang et al. 2014a).

Upon showing positive results in in vitro studies, in vivo work involving KLD-12 also has been promising as reported in rat and rabbit joint OA model, both of which have shown KLD-12 induced full thickness cartilage (Miller et al. 2010, Kim et al. 2014). In fact, cells encapsulated in KLD-12 were seen to migrate towards the culture media containing growth factors, showing it to be capable of delivering cells to the cartilage defects following microfracture procedure (Liebesny et al. 2016). However, a more recent work involving KLD-12 to fill the voids following microfracture procedure in treating joint defects of an equine model was reported to not be
superior than microfracture alone (Miller et al. 2014). This can be explained by the loss of mechanical integrity of KLD-12 in loaded joints.

There have been advances in designing self-assembling peptides that imitate natural ECMs. Besides integrating functional motifs, controlled release of molecular signals incorporated within self-assembling peptides might facilitate peptides mimicking natural ECMs. Smith Callahan et al. (2013b) found low RGD peptide concentrations maintain chondrocyte viability, chondrocyte phenotype and increased ECM contents. RGD binds to α5β1 integrin receptor to initiate cell differentiation and migration (Wang et al. 2014a).

Tissue regeneration is mediated by soluble macromolecules or signals. Thus, embedding self-assembly peptides with controlled release of molecular signals might nearly assimilate natural ECMs (Zamuner et al. 2016). Shah et al. (2010) found that TGF-β1 combined peptides change human mesenchymal stem cells (MSCs) to chondrogenic-like cells.

The ability to bind bioactive molecules to peptide was applied by Grodzinsky and co-workers where the release kinetics of transforming growth factor β-1 (TGF-β1) from SAPH was determined. It was described that TGF-β1 uptake was higher when growth factor was added prior to gelation than immersing assembled KLD in media containing the growth factor. In this study, Kopesky et al. (2014) also found that peptide hydrogel KLD could sustain TGF-β1 release for a more extended period of time, in comparison to agarose which was reported to have initial burst release (Kopesky et al. 2014). This finding agrees with a study published by the same group later on, which incorporated heparin binding-IGF1 to RADA, or commercially available as Puramatrix™ (Florine et al. 2014). Besides KLD and RADA, another group has been investigating the feasibility of conjugating bioactive peptide to another amphiphilic anti-parallel β-sheet SAPH, EAK16 (Zamuner et al. 2016).

Recent advancement of SAPH is its utilisation as bio-ink. Work in our group has shown that our novel designed SAPH encapsulating epithelial cells were feasible to be used as bio-inks for 3D printing in the extrusion-based system (Raphael et al. 2017). A work by another group using CHO1 and CHO2 tetramer SAPH have also shown the same results (Arab et al. 2018).

1.5.5.3. The design principle of SAPH

Most peptide-based hydrogels are low in mechanical stability and susceptible to erosion in water, due to inhomogeneous self-assembly process and low crosslinking density (Li et al. 2014b). Attempts have been made to improve hydrogel peptides’ mechanical stability. It is reported that the elastic modulus of ionic self-complementary peptides increased with peptide concentration but decreased with hydrophobicity (Saiani et al. 2009, Li et al. 2014b).

Inhomogeneity of the self-assembly process can be resolved by developing a system that will assemble and dis-assemble on cue. Ulijn and Smith (2008) suggested three systems that might facilitate controlled self-assembly; pH switch, enzymes and light. Having adverse charges in the peptide design will trigger a pH switch that will initiate self-assembly. Li et al. (2015a) modulated
the peptide fibre morphology which dictates Fmoc-based SAPH mechanical properties by varying the ionic strength and the rate of pH change. Fmoc is another SAPH that forms hydrogel through π–π stacking between the Fmoc groups (Worthington et al. 2015). In another study, enzymes such as protease can be bound to the peptide chain to initiate self-assembly through reversed hydrolysis (Toledano et al. 2006). Haines et al. (2005) designed a peptide chain that contains a cysteine residue with an attached photo-cage, which will begin to fold into β-hairpin structured peptides on the removal of the photo-cage upon light exposure.

Integrating arginine into self-assembly peptide chain also helps to increase its elastic modulus; as arginine is a bulkier side group creating multiple ionic bridges that lead to long twisted fibre bundles (Mohammed et al. 2007). This concept is also applicable for incorporation of inorganic composites or biomacromolecules to improve peptide’s mechanical strength (Li et al. 2014b). Besides enhancing the SAPH stability, its incorporation with inorganic composites was done to enhance the bio-interaction of the scaffold. In an effort to provide heparin-binding site to the scaffold, Recha-Sancho and Semino (2016) added heparin to RADA. When dedifferentiated human articular chondrocytes were cultured in varying ratio of heparin in Hep-RADA, the cells morphology became more stretched as the ratio of heparin increased. Another study by the same group however found the same type of cells remained spherical, produced a higher level of collagen type II and decreased its hypertrophy when cultured in PCL/RADA composite hydrogel (Recha-Sancho and Semino 2016). Another group used RADA in hybrid silk multi-layer scaffold to regenerate the whole thickness of osteochondral tissue (Chen et al. 2016).

Chondroitin sulphate (ChS) was conjugated to P11 SAPH to enhance its efficacy in cartilage TE. Barco et al. (2018) reported that SAPH assembly depended on the charge of the peptide, ratio of chondroitin sulphate and the salt concentration. When ChS was added to +2 charge P11-6 and P11-12, its β-sheet propensity decreased. Conjugating small ratio of ChS to P11-4 (-2 charge) at the ratio of 1:64 also perturbed the peptide assembly, but not at a high ratio (1:16). Besides modulating SAPH’s mechanical properties and its bio-interaction. SAPH functionalised ability has also been utilised to control its biodegradability. Pochan, Schneider and co-workers (2011) carried out a study employing degrading peptides which have MMP-13 cleavable six residues sequence PTG-XKV at the C-terminus of the peptide. When X is substituted with less hydrophobic amino acid, the peptide susceptibility to MMP-13 was also decreased.

### 1.6. Bioreactor

Physiologically, cartilage joints are exposed to mechanical loading. Thus, mechanical stimulation is found to be integral in cartilage development. Even though studies have found that growth factors play an important part in regenerating cartilage, mechanical stimulation remains a significant area to provide an environment that mimics the physiological properties of cartilage (Tuan et al. 2013). A large amount of research is focusing on the physical compressive loading, which has shown that compressive loading initiates other physical mechanical stimulation such as hydrostatic flow and membrane potential.
Mechanical forces and deformations are sensed by cells which are then turned into biochemical signals. The transduction of mechanical stress into biochemical signals by cells is known as mechanotransduction (Varady and Grodzinsky 2016). Such biochemical signals are essential to adjust cellular and extracellular structure, which is vital in regulating chondrocyte function; both in metabolic and catabolic modus operandi.

The use of bioreactors in 3D culture is particularly essential for these following reasons: enabling uniform distribution of cells, efficient transport of biochemical initiators; such as growth factors, and precise control of mass transfer rate, nutrient levels and pH (Yeatts et al. 2013). As stated earlier, in vitro tissue engineering started with 2D cell culture. Yet, researchers have found that 3D cell culturing is essential to reduce their tendency towards plasticity (Nazempour et al. 2017). However, the use of 3D scaffolds has given researchers another hurdle; where cells were not homogenously distributed throughout the scaffolds. As reviewed by Plunkett and O’Brien (2011) and Gelinsky et al. (2015), the encapsulated cells were seen in microscopic images to be found at the periphery of the scaffolds. In-depth study of spatial images of the scaffolds found that cells in the centre of the scaffolds tended to go through apoptosis. It was concluded that necrosis in this study was caused by the inability of the cells to obtain nutrients and to remove waste efficiently.

1.6.1. Perfusion bioreactor

From the biomaterials point of view, infiltration of nutrients throughout the scaffold can be improved by increasing the pore size and porosity of the scaffold. However, this would jeopardise its mechanical strength. Thus, perfusion bioreactor would come to the rescue (Thakurta et al. 2014). Flow perfusion bioreactors were designed to enhance the delivery of nutrients and waste removal in and out of the scaffold as a whole. In a flow perfusion bioreactor, media is forced through each scaffold via tubing using a pump (Yeatts et al. 2013). The velocity of the fluid flow is controllable, and high-velocity fluid flow will, in turn, produce fluid shear stress (Yeatts et al. 2013). Fluid shear stress which is beneficial to the cells will signal the cells to produce extracellular matrix components for the stability of cells’ phenotype (Ogura et al. 2018). The design of a flow perfusion bioreactor includes a circuit that allows recycling of media for a couple of days. This media reuse is essential as the media contains important proteins expressed by the cells that initiate cell proliferation and differentiation (Wendt et al. 2006).

Besides flow perfusion bioreactors, there are other commonly used hydrostatic bioreactors such as spinner flasks and rotating wall bioreactors. Spinner flask bioreactor is the most basic bioreactor, designed using a cylindrical glass container. Scaffolds containing cells are either attached to needles that are secured to the lid or left free floating in the media. The glass container is filled with media. A magnetic bar/rotating impeller and stirrer are used to move the fluid, which produces turbulent flow (Ismadi et al. 2014). Rotating wall vessels (RWV) bioreactor consist of two concentric cylindrical chamber that rotates to balance the gravitational force enhancing fluid transport into the scaffold (Plunkett and O’Brien 2011). The design of these
bioreactors has been improved as with the original models, the oxygen concentration and shear stress were not well regulated due to the bulk mixing. Varley et al. (2017) have improved the design of RWV by using disposable Falcon tubes, reducing the volume and cost of the media and growth factors.

According to Zhang et al. (2014c), the hydrostatic pressure generated by perfusion bioreactors has been proven to regulate the differentiation of MSCs toward chondrogenic fate. However, even after 40 years, work is still going on to determine ideal parameters such as perfusion flow rate and loading time. In a study carried out by Reis and co-workers, the hydrostatic pressure of 5MPa had more benefit to the human adipose stem cells (ADSC) encapsulated in gellan gum than at 0.4MPa (Correia et al. 2012). In the same study, it was found that intermittent pulsatile hydrostatic pressure of 0.5Hz, 4hr/day, 5d/week resulted in higher production of chondrogenic protein by the cells. However, in a separate study by Loboa and co-workers (2013), they reported that the cyclic hydrostatic pressure of 7.5MPa at the frequency of 1Hz given for 4h/day caused a significant reduction in human ADSC viability compared to the control static samples. In their study, cells were encapsulated in 2% agarose hydrogel. The cell-agarose constructs were then sealed in polyethylene bags filled with media. Their report also mentioned that the load was carried out in a temperature-controlled water bath, however, failed to mention details about the oxygen control (not within an incubator). Besides, reduced cell viability due to perfused fluid was not reported elsewhere (Chen et al. 2017, Ogura et al. 2018).

Nazempour et al. (2017) combined the oscillating hydrostatic pressure (4MPa) and fluid-induced surface shear (0.02Pa) onto agarose hydrogel encapsulating bovine chondrocytes. It was found when these two stimulations were used, a 7.6-fold increase of GAG and total collagen was observed compared to 5.9-fold increment when only surface shear was used. The paper also stated that with stimulation, cells produced less collagen type I and X demonstrating phenotypic stability, although the level of collagen type II was not measured. In another separate study, the effect of hydrostatic pressure (HP) and fluid-induced shear, on swine chondrocytes seeded in PLA/PGA scaffold, was compared (Chen et al. 2017). HP generated through completely sealed stainless steel pressure chamber had the highest total GAG and collagen density than shear, but the combination of both stimuli increased the construct’s Young’s modulus.

1.6.2.Compressive loading

Compressive bioreactors, on the other hand, have been used recently to deliver compressive and stress mechanical stimulation to cells (Plunkett and O’Brien 2011). While direct static compressive loading was found to inhibit the anabolic characteristics of chondrocytes, dynamic loading, on the other hand, maintained the chondrogenic stability of chondrocytes and promoted differentiation of MSCs towards chondrogenic phenotype (Grodzinsky et al. 2000, Bian et al. 2012). Immobilisation of chick embryo joints resulted in a decrease in HA content and cartilage volume (Roddy et al. 2011). As stated in the reviews by Fahy et al. (2018) and Zhang et al. (2014c), compressive loading affected cartilage tissue inducing hydrostatic pressure, osmotic
pressure and electric potential gradients. It was reported by DiDomenico et al. (2017), that mechanical loading helped to transport solute through the depth of articular cartilage.

Similar to hydrostatic pressure, the effect of compressive loading has been reported to give mixed output to the chondrocytes. This is due to the numbers of parameters that researchers have to consider in optimising the compressive stimulation. One of the most prominent influences is the loading regime such as the loading frequency, duration and the requirement of resting period (intermittent loading) allowing cells to respond to the stimulus (Anderson and Johnstone 2017). Many studies have also found the importance of pre-culture as delayed initiation of loading permits cells to bind to the newly produced pericellular matrix (Bian et al. 2010a, Nicodemus and Bryant 2010, Pingguan-Murphy and Nawi 2012). Besides the loading element, the state and the source of the cells (passaged/ dedifferentiated/ pre-conditioning) also play a role in the effect of loading. Healthy cartilage had a higher capacity to respond to loading compared to OA cartilage (Wiseman et al. 2004). Others found chondrocytes derived from neonatal and juvenile joints had higher increment of GAG than those from adult joints (Farnsworth et al. 2013, Liu et al. 2013).

Both studies by Lin et al. (2017) and Neumann et al. (2015) (among others), found that mechanical compressive force increased rat MSCs’ (3D, meHA) and human articular cartilage progenitor cells’ (3D, polyurethane-fibrin) capability towards chondrogenicity. Both studies also found the synergistic effect of compressive loading and growth factors such as dexamethasone and TGF-β1.

1.6.3. Compressive and shear multi-axial loading

Besides compressive stress, joint cartilage also experiences shear strain along multiple axes throughout the depth of cartilage. This is especially true at the superficial layer (Zhang et al. 2014c). With depth, cartilage experiences tensile deformation. This heterogeneous and anisotropic ECM structure of cartilage has led the researches toward the development of multi-axial loading bioreactor (Bian et al. 2010a, Yusoff et al. 2011, Meinert et al. 2017). As shown in Figure 1-9 (a), novel-designed bi-axial loading bioreactor consists of chondrocytes encapsulated within agarose hydrogels affixed to the strips of sintered glass, movement of which was controlled by stepper motors either horizontally or perpendicularly (Yusoff et al. 2011). Bader and co-workers (2017) designed a bioreactor where molten cell-seeded agarose constructs were poured in a mould in between nylon endplates (see Figure 1-9 (c)), while another group positioned pre-moulded GelMA-HAMA scaffold in multi-wells (DiFederico et al. 2017, Meinert et al. 2017). Both of these systems utilised polytetrafluoroethylene (PTFE) plungers that are connected to the vertical actuator and a stage that moves in linear direction.
Another bioreactor system that provides multi-axial loading is the commercially available ceramic hip ball that oscillates perpendicular to the scaffold axis providing shear strain and sinusoidal dynamic load of 5-15% of the scaffold height (see Figure 1-9 (b)) (Wang et al. 2013). At 10% compressive loading and 1% shear strain, multiaxial loading was reported to produce higher PGs than those loaded uniaxially (Schatti et al. 2011, Pingguan-Murphy and Nawi 2012). Both DiFederico et al. (2017) and Meinert et al. (2017) found that when the shear strain was present, chondrocytes not only produced higher GAG, they also produced more collagen type II. Wang et al. (2013) reported that when chondrocytes were exposed to hip ball bioreactor for two weeks after pre-culturing for two weeks, even passage three chondrocytes had the same effect as the non-passaged chondrocytes.

1.6.4. Hypoxic condition

Being avascular, the oxygen concentration throughout the depth of native cartilage decreased (Zhou et al. 2004, Fermor et al. 2007). This was found to have a functional effect on the maintenance of chondrogenic phenotype of cartilage. Studies found that hypoxia promotes the chondrogenic potential of ADSC (Portron et al. 2013, Wan Safwani et al. 2017). Mikos and co-workers (2013) co-cultured articular chondrocytes and MSCs at 30:70 ratio in poly(ξ-
caprolactone, PCL) in the presence of TGF-β3. The study found that when cultured in 5% O\textsubscript{2} (hypoxia), co-cultured samples and ART had a higher ratio of collagen type II: type I, in comparison to 20% O\textsubscript{2} (normoxia) condition. Even though the study reported less effect of hypoxia on the quantification of GAG deposition, Alcian Blue histology staining showed otherwise. Also, other studies have reported significantly higher gene expression of SOX-9, ACAN and COL2A1 by MSC pellets upon three weeks of culture within hypoxic condition (2.5% O\textsubscript{2}) (Leijten et al. 2014). More recent work by the same group also found that hypoxia not only increased GAG and collagen type II, it also demoted the level of MMP-1 and -3 (Huang et al. 2018b).

The effect of hypoxia on chondrogenic characteristics was reported to be activated through hypoxia-inducible factor-1 (HIF-1), and hypoxia is capable of protecting the cells from differentiation towards osteogenic phenotype (Thoms et al. 2013, Taheem et al. 2018). In another study, hypoxia was used to enhance the chondrogenic status of MSCs seeded within large alginate constructs (10x6mm, DxH) (Daly et al. 2018). In their study, through histological staining, samples cultured within hypoxic condition had no calcium deposition (signs of osteogenic) but higher type II collagen production than the control samples, only at the peripheral of the constructs. However, when dynamic compressive loading was combined with hypoxia, the chondrogenic protein was observed to be more distributed throughout the samples.

1.6.5. Ultrasound

The research area of utilising ultrasound stimulation on engineered cartilage is still in its infancy. Due to this, researchers are still determining the optimal conditions such as frequency and regime (continuous or pulsed) that would benefit the cells. Having said that, ultrasound is however considered one of the most practical methods to deliver physical stimulation to cells, as it can be provided without having to be in direct contact with the samples. Miller et al. (2017) stated in the paper that the ultrasound frequency applied has to match the elastic modulus of the scaffold to maximise the displacement which will then lead to cellular deformation.

Thakurta et al. (2014) investigated the efficacy of using ultrasound to deliver and direct cells through the depth of the polycarbonate-polyurethane scaffold. In the study, the application of ultrasound improved the distribution of cells through the thickness of the scaffold. The stimuli not only increased the production of COL2A1, SOX-9 and ACAN gene and protein expression, it also enhanced the localisation of collagen fibres.

An interesting approach by Tare and co-workers to control the fluid flow rate through the integration of acoustic waves was recently reported (Li et al. 2014a, Jonnalagadda et al. 2018). The scaffold-free human articular chondrocytes were cultured in a sterilisable rectangular glass capillary containing culture media. The capillary was attached to the resonant chamber which generates and amplifies wave signal of continuous perfusion at 890-910kHz frequency sweeping at a rate of 50kHz. The acoustic stimulation not only induced waves of oscillatory fluid shear perfusion, but cells could also detect this stimulation, showing an increase in SOX-9,
collagen type II and proteoglycan gene expression by the cells (Li et al. 2014a, Jonnalagadda et al. 2018).

Depending on the flow rate and fluid wave, this acoustofluidic stimulation may be beneficial for the regeneration of auricular cartilage since the ear cartilage has the function of detecting and directing sound into the inner ear. This is also based on a study by Garvin et al. (2013) which showed that ultrasound stimulation could be used to control the density and diameter (microstructure) of collagen fibre. It was hypothesised, through the application of ultrasound, that the flexibility and stiffness of engineered tissue could be modulated. The design of ultrasound bioreactor applied by Garvin et al. (2013) and Thakurta et al. (2014), however, is more applicable for scaffold containing cells for functional purposes, compared to the glass capillary design described earlier.

1.6.6. Microgravity

Another stimulation that has a high probability in the regeneration of ear cartilage is microgravity. Studies on the effect of microgravity on cartilage arose when reports showed that upon residing in space for an extended period of time, the cartilage mass of an astronaut reduced significantly. Besides that, culturing primary porcine chondrocytes in the International Space Station (ISS) for 16 days increased the ratio of type II/I collagen, though the aggrecan level and cell density decreased (Stamenković et al. 2010). In another study, human thyroid carcinoma cell formed 3D spheroids when cultured in space for ten days (Pietsch et al. 2013). These studies have proven that the mechanobiology of cells respond to microgravity.

Another work which was carried out on Earth using simulated microgravity was found to be intriguing, with higher amounts of collagen type II, chondroitin sulphate and aggrecan produced by human chondrocytes in microgravity, emphasizing the importance of ascertaining the best magnitude/duration and method in delivering microgravity (Ulbrich et al. 2010, Wu et al. 2013).

As shown earlier by the formation of 3D spheroids of carcinoma cells, microgravity may be highly useful to produce an aggregation of chondrocytes towards spherical morphology which will lead to chondrogenic properties (Daniela et al. 2018). Clinostat, desktop random positioning machine (RPM) and rotating wall vessels (RWV) are the devices that are designed to simulate microgravity on Earth (Grimm et al. 2014). A 2D clinostat is a machine that comprises multiple fast-rotating tubes. The diameter of the tubes is limited to avoid centrifugal forces which will push the cells to the wall of the tube. Thus, the application of 2D clinostat is only for cell suspension (Grimm et al. 2014).

RPM, sometimes known as 3D clinostat, has two pivoted frames, each of which is driven by a separate motor. The speed and direction of the frames are independent to each other (Wuest et al. 2015). Yuge et al. (2006) reported human MSCs cultured in 3D clinostat expressed more of CD44, collagen type II and aggrecan compared to the static samples. A study has claimed that besides microgravity, RPM which contains media also has the effect of fluid shear forces.
(Grimm et al. 2014, Wuest et al. 2017). RPM has the benefit that cells can be cultured in T flasks, multiwell plates and slide flasks. For example, Aleshcheva et al. (2013) cultured human chondrocytes in monolayer in T flasks on Desktop RPM at 60°/s speed at random direction. The effect of microgravity on the cytoskeletal elements was only seen during the shorter duration of exposure (< 4 hr). Cells' morphology for samples in extended exposure were similar to those at 1g control sample.

Similar to RPM, rotary cell culture system (RCCS, Synthecon, Houston, Texas, USA) which has the same design principle as the RWV, provides hydrostatic shear in addition to microgravity (Liu et al. 2016). RWV is one of the first microgravity simulation devices designed by the National Aeronautics and Space Administration (NASA). As the name suggests, RWV has a vessel that rotates continuously, providing fluid motion and microgravity. According to Grimm et al. (2014), when deciding the rotation speed of RWV, the weight of the cells and density and viscosity of the media have to be taken into consideration. Studies done by Liu et al. (2016) and Yin et al. (2016) both agreed that MSCs cultured in RCCS were capable of directing the cells towards chondrogenic differentiation. Yin et al. (2016) however, also found signs of hypertrophy in the samples, though this was not measured by Liu et al. (2016). RCCS was also used to engineer elastic cartilage. Chondrogenic progenitor cells from the perichondrium of microtic patients were seeded on ear-shaped HA/Ch-s scaffold and cultured in RCCS for six weeks (Takebe et al. 2012). Histology staining showed the production of elastin and PGs, even though no proper control static sample was used in their studies. Recently, a separate work culturing bovine chondrocytes in monolayer for the same length of time as in RWV, showed no effect of the stimulation to the cells (Wuest et al. 2017). Chondrocytes, however, besides retaining spherical morphology, produced a higher level of collagen II to I ratio than the other conditions, when left cultured in a static state for the first two days followed by six days in RWV.

1.6.7. Electric and electromagnetic field

In the phenomena of chondrocytes sensing mechanical forces and deformation, this has been shown to lead to multiple events inter- and intra-cellularly. The consequences of physical stress have been observed triggering different signalling pathways. For instance, upon compression, joint cartilage experiences a difference in membrane potential which activates the ionic channels allowing the flow of growth factors that would trigger production of certain genes (Ashraf et al. 2016). This then urged researchers to look at the efficacy of electrical stimulation in cartilage regeneration.

The electric stimulation (ES) itself is a broad area as their application on tissue engineering can be delivered through direct current (DC), alternating current (AC), electromagnetic field (EMF) and pulsed-EMF (PEMF) (Jin et al. 2015). Even though PEMF has been widely used in treating bone fracture, its application in regenerating cartilage is still in its primitive stage (Kenjiro and Hari 2018).
In DC devices, electrical current is converted into ionic current through the oxidation-reduction redox reaction that occurs at the salt bridges connecting two electrodes (Hroník-Tupaj and Kaplan 2012). Hung and co-workers have found that DC ES modulated cells migration and alignment (Gunja et al. 2012, Tan et al. 2015, Vaca-González et al. 2017). It was also found by Tan et al. (2015) DC EF caused synovium-derived stem cells to migrate faster than the non-stimulated samples, regardless of the plasticity of the cells. Also, it was found in the same study, while passage 1 (P1) cells migrated towards the positive node, P4 cells changed their direction (cathodic migration).

Besides the types of ES, other parameters such as intensity, frequency and duration, need to be examined. Vaca-González et al. (2017) observed different intensities and exposure time of ES affecting cell viability and GAG synthesis. Gunja et al. (2012) observed the effect in cell migration when DC EF was applied at the strengths of more than 2V/cm and more cells migrated towards the cathode with increased strength.

In another study, a function generator was integrated to the system generating AC signals (Hiemer et al. 2018). BMMSCs response to AC ES was more prominent than the human articular chondrocytes and the co-culture of both cells. Upon being exposed to sinusoidal ES for 3 times/day at the frequency of 1kHz, BMMCs expressed more collagen type II and less alkaline phosphatase (ALP, osteogenic marker) than other types of cells. The study also found the synergistic effect of ES and hypoxia and suggested that with these double stimulations there is no need for co-culturing of BMMCs and primary chondrocytes.

EMF stimulation can be generated through i) static magnetic field produced by magnetic dipoles or ii) unsteady current passed through a coil known as PEMF. There have been mixed reports on the effect of EMF depending on the intensity, frequency, static or oscillatory, waveform and the state of cells being stimulated (Janson and Putnam 2015). A study carried out by Mayer-Wagner and colleagues found exposing OA chondrocytes to 5mT EMF increased expression of aggrecan and COL2A1, but not at 8mT (Redeker et al. 2017). This was also observed when PEMF was applied. GAG and collagen type II reduced significantly at the intensity of > 2mT and pulse duration of > 10mins (Parate et al. 2017).

Reports have shown that ES resulted in changes in membrane protein intensity, ligand binding, ion channel, and gap junction (Balint et al. 2013). The activation of the voltage-gated calcium channels was reported upon extracellular Ca^{2+} constant influx (Xu et al. 2009). This, as suggested by Parate et al. (2017), may explain the inferior effect of continuous EMF to the PEMF, which perturbed the Ca^{2+} influx. Besides this, Redeker et al. (2017) found EMF had no effect on non-OA chondrocytes. This could be explained by the counteracting effect of EMF/PEMF on the pro-inflammatory cytokine release of IL-1β (Ongaro et al. 2011, Veronesi et al. 2015).
1.7. AIM AND OBJECTIVES

The aim of this project is to engineer functional cartilaginous tissue constructs within self-assembled peptide hydrogels. The feasibility of utilising self-assembled peptide hydrogels will be investigated. Hypothetically, by providing suitable mechanical support, self-assembled peptide hydrogels will enhance the chondrogenesis of chondrocytes. In order to achieve this aim, the project will be carried out with two primary objectives, as explained below.

Objective 1

To develop a three-dimensional scaffold using self-assembled peptide hydrogels that will be able to support chondrocyte encapsulation and ECM synthesis and accumulation. In order to complete this, part of the project is to evaluate and enhance the properties of various formulations/ sequences of peptide hydrogel. Chondrocytes will then be encapsulated within optimised peptide hydrogel to assess cells’ morphology within the hydrogel.

Objective 2

To assess chondrocytes interaction with self-assembling peptide hydrogels, and to characterise the effect of charge and presence of arginine in peptide hydrogels on cell behaviour.
CHAPTER TWO
PHYSICAL CHARACTERISATION AND GELATION
BEHAVIOUR OF SELF ASSEMBLING PEPTIDE HYDROGELS

2.1. INTRODUCTION

Self-assembling peptide hydrogels (SAPH) have a high potential in tissue engineering applications, as the properties of peptide hydrogels can be altered to suit the needs of specific applications (Koutsopoulos 2016). The objective of this chapter is to choose a peptide sequence suitable for auricular cartilage regeneration. Firstly, pH titration of different types of peptide will be studied to see the effect of pH on self-assembly and physical gelation of each peptide. Secondly, this chapter will also layout the degradation assessment of different SAPH.

Novel designed self-assembling peptide hydrogels studied in this chapter are based on an octapeptide sequence with phenylalanine (F), valine (V), glutamic acid (E) and lysine (K). These peptides self-assemble forming stable β-sheets at low concentration of peptides dissolved in deionised water. Table 2-1 shows the different peptide sequences that were initially studied.

<table>
<thead>
<tr>
<th>ID</th>
<th>PEPTIDE SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>F8</td>
<td>FEFKFEFK</td>
</tr>
<tr>
<td>FR8</td>
<td>FEFRFEFK</td>
</tr>
<tr>
<td>F9</td>
<td>FEFKFEFKK</td>
</tr>
<tr>
<td>FR9</td>
<td>FEFRFEFKK</td>
</tr>
<tr>
<td>V8</td>
<td>VEVKVEVK</td>
</tr>
<tr>
<td>VR8</td>
<td>VEVVKEVK</td>
</tr>
<tr>
<td>FK</td>
<td>FKFEFKFK</td>
</tr>
<tr>
<td>FR</td>
<td>FEKFRFK</td>
</tr>
</tbody>
</table>

Table 2-1 Table shows different peptide sequences that were initially studied in this chapter.
The alteration of peptide sequences was done to study the effect of charge and the presence of arginine on the gelation behaviour and mechanical properties of SAPH. The sequences were altered based on our novel-designed octapeptide sequence FEFKFK which was studied by Mujeeb et al. (2013).

A variant of peptide sequences was designed based on the original peptide sequence FEFKFK and FEFKFEFK (F8) that was previously used and was shown to be capable of culturing bovine chondrocytes within this peptide hydrogel (Mujeeb 2013, Szkolar 2015). FTIR analysis showed F8 had the tendency to form β-sheet fibres and TEM images also showed that
F8 formed thick fibre bundles (Saiani et al. 2009). Another peptide sequence that was designed for drug delivery applications, VEVKVEVK (V8), was also characterised in this chapter (Roberts et al. 2012). The peptide sequence V8 has all phenylalanine (F) residues in F8 peptide sequence substituted with valine (V). In order to see the effect of charge on peptide self-assembly behaviour, F9 and FK were designed. With an additional lysine (K) at the end of F9, it is a sequence of nine amino acid residues compared to only eight amino acids for F8. F9 has +1 charge while F8 has zero net charge at neutral pH. In this study, all peptide sequences’ net charges were considered at pH 7, to consider the cell culture condition where cell culture media contains a buffer that will assure the media condition is at roughly neutral pH in 5% CO₂ environment for cell growth.

FK has +2 charge at neutral pH compared to the neutral and +1 charge of F8 and F9, respectively. FK is another variant of octapeptide sequence based on alternating hydrophobic and hydrophilic charged amino acid residues. FK will not only be able to show the effect of higher positive charge on self-assembly behaviour, but it will also explain the importance of amino acid position in the peptide sequence. In FK the peptide sequence is FKFKEFKFK, the position of glutamic acid (E) and lysine (K) at the second and fourth residues were swapped; and at the 6th residue, glutamic acid (E, negatively charged) is replaced with positively charged lysine (K).

Besides assessing the effect of charge, hydrophobicity and location of amino acids, a series of peptide sequences were also designed to incorporate arginine to see the effect of its guanidine side chain on self-assembly behaviour. Lysine (K) at the 4th residue of F8, V8 and F9 was substituted with similarly positive charged arginine (R), resulting to FR8, V8R and FR9 respectively. To see the effect of amino acid position, FR, which is designed based on F8, has arginine (+ve charged) substituting glutamic acid (E, -ve charged) at the 6th residue. FR has the same +2 net charge as FK at pH 7.

This chapter will be outlined in four main parts, i) the gelation properties, ii) hydrogel stability, iii) profile of SAPH’s mechanical properties as well as looking at the iv) effect of the presence of cells and serum in SAPH’s degradation profile. As stated in Chapter 1, the properties of peptide hydrogel can be modulated by changing the peptide sequences, concentration and pH. Thus, it is hypothesised that SAPHs’ gelation behaviour will depend on the peptide overall charges and sequences, and the higher its charge, the higher its mechanical properties and stability in cell culture condition due to its interaction with the ionic solution (culture media).

2.2. MATERIALS AND METHOD

Peptide powders (>95% purity) were purchased from Biomatik Corporation (Cambridge, Canada). All supplements for cell culture work were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. Trypan blue solution (Hyclone; Thermo Fisher Scientific, Loughborough, UK) and Live-Dead Assay (Life-Technologies, Paisley, UK) were used for analytical work. For hydrogel preparation, sodium hydroxide (NaOH; Thermo Fisher Scientific,
Loughborough, UK) was used, and hydrogel was pipetted into 12-well cell culture inserts, with a pore size of 1.0µm (Greiner, Stonehouse, UK).

2.2.1. Preparation of hydrogels

Peptide solutions were prepared by suspending the solid phase peptide at 1-3% final concentration in deionised water. The solutions, prepared in 15ml Falcon tubes (Centristar™, Corning®, Sigma Aldrich, Dorset, UK), were vortexed and sonicated for 30 mins to ensure proper mixing. For example, where 5ml of peptide hydrogel was prepared, 2% (w/v) of peptide powder was dissolved into 4.5ml of filtered sterile distilled water. Using 0.5M NaOH, the pH of the solution was adjusted until gels are formed. The gel was then centrifuged at 5000 RPM for 10 seconds to eliminate bubbles, and deionised water was later added to make up to the final volume. Finally, pH adjusted hydrogels were put in a sonication bath (Elma, Schmidbauer GmbH, Singen, Germany) for 60 minutes to allow homogeneous distribution of electrolytes and pH throughout the hydrogel. Once the solution became a gel, the samples were sterilised under UV for 20mins. The samples were exposed under UV light of the laminar hood (Class II Microbiological safety cabinet, Walker Safety Cabinet, Glossop, UK) (Mujeeb 2013).

2.2.2. pH titration

Gelation properties of the peptides studied in this research were analysed using phase diagrams, where the tilting test tube method was utilised. NaOH was added in step-wise addition, and gelation was studied as a function of pH. Upon the first sonication, initial pH of the peptide solution was measured. The pH was measured using a portable waterproof pH/temperature tester (Oakton, Fisher Scientific, Loughborough, UK) after each addition of 20µL of 0.5M NaOH into the solution. Mixing NaOH within the peptide solution becomes less successful when the solution is more viscous. In order to get more precise pH readings, the solution was either left for a few minutes, or NaOH was added at a lower rate (smaller volume, e.g. 5µL step addition). The tilting test tube method was characterised by defining self-supporting samples upon inversion and gentle tapping as a gel. Whereas if the sample flowed slowly upon inversion, then it was determined as a viscous liquid.

2.2.3. Hydrogel stability test by measuring hydrogel wet weight

Throughout the study, hydrogel samples were immersed in complete media. Complete media consists of Dulbecco's Modified Eagles Medium (Sigma, D5921 – 1000mg glucose without phenol red, DMEM), 2% L-glutamine, 1% sodium pyruvate, 1% pen-strep and 10% (v/v) foetal bovine serum (FBS). Initially, the media was changed three times, every 10 minutes to obtain a consistent pH. For pre-cultured samples, hydrogels were prepared as described in Section 2.2.1 and pipetted into a cell insert within a 12-well plate. Media was changed three times, and the samples were incubated at 37°C overnight. Weighing was carried out the next day on a four-decimal-place analytical balance (TE 1245, Sartorius, Surrey, UK).
For non-pre-cultured samples, hydrogels were weighed 2 hours later (t=0), to obtain \( W_{t=0} \). To get an accurate reading of \( W_{t=0} \), the weight of the inserts was subtracted. Media was changed three times a week. At the end of the week, samples (n=3) were weighed to get \( W_{t=N} \). N is the number of the week.

For each peptide sequence, 12 samples of 500\( \mu \)L hydrogel were pipetted from two separate tubes of hydrogel. The two tubes were prepared independently from the peptide weighing step. Out of the 12 samples, two samples were weighed every week. At every time point, samples were weighed, and weight percentage loss was calculated based on the initial weight. Media for all samples was changed three times a week to mimic cell culture conditions, and samples were kept at 37°C with 5% CO\(_2\) and hydrogels were prepared using sterile-filtered distilled water in a laminar hood.

After these factors were assessed during initial studies, the next study was carried out using the optimised protocol as explained in Figure 2-1. The stability of four peptide sequences was tested in cell culture conditions, without the presence of cells, in a sterile environment and physiological pH. F8, F9, FR and V8 were prepared at 3% (w/v) concentration as explained in Section 2.2.1, then 500\( \mu \)L of the gel was pipetted using a precision positive displacement pipette (Microman\(^\circledR\); Gilson, Bedfordshire, UK) into 12-well Milipore\(^\reg\) cell culture inserts (pore size 0.4\( \mu \)m, Watford, UK), to obtain a cylindrical gel with a height of ~5mm. For all studies in this section, prior to addition of gel, each cell insert was weighed to obtain \( W_{\text{insert}} \). Prior to measuring gels' wet weight 100\( \mu \)L of the media was removed and the inserts were left in an empty well for 5 minutes.
2.2.4. Cell work

2.2.4.1. Cell isolation

The cells were isolated on the same day the 18-24 month old calf was slaughtered. Articular cartilage was dissected from the metacarpal phalangeal joint in a clean environment. The dissected cartilage was washed twice using sterile Dulbecco’s phosphate-buffered saline (DPBS) to remove any foreign particles or synovial fluid from the joints. The dissected cartilage was immersed in complete media to retain the viability of the cartilage. Complete media consists of Dulbecco’s Modified Eagles Medium (Sigma, D5921 – 1000mg glucose without phenol red, DMEM), 1% L-glutamine, 1% sodium pyruvate, 1% pen-strep, ascorbate-2-phosphate (0.086mM) and 10% (v/v) foetal bovine serum (FBS).

Subsequently, the media was substituted with 20U/ml protease and incubated at 37°C in a shaker for an hour. The cartilage dissection went through double digestion steps; with the second step being immersion within collagenase type II (100U/ml) constituted within complete media. The tube containing this suspension was incubated overnight in the shaker at 37°C to ensure complete digestion by the enzyme.

The next day the suspension containing chondrocytes was passed through a 70µm cell sieve (BD Bioscience, Oxford UK) into a sterile Falcon tube. The suspension was washed twice with complete media to stop the reaction of any remaining enzyme and centrifuged at 200G for 5
minutes. Cells were then counted using Trypan blue (0.4% solution, HyClone, Fisher Scientific, Loughborough, UK) and haemocytometer passaged at 0.05 x10^6 cells per cm^2 in vitro to increase the number of cells.

2.2.4.2. Cryopreservation method

After expansion, cells were detached from T flask using Trypsin (2ml to cover a T75 flask) for less than 5 mins. Detached cells were re-suspended in complete media. Cells were counted using a haemocytometer and aliquoted into cryogenic storage vials in 10% dimethyl sulfoxide (DMSO) and complete media at 1x10^6 cells per ml. Vials were transferred into a Mr Frosty™ Freezing container (Thermo Fisher Scientific, Loughborough, UK) and left overnight at -80°C, before being transferred to liquid nitrogen storage.

2.2.4.3. Monolayer passaging

A vial of cryopreserved bovine chondrocytes was resuscitated in 10ml complete media (DMEM (Sigma, D5921 – 1000mg glucose without phenol red), 2% L-glutamine, 1% sodium pyruvate, 1% penicillin-streptomycin-fungizone (PSF), ascorbate-2-phosphate (0.086mM) and 10% (v/v) FBS. After centrifugation (300G, 5 mins), the supernatant containing DMSO was replaced with fresh media. Re-suspended cells were cultured in a T75 flask (Corning®, Fisher Scientific, Loughborough, UK) in complete media. DMEM without phenol red was used to enhance visibility when samples were stained fluorescently and viewed using a fluorescent microscope.

When cells reached 90% confluence (generally after culturing for six days) they were split in a 1:6 ratio. Upon detaching using trypsin, media containing FBS was then used to stop trypsinisation and centrifuged (300G, 5 mins) to wash away trypsin prior to cell counting. Cell count was ascertained using a haemocytometer and trypan blue dye exclusion, where 10µl samples of the cell suspension were pipetted and mixed with trypan blue solution at a ratio of 1:1 (v/v).

2.2.4.4. Cell encapsulation

Bovine chondrocyte suspension (100µL) was physically mixed into 1ml of prepared hydrogels in 50µL steps, giving a final cell density of 1 million cells per ml. Cell-gel mix was then aliquoted at 200µL into each 12-well insert, using a positive displacement pipette (Microman®, Gilson, Bedfordshire, UK).

Hydrogels mixed with 100µL of complete media without any cells were used as a control.

2.2.5. Cell viability assay

Live/Dead viability kit for mammalian cells was used to assess the viability of chondrocytes encapsulated in hydrogels. Live/Dead assay contains ethidium homodimer (EthD, high-affinity nucleic acid stain) which penetrates through a broken cell membrane and fluoresces upon binding to DNA to emit red fluorescence (Kummrow et al. 2013). The second part of the Live/Dead assay is the Calcein AM. Upon entering the cell, acetoxymethyl ester is hydrolysed
by intracellular esterases, which then converts calcein AM to green-fluorescent calcein (Gillissen et al. 2016). Different sequences of peptide hydrogels (F9, FK and FR) were encapsulated with 1 million cells per ml of bovine chondrocytes. At each time point, (0, 7, 14 and 21 days of culturing), samples were washed at least 3 times with DPBS (15-20 mins for each wash). Samples were then incubated with Live/Dead viability kit (5µM EthD-1 and 2µM of Calcein AM) for 30 mins at room temperature. Images were then obtained using a confocal microscope (Leica, Milton Keynes, UK). Calcein fluorescent signal was observed at 525nm EX, 580-640nm EM and EthD-1 at 625nm EX, 660-725nm EM wavelength. Presented figures were attained from z-stack images of a hydrogel thickness of around 500µm, and these images were then stacked together into a maximum projection.

2.2.6. Small amplitude oscillatory shear (SAOS) rheology

Upon preparation (see Section 2.2.1), hydrogels were left overnight at 4°C to allow for homogenisation of pH and stabilisation of the self-assembly reaction. Then, 200µL of the hydrogels were pipetted into a 12-well insert (Greiner, Stonehouse, UK). After washing with media three times, hydrogels were incubated in complete media. The elastic modulus (G') of each hydrogel sample was measured upon preparing the hydrogel solution and after immersing these hydrogels in complete bovine chondrocyte culture media overnight at 37°C, 5% CO₂ incubator. Samples were assessed using a rheometer (Discovery HR2, TA Instruments, Herts, UK) and 20mm parallel plate geometry. The gap size was set at 500µm and temperature was maintained at 37°C during testing. In order to control the temperature and minimise evaporation, a solvent trap was used.

The rheological test was run in two stages. First, amplitude sweeps were performed at a fixed frequency of 1Hz at 0.1-10% strain range. Amplitude sweep was carried out to determine the linear viscoelastic region (LVR). Based on the LVR value, frequency sweep was carried out at 1% strain to restrain from breaking the hydrogel fibril network. This value was determined through strain sweeps of the sample, and the G' value at 0.2% shear strain, 1Hz frequency was recorded.

Samples were also tested before exposure to cell culture media to assess the effect of media.

2.2.7. Hydrogel degradation profiles

The rate of degradation of a scaffold upon in vitro or in vivo implantation is important (Dhandayuthapani et al. 2011). Abrupt or fast complete degradation is undesirable as implanted cells may need some time to build their own ECM. The purpose of this section is to evaluate the factors that may promote degradation of SAPH. Factors such as the presence of cells and serum, which would best simulate the human physiological environment, were studied in this project. To measure the rate of degradation, samples’ rheological properties (see Section 2.2.6), as well as monomeric quantification using size exclusion chromatography, were analysed.
2.2.7.1. Sample preparation

Hydrogels were aliquoted into four different Falcon tubes with 1 ml of gel in each tube. The setup for this study is summarised in Table 2-2. Tubes 1 and 2 did not contain any cells. For tube 1, 100µL of media without FBS was mixed into the hydrogel, while media with FBS was used for tube 2. Tube 4 was used as the standard culture of bovine chondrocytes. Encapsulation of 4 million bovine chondrocytes per ml was achieved by resuspension of cells into 100µL of complete media and then mixed gradually into the hydrogel. Tube 3 also contained bovine chondrocytes at the same seeding density as tube 4, but cells in tube 3 were washed to remove serum prior to seeding into hydrogels. The experimental setup is pictured in the schematic diagram Figure 2-2.

<table>
<thead>
<tr>
<th></th>
<th>FBS</th>
<th>Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1</td>
<td>×</td>
<td>×</td>
</tr>
<tr>
<td>Tube 2</td>
<td>√</td>
<td>×</td>
</tr>
<tr>
<td>Tube 3</td>
<td>×</td>
<td>√</td>
</tr>
<tr>
<td>Tube 4</td>
<td>√</td>
<td>√</td>
</tr>
</tbody>
</table>

Table 2-2 Experimental setups to assess the effect of serum and cells in SAPH degradation behaviour. Samples for this study were treated in four different conditions to assess the effect cells and serum in SAPH's degradation profile.

![Schematic diagram](image)

Figure 2-2 Schematic description of the experimental setup to assess self-assembled hydrogel degradation profiles. Media containing +/- FBS and +/- bovine chondrocytes were assessed to observe the effect of the presence of cells and serum in the degradation profile, to simulate the human physiological environment. Samples 1 and 3 were immersed in DMEM without FBS, while samples 2 and 4 were cultured in DMEM with FBS. Additionally, 4 million cells per ml (in 100 µl) were encapsulated within SAPH in samples 3 and 4. Media without cell (100 µL) was mixed in gels for samples 1 and 2.

2.2.7.2. Measuring intact peptide using high-performance liquid chromatography (HPLC)

Two different samples were measured at each time point (day 3, 7, 14 and 21, two independent replicates), with the measurement taken at day 0 considered as the baseline. At each time point, samples were dissolved in water/acetonitrile (50/50 v/v) with 1% trifluoroacetic acid (TFA) in mass spec vials, diluting to 1mg/ml concentration and kept at -20°C until measurement.
An Ultimate 3000 high-performance liquid chromatography (HPLC) system (Dionex, Thermo Fisher Scientific, Loughborough, Leicestershire, UK) equipped with LPG-3400SD pump, WPS-3000SL autosampler and diode array detector DAD-3000 (Thermo Fisher Scientific, Loughborough, Leicestershire, UK) was used to measure intact peptide.

The stationary phase consisted of reversed-phase 4-µm Jupiter® 90Å (250 x 4.66 d.i. mm), provided by Phenomenex (Macclesfield, Cheshire, UK).

2.2.7.2.1. Chromatographic condition

Elution was achieved by a gradient mobile phase composed of acetonitrile (ACN), HPLC water and trifluoroacetic acid (TFA); at 1 ml/min flow rate.

The peptide was eluted with a linear gradient that went from 90% solvent A/10% solvent B to 30% solvent A/70% solvent B in 45 min. Solvent A was H₂O, and solvent B was ACN, both containing 0.05% TFA. The amount of non-degraded peptide was expressed as a percentage of intact peptide using Equation 1, which indicated the peptide stability in different culture conditions.

\[
% \text{intact peptide} = \left( \frac{AUC_t}{AUC_{t0}} \right) \times 100\%
\]

Equation 1 The equation is used to calculate intact peptide.

The intact peptide being calculated at time points t=3, 7, 14 and 21 days, where t₀ is day 0. AUCₜ and AUC₋₀ are obtained from the peak area of the peptide as indicated by Chromeleon software (Thermo Fisher Scientific, Loughborough, Leicestershire, UK).

Peak area at each time point, as calculated by Dionex Chromeleon 6.70 software was considered as the area under the curve (AUCₜ, t is either day 3, 7, 14 or 21), while AUC₋₀ was taken at day 0, where samples were incubated for less than 60 mins.

2.2.8. Statistical analyses

Data were analysed using the Kruskal-Wallis significance test with the significance level of \( p=0.05 \). A non-parametric t-test was used to compare the significance between groups/samples.

2.3. RESULTS AND DISCUSSION

2.3.1. Gelation behaviour depends on overall charges of peptide sequence and the hydrophobicity of amino acid residues

The aim of this part of the study was to correlate the pH to the theoretical net charge of each peptide sequence. In addition to this, pH titration was performed to observe the gelation and self-assembly of different peptide sequences. Peptides’ net charge depends on the number of charged amino acids contained in the peptide and the pH of the environment. The net charge of the peptide was calculated based on Equation 2 (Moore 1985). These equations are derived from the Henderson-Hasselbalch equation.
\[ Q_{\text{peptide}} = \sum Q^- + \sum Q^+ \]

\[ Q^- = \frac{(-1)}{1 + 10^{-(p\text{H} - p\text{K}a)}} \]

\[ Q^+ = \frac{(1)}{1 + 10^{(p\text{H} - p\text{K}a)}} \]

**Equation 2:** Formula used to quantify net charges of the peptide, \( Q \), based on the Henderson Hasselbalch equation.

\( Q^- \) is the charge for negatively charged side chain and \( Q^+ \) is for the positively charged side chain. \( p\text{H} \) is determined through measurement using portable \( p\text{H} \) meter, and \( p\text{K}a \) values in Table 2-4 are referred.

Isoelectric point (pI) is the \( p\text{H} \) at which the net charge on a molecule is zero. The calculation of net charge of these peptides includes the charges of N and C termini. Neutralisation of charged amino acids also depends on the \( p\text{H} \) of the environment (see Table 2-3).

<table>
<thead>
<tr>
<th>Low pH</th>
<th>High pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>-COOH</td>
<td>-COO- + H+</td>
</tr>
<tr>
<td>-NH3+</td>
<td>-NH2 + H+</td>
</tr>
</tbody>
</table>

**Table 2-3 pH of the environment governs the neutralisation of charged amino acids.**

Number of moles of the peptide in each sequence was calculated based on the total molecular weight of all amino acids in each peptide. Table 2-4 shows the molecular weight of each amino acid used in this study.

Figure 2-3 shows the correlation of \( p\text{H} \) to the net charge of the peptide. For example, to calculate the molecular net charge of V8 the ionisable groups N terminus –NH\( _3^+ \) (pKa=9.13), C terminus –COOH (pKa=2.8), two glutamic acid (Glu, E) –COOH (pKa=4.25) and two lysine (Lys, K) –NH\( _3^+ \) (pKa=10.53) were taken into account (See Table 2-4).

Theoretically, F8 and V8 contain no charge at neutral \( p\text{H} \). In the experimental setup, F8 peptide solution started to gel at \( p\text{H} \) 3.5 (see Figure 2-3 (a)). Further addition of NaOH resulted in the gel becoming cloudy. However, this was not observed for V8 peptide solution where it formed a gel from \( p\text{H} \) 5.0 and remained clear up to \( p\text{H} \) 8.0 (see Figure 2-3 (c)). Inside aqueous media, two \( \beta \)-sheet fibrils come together to form a single fibril to bury hydrophobic side chains. Phenylalanine (F), which is more hydrophobic, has one of the larger amino acid side chains. Phenylalanine’s rigid aromatic side chain contributes to its hydrophobicity and tends to result in it being buried in the interior of a folded peptide. This may have explained the cloudiness of the F8 hydrogel.
<table>
<thead>
<tr>
<th>Structure</th>
<th>Amino Acid</th>
<th>1 letter code</th>
<th>Molecular weight (g/mol)</th>
<th>pKa of charged side chain</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Phenylalanine" /></td>
<td>Phenylalanine</td>
<td>F</td>
<td>165.2</td>
<td>NA</td>
</tr>
<tr>
<td><img src="image2.png" alt="Glutamic acid" /></td>
<td>Glutamic acid</td>
<td>E</td>
<td>147.1</td>
<td>4.25 (–COOH)</td>
</tr>
<tr>
<td><img src="image3.png" alt="Lysine" /></td>
<td>Lysine</td>
<td>K</td>
<td>146.2</td>
<td>10.53 (–NH₃⁺)</td>
</tr>
<tr>
<td><img src="image4.png" alt="Arginine" /></td>
<td>Arginine</td>
<td>R</td>
<td>174.2</td>
<td>12.48 (–NH⁺=C- (NH₂)₂)</td>
</tr>
<tr>
<td><img src="image5.png" alt="Valine" /></td>
<td>Valine</td>
<td>V</td>
<td>117.1</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 2-4 Molecular weight in g/mol for each amino acid are stated in this table. Total molecular mass was calculated and taken into account for the molar ratio of NaOH: peptide. The table also shows pKa values of the charged side chain of each amino acid, which are used to calculate net charge at specific pH using Equation 2.
Figure 2-3 pH titration and the theoretical value of net charge of different peptide sequence.

Figure shows pH titration for F8 (a), FR8 (b), V8 (c), VR8 (d), F9 (e), FR9 (f), FK (g), and FR (h). Graphs show the theoretical value of net charge based on pKa value of each charged amino acids and the experimental pH titration as a function of molar ratio of NaOH to peptide, pH titration as a function of peptide net charge (orange line) and molar ratio of NaOH to peptide (blue line). The dotted line shows the theoretical value of pKa for N terminal and solid line for carboxylic chain of glutamic acid, while shaded areas signify the pH where the peptide solution formed a gel.
In F-moc dipeptide systems as reported by another group, rheological analysis showed that when phenylalanine (F) was used instead of alanine (A), hydrogel strength increased. It was also shown in the study that increased overall hydrophobicity would expedite self-assembly and later in forming a gel (Nguyen et al. 2014). Increased mechanical properties may be explained by π stacking of the aromatic ring of phenylalanine. Besides that, the aromatic structure of phenylalanine side chain has been shown to increase the β-sheet propensity (Lakshmanan and Hauser 2011, Micklitsch et al. 2015).

Referring to Figure 2-3 (e), F9 started to gel at around pH 5.0, yet the pH could be pushed further to neutral pH by adding NaOH. F9 gel was also stable without exhibiting a cloudy phase. The addition of another charge results in the peptide having an overall +1 charge at pH 7. The additional hydrophilic amino acid residue reduces the overall hydrophobicity of the peptide which prevents F9 from precipitating at pH 7.0.

The difference in peptide length of F9 (nine amino acids) and F8 (eight amino acids) will contribute to the increase of the diameter of the fibril, by 0.5nm. F8 has 3.5-4.0nm diameter, while F9 has 4.0-4.5nm fibril diameter (Guilbaud et al. 2013, Gao et al. 2017). It has previously been shown that thinner (and longer) fibril leads to higher entanglement and contributes to higher mechanical properties (Micklitsch et al. 2015). Micklitsch et al. (2015) found VK13 to not only have faster gelation than VK11, but G’ of VK13 was also higher. As the names suggest, β-hairpin forming peptides VK11 and VK13 have 11 and 13 amino acids, respectively, with valine at the beginning and the end of the peptide sequence (Geisler and Schneider 2012). The study suggests that the longer a peptide is, the higher its surface area, which would then lead to the increased formation of crosslinks within the network.

The net charge of these peptide sequences may contribute to self-assembly pattern. F8 gels at a lower pH and turns cloudy as pH increases. Cloudiness is an indication of aggregation of the fibril. Cloudiness or fibril aggregation may be a sign of inhomogeneous peptide assembly throughout the gel or initial sign of precipitation. When the hydrogel is cloudy, the mechanical properties may be different throughout the whole sample, and the gel may precipitate when culture media is introduced. Besides, cloudy hydrogel will perturb imaging of the cells or protein in the hydrogel. Cloudiness is defined as reduced light transmittance through the peptide solution. This may be explained by large amorphous aggregates which stop the light from being transmitted through the solution, thus observed as a cloudy solution. The cloudy solution would usually lead to precipitation of peptide solution when pH of hydrogels was further increased. Thus, it is essential to assess any aggregation through dynamic light scattering (Saiki et al. 2015). Our finding agrees with results reported from other literature (Branco et al. 2009, Rajagopal et al. 2009, Owczarz et al. 2015). Lower net peptide charge of MAX8 contributes to faster folding and assembling of the peptide (at lower pH), in comparison to higher net charge MAX1 (Branco et al. 2009, Rajagopal et al. 2009).
Similar to F8, the theoretical net charge of FR8 and VR8 is around 0 at pH 7. FR8 also starts to gel at around pH 4.0 and tends to become cloudy at pH 4.5 (see Figure 2-3 (b)). This suggests that replacing lysine (K) with arginine (R) does not prevent cloudy formation, as R is also basic and will only be deprotonated at higher pH (pKa: 12.48). Even though V8 can be made up to pH 7.0 without forming a cloudy gel (most likely due to the lower hydrophobicity of valine compared to phenylalanine), when K is replaced by R in VR8, the gel became cloudy when the pH was over 5.0 (see Figure 2-3 (d)). This is probably due to the higher hydrophobicity of arginine compared to lysine. This effect of replacing K with R was also seen in +1 charged peptide hydrogel FR9. Whereas F9 could go to pH 7.0, FR9 was observed to form a cloudy gel when the pH was higher than 4.5 (see Figure 2-3 (f)). The effect of the presence of R in both neutral and +1 charge peptide (FR8 and FR9) hydrogel were observed as these hydrogels gel at pH 4-5 and cloudiness in FR9 occurred earlier than F9 (with the presence of only K). Another previous report from our group showed that introduction of arginine (R) did not affect β-sheet conformation tendency at the molecular level (at pH <3) (Gao et al. 2017). The study found that when arginine replaced lysine in the non-alternating version of F8 (FEFEFKFK), to produce FEFEFRFK, large bundles and heterogeneous fibre morphology was formed. These findings were based on images obtained from transmission electron spectroscopy (TEM) and small angle neutron scattering (SANS) scattering results. This also agrees with the study by Owczarz et al. (2015) where peptide with lower net charge favoured the formation of amorphous aggregates compared to the peptide with higher net charges which promoted fibrillar structure. RADA-1 (contains arginine) was prepared at different pH and through the measurement of monomeric peptide using size exclusion chromatography; the closer the peptides’ pH to isoelectric point (pl), the higher its tendency to form amorphous aggregates (Owczarz et al. 2015). It was found that peptide net charge correlates with fibril stability, peptide conformation and surface charge distribution. This current study has observed that FR8, FR9 and VR8 formed amorphous aggregates as pH gets closer to pl. The finding of this current study may be explained by this, where arginine incorporation in F8 and V8 (both 0 charged) led to cloudy formation. Both FK and FR start to form gels at pH 4.0. These peptides stay as gel up to neutral pH (see Figure 2-3 (g) and (h). These figures also show that the net charge of FK and FR was +2 when the pH was 7.0. As mentioned before, the presence of R in +1 charged FR9 made the gel form a cloudy solution at a lower pH than F9. However, R replacing K in +2 charged FR, did not give any effect of the pH of peptide gelation (no cloudy formation).

Effect of pH on self-assembly may also be explained by the charge distribution of amino acid residues in these peptide sequences. Based on the EAK16 model peptides, Jun et al. (2004) studied different peptide sequences that had the same net charge, peptide length and stiffness. It was found that each peptide sequence had different morphology, forming either fibrillar or globular nanostructure based on the charge distribution of the peptide.
pH titration of peptides in this chapter is essential as environmental pH of these peptides governs self-assembly. pH titration was performed by recording the pH as a function of NaOH: peptide molar ratio upon addition of 0.5M NaOH. NaOH titration was stopped upon gelation for each peptide hydrogel. Gelation of each peptide hydrogel was judged by either ‘flick-and-tilt’ test, or prior to physical appearance (cloudiness) of the gel. Even though this ‘flick-and-tilt’ test is an easy and quick way to test gelation, this method has its limitations as it may not be reproducible. Gelation would be better tested by measuring the rheological properties of the hydrogel.

In addition, modifying the order of amino acid sequence would impact the self-assembly of the peptide. For instance, the effect of R on the self-assembly of a peptide sequence depends on the location of R within the sequence. pKa shifts were seen in FR8, FR9 and VR8 pH titration. Unlike FR where R is protected between the lysine, the bulky side group of arginine in FR8, FR9 and VR8 has electrostatic interaction with neighbouring glutamic acid. This interaction may have contributed to the pKa shift of these peptides (Aggeli et al. 2003, Tang et al. 2011).

Another explanation for this can also be related to the higher charge of FR in comparison to FR8. A study of charged self-assembling peptides designed based on RADA16-I has shown that +5 charge peptide sequence only form fibres when it is close to the isoelectric point (pl, pH where the net charge is zero) (Zhang et al. 2017). This might explain why the pH of higher charge peptide sequence (FK and FR) can be pushed further (closer to pl) without these hydrogels forming precipitation.

In addition to the change in pl and net charge, it can be concluded from Figure 2-3 that each peptide sequence forms a hydrogel at a different pH value. Some peptide hydrogels precipitate at lower pH than others. The aim in preparing hydrogels is to have them as close as possible to physiological pH (pH 6.5). Even though this value is lower than the physiological pH, if the gels were prepared at higher pH (e.g. 7.0), the gels tend to become cloudy when culture media was introduced. Besides that, the gels’ pH became ~7.0 upon media exposure. Even though F9 can go up to pH 6.8 before precipitating, FEFRFEFK (FR9) could only go to pH 4.8. The pH of FEFRFEFK (FR8) could not be pushed further than pH 4.6. In another study, pH of RADA-1 was modulated using buffers to see the effect of global surface net charge. Quantification of the monomeric peptide by size exclusion chromatography showed that as the net charge approached pl, RADA 16-I formed amorphous morphology at pH 6.0 (Owczarz et al. 2015).

Furthermore, two different peptide sequences were combined to see if it would affect the self-assembly behaviour of these peptide hydrogels. F8 was combined with either FR8, FK or FR. The combination of peptide hydrogels were prepared based on 50% of the whole weight of each combined peptide sequence. Referring to Table 2-5, all of these combined peptide sequences could be prepared up to pH 3.8-4.8 before starting to precipitate. Interestingly, it was found that the presence of F8 in FK and FR reduced the pH of hydrogel formation, even though FK and FR by itself did not form a cloudy solution until pH 6.7 and 6.6, respectively.
Based on all of these peptide sequences, four peptide sequences were chosen for further studies. V8, F9, FK and FR could be made up to a pH near to 7; so, these peptide sequences were used for studies in the next section. V8 has 0 charge, and F9 has +1 charge, while FK and FR have +2 charge at neutral pH.

<table>
<thead>
<tr>
<th>ID</th>
<th>Peptide sequence</th>
<th>Maximum pH ±SD</th>
<th>Net charge at this pH</th>
<th>Corresponding net charge at pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>F8</td>
<td>FEFKFEFK</td>
<td>3.81 ± 0.25</td>
<td>+1</td>
<td>0</td>
</tr>
<tr>
<td>FR8</td>
<td>FEFRFEFK</td>
<td>4.41 ± 0.35</td>
<td>+1</td>
<td>0</td>
</tr>
<tr>
<td>F9</td>
<td>FEFKFEFKK</td>
<td>5.43 ± 0.49</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>FR9</td>
<td>FEFRFEFKK</td>
<td>4.57 ± 0.19</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>V8</td>
<td>VEVKVEVK</td>
<td>7.26 ± 0.63</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VR8</td>
<td>VEVREVK</td>
<td>4.77 ± 0.50</td>
<td>+1</td>
<td>0</td>
</tr>
<tr>
<td>FK</td>
<td>FKFEFKFK</td>
<td>6.77 ± 0.51</td>
<td>+2</td>
<td>+2</td>
</tr>
<tr>
<td>FR</td>
<td>FEFKFRFK</td>
<td>6.64 ± 0.64</td>
<td>+2</td>
<td>+2</td>
</tr>
<tr>
<td>COM-8R</td>
<td>F8 + FR8</td>
<td>4.70 ± 0.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COM-8K</td>
<td>F8 + FK</td>
<td>4.18 ± 0.08</td>
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</tr>
<tr>
<td>COM-R</td>
<td>F8 + FR</td>
<td>3.76 ± 0.15</td>
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<td></td>
</tr>
</tbody>
</table>

Table 2-5 Peptide sequences for further studies were chosen based on the final pH where gels were formed. Table shows final pH of different peptide sequences after adding 0.5M NaOH. Data are pooled from at least 5 samples, prepared independently. For guidance, net charge and abbreviation code (ID) of each peptide sequence is shown. COM-X represents two peptide sequences combined.

2.3.2. Physical properties of peptide hydrogels

2.3.2.1. Some peptide hydrogels are less stable than others

Previous works have shown that SAPH would exhibit abrupt degradation and less stability compared to other types of hydrogels (Jung et al. 2008, Nagy et al. 2011, Goktas et al. 2015). However, in order to culture cells within these scaffolds, SAPH would need to be stable for at least 5 weeks to support the cells while they produce extracellular matrix (ECM).

Stability of hydrogels is even more important when the application of mechanical bioreactor is considered. A stability study of these hydrogels was investigated by analysing the weight changes of each sample of SAPH. Thus, the stability of four peptide sequences was tested in cell culture conditions, without the presence of cells, in a sterile environment. Upon preparing, the gel was immersed in media to adjust to pre-determined pH (see Table 2-5). F8, F9, FR and V8 were prepared at 3% (w/v) concentration as described in Section 2.2.1. F8 was included in this study for comparison. For this study, 500µL of hydrogel was pipetted into a 12-well cell insert using a 1000 µL-tip with a positive displacement pipette. Physical degradation of the gels was investigated based on gel weight. The values in Figure 2-4 were taken from the measured weight of hydrogels in comparison to their initial weight (t=0).

F8 showed non-significant decreases in weight percentage until week 4, where F8 samples reduced to 21.7 ± 13.0% of its original weight (p<0.05). V8 samples, on the other hand, lost
more than 95% of their initial weight, even after 1 week of study. Afterwards, all V8 samples were degraded. This might be due to the substitution of phenylalanine (F) with less hydrophobic amino acid, valine (V).

Compared to neutral charged F8, +1 charged F9 samples did not have significant changes to their percentage weight throughout the 6 weeks of study. This trend of stability was also observed in +2 charged FR. This suggests that the presence of charge in the peptide sequence may contribute towards the stability of peptide hydrogels. FR, however, reduced significantly at 6th week (26.3 ± 37.2%) in comparison to the previous week weighing 83.4 ± 36.0% of initial weight.

It was also observed from Figure 2-4 that FR had significantly higher weight percentage compared to F8 at both week 3 and 4 (besides V8). This difference of stability may be due to either differences in charge or presence of arginine. At week 5, F8 had 30.9 ± 11.5%, while F9 and FR had 90.1 ± 0.1% and 83.4 ± 36.0% of initial weight, respectively. At week 6, when the study ended, each peptide sequence (F8, F9 and FR) only had one sample left, while the other sample for each peptide sequence were degraded.

![Figure 2-4 Percentage weight loss of peptide hydrogels](image)

Figure 2-4 Percentage weight loss of peptide hydrogels FEFKFEFK (F8), FEFKFEFKK (F9), FEFKFRFK (FR) and VEVKVEVK (V8) measured each week.

Media was changed 3 times a week. At each time point, different samples were weighed (n=2), and the weight difference was based on the initial weight of each sample. The total number of samples for each peptide sequence was 12 samples. Significant changes in weight percentage in comparison to the previous week for V8 (*) and FR (**) (p<0.05). (***) denoted significant reduction of weight percentage in F8 at week 4 compared to week 0. (#) defined that V8 was significantly lower than other samples (marked by #), F9 and FR (##) and F9 (###) at the specific time point. At week 3 and 4, F8 was also significantly lower than FR (marked by $). Error bars represent standard deviation of two samples at each time point.

This long-term stability study provides information on the stability of these hydrogels and will allow development of new formulations enabling longer cell culture durations. The work shows that up to 5 weeks, F9 had the least weight loss or the highest stability, and V8 had the least stability. Based on this initial work, V8 was shown to be not as stable as the rest of the peptide sequences. To encapsulate cells, long-term stability is important, especially when mechanical stimulation work such as perfusion work would be applied in the future. It is however noted that this result is based on n=2, which is considered a small number of samples. Higher sample size will be considered in the future to validate this result.
Figure 2-5 shows images of F8, taken at week 6 (a), and F9 (b), FR (c) and V8 (d) taken at day 11. On day 11, F9 and FR were still present, but not V8.

Note that all samples were prepared at the same concentration. The presence of bulkier arginine (R) amino acid within the peptide potentially results in a higher elastic modulus than a peptide without arginine. Further studies need to be carried out to examine hydrogel stability for different peptide sequences. This preliminary result may suggest that peptide sequences with a higher charge, containing R, potentially increase the stiffness of hydrogel and allow a longer duration of culture.

Figure 2-5 Hydrogel stability testing for different types of peptides. (a) shows 12 well-plate where all media was drained during media change which is carried out 3 times a week. This image was taken at Week 6 where only 7 samples remained as those samples in well A2, A3, B3, C1 and C2 had dissipated. (b) FEFKFEFK (F9) at day 11 in cell insert, (c) FEFKFRFK (FR) at day 11 in cell insert and (d) shows two replicates of VEVKEVK (V8) that were no longer present on day 11.

The decreased stability of V8 observed could be explained by its critical gelation concentration (CGC). CGC is the concentration threshold of peptide hydrogel where it will start to gel. This, as explained by Fung et al. (2003), is due to hydrophobic and hydrophilic characteristics of the fibre. When V8 hydrogel is prepared at a concentration below CGC, thin filaments are formed instead of fibrils (Fung et al. 2003). Thus, to further assess V8 as a potential peptide hydrogel, preparing this peptide hydrogel at a higher concentration would increase hydrogel stability and increase its mechanical properties (Hickling et al. 2014). However, this may not be cost-effective.

In terms of the amino acid itself, valine is less hydrophobic than phenylalanine. A study that was carried out using the coarse-grained model by Monte Carlo simulations has shown that peptide
fibril formation capability is weakened when a less hydrophobic amino acid is being used (Mu and Yu 2014). In their study, substituting glycine (G) with phenylalanine (F) showed that the hydrogel formed was more elastic when F was used. This may be contributed by F being a rigid amino acid due to bulky phenyl ring side group, and F is very well known for its β-sheet propensity (Tang et al. 2011). In addition to the bulky side group, the better stability of phenylalanine-containing peptide sequence can also be explained through π-π interactions between the aromatic ring of phenylalanine of neighbouring β-strands (Lakshmanan and Hauser 2011, Tang et al. 2011).

On the other hand, another study was carried out by Bowerman et al. (2011) on Ac-(XKKK)2-NH2 with X being either valine, isoleucine, phenylalanine, pentafluorophenylalanine or cyclohexyl alanine (Bowerman et al. 2011). It was observed through Fourier transform infrared spectroscopy (FTIR) and circular dichroism spectroscopy that while the others formed β-sheets, only the valine peptide produced random coils. Their study also showed that when two amino acids with the same hydrophobicity are being compared (Ile versus Phe), aromatic containing peptides produce hydrogels with higher mechanical properties. The aromatic side chain may offer more surface area for a higher occurrence of fibril entanglements and determine the fibre morphology.

As mentioned above, these patterns of stability may change in the presence of cells. Shoichet et al. (1996) have reported decreased stability of hydrogel in the presence of cells. Within the same study, they also observed that changing media frequently reduced hydrogel stability, compared to their controls where media was not changed over 21 days. Not changing media for a period of time, however, could then lead to infection, where the presence of bacteria would produce enzymes that will accelerate hydrogel degradation. Throughout these studies, all media used contained Pen-strep to control contamination.

From this study, V8 was discounted and would not be studied further.

2.3.3. Rheological properties of peptide hydrogels

2.3.3.1. The effect of culture media on storage modulus of peptide hydrogels

Based on the final pH (see Table 2-5), three different types of peptide hydrogels were assessed in this study. F9, FK and FR were chosen as they can be prepared up to physiological pH. Cells sense and respond to mechanical properties of hydrogels (Bryant et al. 2004, Vichare et al. 2014). The viscoelastic properties of these peptide hydrogels can be changed by modulating their concentrations (Mujeeb et al. 2013, Boothroyd et al. 2014). Effect of concentration of peptide hydrogels on their mechanical properties was analysed. All samples were prepared as described in Section 2.2.1, at three different concentrations; 10, 20 and 30mg/ml. Samples were incubated overnight in complete media at 37°C, 5% CO₂ before their rheological properties were assessed for t=0 (with media).
Figure 2-6 shows storage modulus (G') of peptide hydrogel prepared at pH 5.5. Upon preparing hydrogels and adding NaOH, samples were analysed for their viscoelastic properties. Before adding media, storage modulus for hydrogel increased with increasing hydrogel concentration, regardless of the peptide sequence. The low G' of these samples before adding media suggests that these peptide hydrogels have shear thinning properties and ability to recover upon injecting.

After being exposed to cell culture media overnight (LG DMEM + 10% FBS), the effect of the presence of salt within media on the mechanical properties of the hydrogel was assessed again using a rheometer. For all peptide sequences, it was observed that increasing peptide concentration increases its mechanical properties. Thus, this study shows that viscoelastic properties of peptide hydrogels can be controlled by their concentrations.

It can also be seen from Figure 2-6 that there is a correlation in the mechanical properties of hydrogels before and after adding media. The effect of exposure to media increased the storage modulus of all three peptide hydrogels by two orders of magnitude, regardless of the peptide sequence. The increase in storage modulus of these peptide hydrogels upon adding media can be explained by the change of pH, from 5.5 to 7.2 in media as well as the increase in salt concentration. pH change may affect the repulsion of peptide random coils which consequently form peptide fibrils (Schneider et al. 2002, Aggeli et al. 2003).

This effect of media on storage modulus can also be explained by the salt content of media contributing to increasing storage modulus of peptide hydrogels (Jonker et al. 2012). Low glucose DMEM (D5921, Sigma Aldrich, Dorset, UK) contains inorganic salts. The effect of salt addition on the modulus of peptide hydrogels was also seen by Ozbas et al. (2004). In their study, MAX1 was first dissolved in deionised water, and the concentration of salt was manipulated by adding buffer/salt solutions. They found that the ionic content and salt in culture media increased storage modulus of MAX1. Upon self-assembly into β-sheet fibrils, hydrophobic and charged amino acids superimposed with adjacent peptide chains through van der Waals and electrostatic interactions (Wang et al. 2014a).
Effect of concentration and presence of culture media in SAPHs’ rheological properties.

Figure 2-6 Effect of concentration and presence of culture media in SAPHs’ rheological properties. Rheological properties of F9, FK and FR upon triggering gelation by adding 0.5M NaOH (open symbols) and upon exposing to cell culture media for 24 hours (closed symbols). Data is the mean of 3 samples in 2 separate repeats (total n = 6) and presented in log value. Error bar represents the standard deviation of all samples at each concentration. (*) denoted significant different in stiffness of each SAPH compared to other samples at different concentrations. Kruskal-Wallis test showed that G’ for F9 increase significantly with the concentration for both conditions, however for FK and FR, only after being conditioned with media, the hydrogel stiffness increase significantly with increased concentration. After exposing hydrogel to culture media for 24 hours, storage modulus was measured. For the rest of this study, peptide hydrogels with G’ of 10kPA will be used (10mg/ml F9, 20mg/ml FK and FR, dotted line).

Effect of ionic strength on hydrogel mechanical properties/elasticity was also assessed in a different study by Feng et al. (2012), which measured rheological properties, small-angle X-ray scattering (SAXS) and nuclear magnetic resonance (NMR) spectroscopy. Their study showed that as the ionic strength increases, the assembly of peptides becomes slower, producing closely packed thinner and rigid fibres.

Figure 2-6 also shows G’ for hydrogels after addition of media. For these peptide sequences, increasing the concentration from 10 to 30 mg/ml increases their storage modulus. The ability to tune peptide mechanical properties by modulating concentration is an advantage for the application of peptide hydrogels. This phenomenon can be interpreted as the relation of peptide concentration and fibre density. As reported by other studies, scanning electron microscopy (SEM) examination of a cross-section of different concentrations of hydrogel has shown highly dense packed fibrils at higher concentration (Sieminski et al. 2007, Hui et al. 2008, Hule et al. 2009). For this present work, an attempt was made to image the peptide fibre using SEM. However, it was proven to be challenging to prepare the samples, especially with the highly charged SAPH.

With increasing peptide concentration from 10 to 30 mg/ml, storage modulus (G’) for all peptide sequences were found to increase gradually. However, the rates of increase (slope) for G’ between F9, FK and FR were not parallel. A trend of storage modulus was seen for different peptide sequences, regardless of the concentration, ranking F9 > FK > FR. This suggests that the peptide hydrogels’ mechanical properties are not only controlled by their concentrations, it also differs between each sequence. In a study by Sieminski et al. (2008), fibre morphologies of different peptide sequences (RAD16-I, RAD16-II, KFE8 and KLD12) were examined using
transmission electron microscopy (TEM) (Sieminski et al. 2008). The study found that among those sequences being studied, the fibre network in KFE8 is the thickest and longest. Thus, differences in fibre morphology as explained by the previous study may affect the physical properties of different peptide sequences.

For the purpose of comparing different peptide sequences effect on the chondrogenic behaviour of chondrocytes, different concentrations of peptide hydrogels were chosen so that initially these hydrogels would have a similar storage modulus (10kPa). Peptide hydrogels with a storage modulus of 10kPa have previously been shown to be feasible for supporting chondrocytes for cartilage regeneration, even though the stiffness of cartilage was measured to be higher than 10kPa (Mujeeb et al. 2013). This previous study from our group saw chondrocytes with rounded morphology and positive staining of collagen type II (via immunofluorescence) when 3D encapsulated in F8 with initial G’ of 10kPa. As can be seen from Figure 2-6, 10mg/ml F9, 20mg/ml FK and FR all have a storage modulus of 10kPa, and thus these concentrations were used for further tests.

2.3.3.2. The effect of cells encapsulated within peptide hydrogels on their stiffness

We have previously shown that G’ of SAPH changes with its concentration. In this section, we will assess if the stiffness changes in the presence of cells. F9 was prepared at 1% (w/v), and FK and FR were prepared at 2% (w/v) concentration as described in Section 2.2.1. For this experiment, 100µL of bovine chondrocytes suspension or media only were physically mixed into 1ml of SAPH (1 million cells per ml, see Section 2.2.4.4). Cell-gel mixture (volume 200µl) was then pipetted into 12-well inserts and treated with normal cell culture conditions, with media changed 3 times a week.

After culturing in culture media at 37°C, 5% CO₂ for a week, control samples of F9, FK and FR which were mixed with complete media without the presence of cells, were measured to be around 10.3±3.3kPa.

As depicted in Figure 2-7, SAPH encapsulating cells were found to have a higher storage modulus. G’ for all samples appeared to be twice as much as those hydrogels without any cells. FK has G’ of 21.7±4.9 kPa, and FR has a storage modulus of 19.0±2.6 kPa. F9 yielded G’ of 22.2±6.1 kPa. This difference in storage modulus is hypothesised to be caused by the interaction of cells with the fibre. As claimed by Jansen et al. (2013), cells can function as crosslinker that enhances the stiffness of the hydrogel. Also, the increase in stiffness may also be explained by the production of collagen by cells encapsulated within F9 compared to those within FR.
Rheological properties of hydrogels prepared from different peptide sequences, with or without cells.

Each self-assembled peptide hydrogel was encapsulated with bovine chondrocytes at a seeding density of 1 million cells per ml of chondrocytes. Storage modulus of each sample was measured using a rheometer after culturing for 7 days. At this time point, $G'$ of all SAPH encapsulating cells were higher than those not seeded with any cells. For each peptide hydrogel, data was taken from 4 samples. Error bars represent standard deviation of all samples for each condition. (*) shows that all samples encapsulating cells were significantly different to unseeded samples ($P$ value < 0.05).

Cell-mediated stiffening of SAPH as shown in this study may be explained by cell network formation or collagen deposition by encapsulated cells. One way to study cell network formation within SAPH is to examine the cells’ morphology. This is extensively studied in Chapter 3 and Chapter 4. However, it is also worth noting that morphology of cells encapsulated within SAPH might change upon exposure to strain under rheometer, as cells resist forces (Jansen et al. 2013). Besides, imaging work showing cell interaction with the fibre may help to give more insight into the increased stiffness of SAPH-cell.

Another factor to be considered would be the gap size between the rheological plate. For this study, gap size 500 $\mu$m was used. Theoretically, gap size should be set at $\geq 10$ times the size of particles of the tested sample. To address the concern of whether this rheological measurement is quantifying the bulk properties of the cell-hydrogel mix or encapsulated cells only, p-value comparing values of $G'$ across samples with cells was calculated. It was found that samples (p-value < 0.05) were significantly different compared to other samples. If this gap size is measuring only cells, then the reading for all peptide hydrogels should be the same. However, as shown in Figure 2-7, this was not the case. Thus this shows that the gap size in this study is valid for this measurement.

Also, results obtained in this section agree with a study reported by Aurand et al. (2014). The paper described that storage modulus of hyaluronic acid (HA) and poly(ethylene glycol) (PEG) increased in the presence of cells when measured at three weeks point of culture. In the future, the rheological properties of peptide hydrogels would be measured upon encapsulation with different cell seeding density. For this chapter, cells were seeded at 1 million cells per ml seeding density. This seeding density was based on previous works (Mujeeb 2013, Markey 2014, Szkolar 2015).
Furthermore, F9, FK and FR samples that were mixed with complete media without the presence of cells, produced an initial storage modulus of 10kPa, which is parallel with the results obtained from the previous section. As shown in Figure 2-6, F9, FK and FR would give a storage modulus of 10kPa when prepared at 1%, 2% and 2% respectively.

2.3.4. Degradation profile of peptide hydrogels in cell culture condition over time

2.3.4.1. Rheological measurement

In terms of cell-scaffold interactions, researchers have shown that cells not only respond to chemical cues such as the presence of ligand and physical cues (matrix stiffness); cells are also dependant on the degradability of the hydrogel system. The rate of biodegradation and de novo ECM production should be equivalent (Zhu 2010, Zhu and Marchant 2011). As the biomaterial scaffold degrades, it leaves space which would then be filled with newly produced ECM. Thus, in this section, degradation profiles of three different sequences of self-assembled peptide hydrogels were assessed over the culture period of 21 days.

In order to test the effect of different culture conditions on degradability of F9, FK and FR, these gels were cultured in DMEM with/without serum and with/without bovine chondrocytes seeded in each hydrogel. As explained in Section 2.2.7, 4 million cells per ml were encapsulated in the hydrogel and cultured in cell culture conditions.

The degradation profile of F9 in the absence of both cell and FBS (-FBS/-cell) as shown by the rheological properties showed a significant increase to 10.9±1.8kPa at day 21 (p<0.01). As depicted in Figure 2-8 (a), G′ for F9 decreased insignificantly at day 14. At day 21, its stiffness was similar to the same sample measured at day 0 (10.1±0.86kPa, p<0.01). The increasing G′ may be explained by the addition of salt contained in DMEM when DMEM is replaced 3 times a week. As made clear in section 1.5.5.1, mechanical properties of self-assembled peptide hydrogel can be modulated by pH and presence of salt. The stiffness of FK (-FBS/-cell) was significantly decreased at day 14, and this value was constantly decreased until day 21. FR in the same condition also had a constant value up to day 14, and its G′ significantly decreased at day 21. With the presence of neither cell nor FBS, the SAPH was not expected to degrade. However, the decrease of G′ in FK and FR may be due to hydrolytic degradation. The hydrolytic degradation rate depends on the rate of peptide monomer uptake which from this result, suggests that the rate of monomer uptake may differ for each peptide sequence. This also suggests that the off-rate of peptide monomer varies depending on the peptide sequence or charges.

The second set of the sample was cultured in media containing FBS (+FBS/-cell). As seen from Figure 2-8, when FBS was present, the storage modulus of F9 reduced by 21% at day 3, FK decreased by 64% at day 7 and 35% at day 14 for FR. These reductions of G′ were found to be statistically significant. FK in this condition behaved in a similar pattern to the FK (-FBS/-cell) condition (reduced significantly at day 14). Significant reduction in storage modulus at day 14
was also observed in FR (+FBS/-cell) samples. For both FK and FR (+FBS/-cell) samples, the values at day 14 and day 21 were significantly lower than those measured at day 0, 3 and 7. At the end of the experiment (Day 21), $G'$ for F9 was $8.1\pm1.1\text{kPa}$, while FK and FR had $3.6\pm1.9\text{kPa}$ and $4.6\pm1.6\text{kPa}$ correspondingly. One-time reduction of $G'$ for each peptide hydrogel may also suggest that each peptide has a different rate of peptide monomer uptake.
Half of the samples were encapsulated with bovine chondrocytes (4 million cells per ml) and were cultured in media with/without FBS. At each time point, rheological properties of each sample were measured. (*) denotes the values between the samples are significantly different. (#) shows the value is significantly higher than samples in other conditions at that time point (p<0.01). Error bar defines mean ± standard deviation.

Figure 2-8 (b) and (c) show a significant reduction of G’ of the FK and FR samples in this condition compared to the (-FBS/-cell) samples, even at the start of the experiment (day 0, p<0.01). F9, however, did not show a significant difference between -FBS/-cell and +FBS/-cell on day 0. All of the samples measured at day 0 were immersed in the media for two hours.
accommodating the time from setting up the experiment to the point of rheological measurement. The reduction of G’ at day 0 in FK and FR between -FBS/-cell and +FBS/-cell after 3 hours of introduction to media+FBS may be enlightened by the self-assembly rate which leads to gelation. SAPHs’ gelation rate depends on the amino acid sequence, the charge of the sequence, the concentration and type of electrolyte (Koutsopoulos 2016). As shown in our previous work, SAPH’s gelation rate of one peptide sequence is longer than another (Szkolar et al. 2014). FK and FR have +2 charges and consist of eight amino acids, while F9 has +1 charges with nine amino acids. The presence of FBS may have changed the concentration and the osmolarity of DMEM with and without FBS. It can also be assumed that with the presence of FBS, self-assembly of these higher charged SAPHs takes longer than in media without FBS.

Bovine chondrocytes were encapsulated in F9, FK and FR. The samples were cultured in DMEM without FBS to assess the degradation profile of SAPH in the presence of cells. For this set of samples at day 0, FK(-FBS/+cell) had G’ of 8.9±1.7kPa, almost half of the control sample (-FBS/-cell) which had G’ of 16.2±1.2kPa. However, for this condition, out of the three peptide hydrogels, only FK had non-significant changes throughout the study. Both F9 and FR had a significant decrease in storage modulus as FR reduced by 30% (at day 3) while F9 reduced by 38% (at day 7). At later time points, no significant changes in G’ were observed in all SAPH, except for FR where the value measured at day 21 was significantly lower than those at day 3.

The effect of having both cell and FBS on degradation profile is assessed in the fourth set of samples. At day 0, the presence of both cell and FBS did not significantly change the value of G’ measured on F9 and FR, in comparison to other conditions. It did, however, affect FK samples. FK(+FBS/+cell) had significantly lower G’ than FK(-FBS/-cell) and FK(+FBS/-cell) on day 0. This finding suggests a requirement of both FBS and cell in degradation of SAPH. Interestingly, its value had no significant difference to FK(-FBS/+cell) suggesting no necessity of FBS for cell at day 0 (or earlier point of study). The effect of FBS and cell was emphasised as the G’ of FK(+FBS/+cell) was lower than FK(-FBS/-cell) at day 14 and day 21. This effect, however, was not observed in F9 and FR samples at the same time point. The G’ for F9(+FBS/+cell) samples did not change significantly from day 0 to day 21. These measurements, in comparison to F9(-FBS/-cell), suggests that F9 may be less affected by proteolytic degradation. When both cell and FBS are present, cells are expected to produce proteolytic enzymes which will accelerate the rate of SAPHs’ degradation.

Also, it is noted that at day 14, FK samples encapsulated with bovine chondrocytes and cultured with media complete with serum, were measured to be around 1.0kPa. It was observed that the formation of a thick layer at the interface of these hydrogels might have affected the measurement; however, samples were observed to be in good condition. The reason for this value could also lie on the rheology measurement which relies heavily on parameters such as percentage strain and gap size.
Degradation profile of FK shown by the rheological measurement in this study is somewhat expected from a peptide hydrogel in the effect of the presence of cells and serum. In general, if we look at each peptide sequence at each single time point, there is a trend of the synergistic effect of serum and cells, which contributes to the faster degradation rate of each hydrogel. The effect of proteolytic degradation may have also been supplemental to the physical degradation that occurs due to hydrogel erosion caused by media changes. Media was changed three times a week to keep the cells alive. As such, media changes might have affected hydrogels’ stability and integrity.

As depicted in Figure 2-8(a), it was found that the storage modulus of F9 was not significantly different across all conditions at all time points except for day 21. G’ for F9 after being cultured for 21 days was $11000 \pm 3000$ Pa for (-FBS/-cell), significantly higher than samples with cell and/or FBS.

In the presence of bovine chondrocytes, the measurements show that the mechanical properties of F9 after being cultured in DMEM (without serum) for 21 days were similar to the same hydrogels at day 0. This may be due to an inability of cells to produce protease in the absence of serum. Figure 2-9 shows the viability study on cells 2D cultured on SAPH with and without serum. Reduced cell viability may explain the phenomena behind FK and FR samples treated in the same conditions.

No significant changes were seen from F9 samples indicating that either cells did not produce any biodegradable enzymes or F9 is so highly resistant to cell culture conditions that no effect was observed in terms of its rheological properties. Figure 2-9 which shows that bovine chondrocyte (BC) cells cultured in F9 were alive up to 21 days of culture suggests that F9 is resistant to proteolytic degradation. It is also noted here that the value of storage modulus of these hydrogels was different than shown in Figure 2-7. The difference can be explained by the higher cell density (4 million cells per ml) used in this particular study compared to the previous section (1 million cells per ml). Higher cell density used in this study was purposely done to emphasise the effect of cells on the degradation of hydrogels.
### Sample Table

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</table>

### Figure 2-9

Cells cultured with/without serum were stained with live/dead assay.

a) Human chondrocytes were encapsulated on F9, FK and FR in the presence/absence of serum. Images were obtained after 24 hours of seeding. (b) Bovine chondrocytes were cultured in 3D at 4 million cells per ml in F9, FK and FR and images were acquired within the same day of seeding.

Interestingly for FK hydrogels, storage modulus for un-seeded samples that were cultured in the presence of serum was reduced, even without the presence of cells. This could be due to FK being more susceptible to hydrolytic degradation, or its peptide off-rate is higher than other samples. In the next section, further investigation quantifying intact peptide within these samples would help to justify this outcome.

In general, if we look at each peptide sequence at each single time point, there is a trend of the decreased stiffness for FK and FR samples with both serum and cells, which contribute to faster degradation rate of each hydrogel. The effect of hydrogel degradation may have also been supplemental to the physical degradation that occurs due to hydrogel erosion caused by media changes which is closely related to each SAPHs’ peptide off-rate. Media was changed three times a week to assimilate real cell culture conditions. As much as media changes might have affected hydrogels’ stability and integrity, this step is required to provide cells their necessary nutrients and for waste removal.

#### 2.3.4.2. Quantification of intact peptide using HPLC

In tissue engineering applications, hydrogels need to fulfil a few criteria such as being biocompatible, have sufficient mechanical properties, ideal biodegradability, allowing mass
transport across hydrogel and interaction with the microenvironment. According to Zhang et al. (2014a), indicators of degradation of hydrogel that have been used in studies are measuring weight (mass), topology, geometry and chemical properties. Previously, hydrogel wet mass and rheological measurement of hydrogel have been analysed and shown that there was a percentage of loss of mass when SAPH was cultured in different conditions and that each SAPH behaved differently. In this section, HPLC was used to measure the presence of intact peptide. The amount of intact peptide and storage modulus of the peptide hydrogel can be used to support each other, and this measurement describes the degradation profiles of each peptide hydrogel. Values in Figure 2-10 were obtained from the measured area under the curve.

Referring to Figure 2-10(a), omnibus Kruskal-Wallis significance tests have shown that there were no significant changes of the amount of intact peptide in F9(-FBS/-cell) throughout the experiment, except there was a considerable reduction of 32% in this sample from day 0 to day 3. The one-time reduction at an early time point may be explained by the peptide uptake rate due to the equilibrium state of peptide monomer in the media, which is expected for peptide hydrogels especially when fresh media is introduced to the sample. The reduction of the amount of intact peptide was also observed in the FK(-FBS/-cell) sample, at day 3, by 30% (p<0.01, see Figure 2-8(b)). However, the amount in this sample reduced further, by addition of 50% reduction at day 21 (p<0.01). This further reduction at the later time points may be due to hydrolytic degradation of FK samples, which was also observed in the rheological measurement of FK (see Figure 2-8(b)). Kruskal-Wallis test has also shown that there is a significant reduction in the percentage of peptide monomer throughout 21 days of culture in FR(-FBS/-cell). The amount measured at day 21 for FR(-FBS/-cell) was significantly lower than those measured at day 0 and day 3.

When F9 samples were cultured in media with FBS, there is no significant reduction between two adjacent time points. The FK- and FR(+FBS/-cell) reduced significantly from day 0 to day 3 and also the amount of intact peptide in FK(+FBS/-cell) continued to decrease until day 21. The decrease in degradation profile of FK(+FBS/-cell) at day 21 was also significant as observed in rheological measurement as described earlier. For those samples with (+FBS/-cell) condition, FK samples were shown to be more prone to degradation compared to FR samples which were shown to be stable from day 3 onwards. As stated above, this may imply that the synergistic effect of cells and serum is not necessary for degradation to happen in FK. This could also mean, not only that proteolytic degradation occurs in FK, but physical degradation such as by surface and bulk erosion may also accelerate the degradation rate of FK with/without cells and/or serum.
Figure 2-10 HPLC characterisation quantifying intact peptide within SAPH in different

Upon dissolving samples in a solvent containing water/acetonitrile (50v/v) and 1%(v/v) TFA, samples we analysed through HPLC column quantifying intact peptide. All samples show a reduced value of intact peptide monomers for F9, FK and FR at day 3. F9 samples (a) maintained their number of intact peptide, while a reduction of the intact peptide was observed for FK (b) and FR (c) samples. At least 7 samples have been analysed for each condition, with error bar representing standard deviation at each condition. (*) signifies the values were significantly different to the other sample. (**) shows the value is significantly higher than (-FBS/-cell) and (+FBS/-cell). (***) shows the value is significantly lower than (+FBS/-cell) and (+FBS/+cell). (#) means the value is significantly lower than other conditions at the specific time point. (##) means the value is significantly higher than other conditions at the specific time point.

At day 0, the values of F9, FK and FR were similar to all conditions, showing that the initial peptide concentration for all peptide hydrogels was equal. From day 0 to day 3, there is a
significant reduction in the amount of intact peptide for all samples FK and FR(-FBS/+cell). This again may be explained by the need to maintain the equilibrium of peptide monomer in hydrogel and media. At day 21, there is a significant decrease of intact peptide for FK and FR, and non-significant increase for F9 for this condition was also observed.

Earlier, it was hypothesised that these gels would only degrade through proteolytic degradation. Since no enzymes will be produced to initiate proteolytic degradation in the case of absence of serum or cells, the percentage of intact peptide in samples cultured in DMEM only was expected to be constant throughout day 3 to day 21 of culturing. Prior to day 3, the amount of intact peptide was expected to reduce due to the equilibrium of peptide monomer upon first introducing to cell culture media. However, as can be seen from Figure 2-10(b) and (c), the reduction (28.5% and 26.4%, p-value ≤ 0.01) of intact peptide at day 21 may suggest that for FK and FR, hydrogel degradation may also be triggered by physical degradation such as by surface and bulk erosion, or chemically (on-off rate of peptide monomer).

When both cell and FBS were present, FK- and FR(+FBS/+cell) had a significant reduction at day 3. F9(+FBS/+cell) had no significant changes at one time point from the previous/ adjacent time point. FK and FR also had a decrease in intact peptide amount at day 21 (see Figure 2-10(b) and (c)). The effect of both cells and serum show the same effect for all samples and is parallel with rheological measurement (refer to Figure 2-8). When the same conditions where both cells and serum were present, across all three peptide sequences, FK was seen to be the most affected by proteolytic degradation where only 15.9±4.4% of intact peptide was detected in FK samples at the end of the study; compared to 61.9±15.1% and 34.3±3.1% for F9 and FR respectively. The effect of both serum and cell was also reported in previous work from our group. Significantly reduced amount of intact peptide was observed in FEFEFKFK (0 charges at pH 7) in the presence of both cell and serum, than in the presence of cell only (Castillo Diaz et al. 2016).

When peptide hydrogels were encapsulated with bovine chondrocytes, both FK and FR showed a reduced amount of intact peptide within the hydrogel, with or without the presence of serum. Looking at the viability assay in Figure 2-9, this suggests that chondrocytes need serum to survive, especially in long-term culture. As shown in this figure, cells were found to be alive in the presence of serum, indicating cell necrosis may not be responsible for the reduced amount of intact peptide. Besides, the reduction cannot solely be accounted for by cell necrosis due to lack of serum as it happened in both +/- serum conditions. Further analysis such as quantifying the production of MMPs or ADAMTS may help to explain this constant value of intact peptide within F9 samples.

Different degradation pattern was also observed by a work carried out by our group, where the proteolytic degradation effects of different types of commercially available enzymes were assessed (Burgess et al. 2018). It was reported from the work that each enzyme had a distinct affinity to degrade different peptide hydrogels, thus leading to the assumption that each peptide
hydrogel degrades at a different rate. In this current study, from both the HPLC quantification of intact peptide and rheological measurement, it was shown that F9 has the slowest degradation profile compared to FK and FR. This could be explained by both FK and FR containing peptide sequences that are recognisable by the protein convertase, furin. As stated in Section 1.4.6, upon binding to (R/KX,R/K↓R) binding site, furin would activate the MMP. Thus, further analysis such as quantifying the production of MMPs or ADAMTS may help to explain this constant value of intact peptide within F9 samples.

Samples (n≥6) analysed for rheological properties, and measurements of the intact peptide were prepared over a span of 5 months to monitor reproducibility. All HPLC samples had TFA added and were kept in the freezer to stop further degradation of the samples. However, the large error in the data may have come from the dissolution of peptide hydrogel within the solvent, in terms of homogeneous integration of peptide within the solvent. This protocol may need to be optimised in terms of designing a solvent gradient specific for each peptide sequence.

Future work will include analysing the immunofluorescence staining of MMPs and ADAMTS, which possibly induce the degradation within each peptide hydrogel. As stated earlier, degradation can be categorised to either surface or bulk degradation. It is suggested that longer term parallel work to measure the rheological properties, intact peptide, weight mass and physical appearance may be essential to analyse the mechanism behind biodegradability of these peptide hydrogels. It would also be suggested that the effect of serum and cells within SAPH can also be accomplished by modulating two parameters within this study – concentration of FBS and cell seeding density. In addition, the remodelling events such as ECM turnover, cell metabolism, cell proliferation and their impact on mechanotransduction in 3D cell culture needs to be studied further.

Besides rheological properties and HPLC quantification of the intact peptide, another method to characterise dynamic changes in mechanical properties of hydrogels is through measurement of water content within the peptide hydrogels. Within hydrogels, water can be found in different states; free water, bound water and intermediate water. According to Guan et al. (2011), free water has the same behaviour as the bulk water and only has water-water interaction. While intermediate water has both weakly water-water and water-polymer chain interactions, bound water, on the other hand, forms hydrogen bonds with the polar polymer chains (Sekine et al. 2014). Analysis of the different state of water in hydrogels can be done through characterisation of the water molecular structure and its enthalpy.

As stated earlier, bound water molecule forms hydrogen bond with the polymer chain, the –OH stretching band quantifying water molecules with four hydrogen bonds can be done through Raman and Fourier Transform Infrared (FTIR) spectroscopy at the spectral band of 2500-3600cm⁻¹ (Pasqui et al. 2012, Sekine et al. 2014). An investigation of water molecules in different hydrophilic polymers by Guan et al. (2011) detected free water at the 3380cm⁻¹ band
and bound water at the 3220 cm\(^{-1}\) band. Besides Raman and FTIR, other studies have been applying Differential Scanning Calorimetry (DSC) to measure the amount of bound and free water molecules in hydrogels (Ikeda-Fukazawa et al. 2013, Zhao et al. 2016). In DSC analysis, hydrogels will be frozen prior to the analysis. The application of DSC in this analysis is based on the assumption that only free water molecules will be frozen and the bound water molecules will have different endotherm energy when the frozen gel is being warmed up than the frozen free water (Gulrez et al. 2011). The form and structure of water molecules within hydrogel would give insight on gel permeability.

Earlier in this study, a diffusion study was carried out using rhodamine dye and UV spectroscopy. The study was performed as described in detail in Section 6.1 (Supplements). As can be seen from Figure 6-1 (b), the diffusivity of SAPH sequence was different from each other. It was also noted from a previous study; the phenol red was trapped in the charged peptide hydrogels, doubting the result of this diffusion study, thus was not continued/ repeated. As stated earlier in section 1.5.3, the diffusivity of the hydrogel is essential as it allows transport of nutrients and waste to the cells encapsulated within the hydrogels.

This current study looked into the degradation profile of different peptide hydrogels in vitro. Degradation tests of these hydrogels when implanted in vivo may reveal a more pronounced effect of the microenvironment of ECM interaction and molecular production physiologically. It has been seen from the literature that both destructive and nondestructive in vivo degradation tests are applicable for future work. In addition, the application of mechanical bioreactor may also become useful to observe the effect of physical loading and hydrodynamic stress that would mimic the physiological environment. Another approach that may be applicable is through the modelling of SAPH which could predict the degradation behaviour of these hydrogels.

2.4. CONCLUSION

This chapter has outlined the characterisation of self-assembling peptide hydrogels that have the potential for cartilage engineering applications. The variation of peptide design was performed by replacing phenylalanine (F) with valine (V), substituting lysine (K) with arginine (R), switching the location of lysine and glutamic acid (E); and adding another lysine (K) at the end of the peptide sequence. The effect of modulating the peptide sequences has been studied in terms of their gelation properties, mechanical properties, and stability and degradation profiles. Earlier in the chapter, it was shown that altering the overall charge and hydrophobicity of the peptide changed the hydrogel gelation behaviour and mechanical stability. Also, the effect of substitution of lysine with arginine depends on the presence of the overall charge of the sequence. Subsequently, both rheology and HPLC analysis have revealed that while FK and FR degrade significantly with time, F9, however, does not. As suggested earlier, the difference in degradation profile of each peptide sequence could be due to the different on-off peptide monomer rate in each SAPH.
CHAPTER THREE
THE PRESENCE OF CHARGE AND ARGinine IN
SELF-ASSEMBLED PEPTIDE HYDROGELS
AFFECTS CELL MORPHOLOGY AND
CHONDROGENIC MARKERS

3.1. INTRODUCTION

One of the earlier concerns in cartilage tissue engineering is the differentiation of chondrocytes upon monolayer expansion. In 1982, Benya and Shaffer were able to redifferentiate passaged chondrocytes in 3D agarose. However, agarose, as stated in Section 1.5.2.4, lacks cell binding sites. Thus, in the past few decades, researchers have been pioneering the feasibility of using and modifying hydrogels as a 3D platform to regenerate cartilage (Oh et al. 2016, Sarem et al. 2018).

Besides assessing different types of hydrogels, researchers have also been altering the composition and mechanical properties of the hydrogels. Modifications to the biomaterial scaffold were done mainly to direct cells toward spherical morphology which would lead to chondrogenic gene expression and matrix protein production. Studies have found that cytoskeletal arrangement plays a significant role in mechanosensing and mechanotransduction, which govern cell fate (Mathieu and Loboa 2012). Buitrago et al. (2018) observed changes in cell morphology when cells were encapsulated in varying mechanical properties of silk fibroin/collagen hydrogel. This and other studies have also found that hydrogel stiffness dictates the level of GAG and collagen type II productions (Oh et al. 2016, Wang et al. 2016, Park et al. 2017a, Schneider et al. 2017, Buitrago et al. 2018).

Other than initial mechanical properties, most hydrogels also go through the process of degradation, be it hydrolytically or proteolytically. These mechanical properties of hydrogel, which change dynamically, depend on the microstructure of the hydrogels and enzyme production of the cells cultured within. Different biodegradation rates of gelatine-based hydrogels affects the chondrogenicity of cells seeded in it, as shown in both in vivo and in vitro studies (Sarem et al. 2018).

As stated in Section 1.5.5.2, self-assembled peptide hydrogels (SAPHs) have been utilised and studied for cartilage development, in terms of promoting desired phenotype and regeneration of tissues. Grodzinsky and co-workers have shown that when cells were encapsulated in KLD and RADA, the cells produced a higher level of GAG and PGs than those cultured in agarose.
hydrogel (Kisiday et al. 2002a, Florine et al. 2013). Previously in our group, Mujeeb (2013) investigated the injectability and suitability of using non-charged SAPH FEFKFKFK in encapsulating bovine chondrocytes. This peptide has zero charge at pH 7. Another study in our group also found that bovine chondrocytes remained viable and retained morphology when seeded within an alternating version of non-charged SAPH, FEFKFKFK (F8). It was also reported that bovine chondrocytes acquired elongated morphology after being cultured within +1 charged SAPH FEFKFKFKK (F9) for five days (Szkolar 2015). More recent work by another group has shown that hMSCs differentiated into either adipocytes, osteoblast or chondrocytes when 3D cultured in different stiffness of SAPH, KFE-8 (Hogrebe and Gooch 2016). The authors also found in a separate study that incorporation of RGD in KFE-8 caused cell spreading (Hogrebe et al. 2018).

There are a few advantages of using peptide hydrogels in tissue engineering; hydrogels are biocompatible, biodegradable, tunable for desired mechanical properties, injectable and can be functionalised. In the previous chapter, we have shown that the mechanical properties of these peptide hydrogels can be tuned by changing the concentration and the pH. It was also found that these SAPHs have long-term stability. As reported in the previous chapter, the SAPH have different degradation profiles. Thus, in this chapter, SAPHs were encapsulated with bovine chondrocytes and cultured for 21 days. This chapter discusses the effects of charged peptide hydrogels (FEFKFEFKK, FKFEFKFK and FEFKFRFK) and SAPH’s degradability on cell viability, cell proliferation and production of glycosaminoglycans (GAG) and collagens. We hypothesised that SAPH sequence which has a slower rate of degradation or higher stability would support the regeneration of cartilage and that cells would not be affected by the different charge of each SAPH.

In addition, this current chapter describes the potentiality of combining two different charged peptide sequences, by encapsulating cells within one peptide hydrogel and physically incorporating this cell-SAPH mixture into another peptide hydrogel. In cartilage engineering, the rise in hybrid/composite hydrogels is due to the i) zonal arrangement of articular cartilage where the cell morphology and density and collagen fibre arrangement and density are different in each zone (see Section 1.3) and ii) the highly flexible auricular cartilage. Recently, Owida et al. (2018) incorporated electrospun PLA nanofibers which are aligned in different zonal arrangement in HA hydrogel. The study found bovine chondrocytes cultured in the composite hydrogel had different morphology, cell and aggrecan density in each layer.

3.2. MATERIALS AND METHODS

FEFKFEFKK, FKFEFKFK and FEFKFRFK peptide powders (>95% purity) were purchased from Biomatik Corporation (Cambridge, Canada). All supplements for cell culture work were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. Trypan blue solution (Hyclone, Thermo Fisher Scientific, Loughborough, UK) and Live-Dead Assay (Life-Technologies, Paisley, UK) were used for analytical work. For hydrogel preparation, sodium
hydroxide (NaOH; Thermo Fisher Scientific, Loughborough, UK) was used, and hydrogel was pipetted into 12-well cell culture inserts, with a pore size of 1.0µm (Greiner, Stonehouse, UK). CellTiter- Glo 3D cell viability assay (Promega, Southampton, UK) was used to assess ATP production by cells cultured in peptide hydrogels. Blyscan GAG assay (Biocolor, Northern Ireland, UK) was used to measure GAG in samples.

3.2.1. Hydrogel preparation

Peptide solutions were prepared by suspending the solid phase peptide in distilled water. FEFKFEFKK (F9), FKFEFKFK (FK) and FEFKFRFK (FR) were prepared at specific concentrations known to produce a storage modulus of 10kPa (see Section 2.2.1). Using 0.5M NaOH, the peptide solutions were neutralised to pH where gels start to form. Table 3-1 outlines the peptide sequences tested in this study. The table also shows the net charge of the peptide upon reaching pH 7.

<table>
<thead>
<tr>
<th>ID</th>
<th>Peptide sequence</th>
<th>Net charge at pH 7</th>
<th>Concentration at 10kPa (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F9</td>
<td>FEFKFEFKK</td>
<td>+1</td>
<td>1%</td>
</tr>
<tr>
<td>FK</td>
<td>FKFEFKFK</td>
<td>+2</td>
<td>2%</td>
</tr>
<tr>
<td>FR</td>
<td>FEFKFRFK</td>
<td>+2</td>
<td>2%</td>
</tr>
</tbody>
</table>

Table 3-1 Peptide sequences used in this study with abbreviated ID used throughout this chapter. The net charge on each peptide sequence at neutral pH is also shown. These SAPHs were prepared at these concentrations providing 10kPa of initial storage modulus.

3.2.2. Bovine chondrocytes culture

3.2.2.1. Cell isolation

See Section 2.2.4.1.

3.2.2.2. Cryopreservation method

See Section 2.2.4.2.

3.2.2.3. Monolayer passaging

See Section 2.2.4.3.

3.2.3. Cell seeding methods

These methods were designed to 1) increase the number of cells successfully encapsulated and 2) improve cell homogeneity and viability. We investigated two seeding methods, in-plate seeding and in-tube seeding (see Figure 3-1).

3.2.3.1. In-plate seeding

Using a 1000µl positive displacement pipette (Microman®, Gilson, Bedfordshire, UK), 350µl of hydrogel was transferred into each 12-well insert (pore size 0.4µm, Milipore®, Watford, UK).
Cells were re-suspended in fresh medium at the desired stock cell concentration of 8 million cells per ml. From this stock, 50µl was pipetted and mixed into each hydrogel giving a final concentration of 1 million cells per ml.

3.2.3.2. In-tube seeding

After counting cells using Trypan blue exclusion, 2 million chondrocytes were resuspended in 200µl media. This cell suspension was then physically mixed into 1.8ml of the prepared hydrogel. Subsequently, 400µl cell-hydrogel mixture was pipetted into each 12-well insert using a positive displacement pipette, giving a final concentration of 1 million cells per ml.

Samples prepared using these cell-seeding methods were then treated as follows: immersed in 1.5ml media, cultured within a 12-well plate (Cellstar®, Greiner Bio-one, Sigma Aldrich, Dorset, UK), kept within a humidified 37°C, 5% CO₂ environment and media changed twice a week.

![Diagram of cell encapsulation method](https://example.com/diagram.png)

**Figure 3-1 Cell encapsulation method.** In order to ensure homogeneity in cell distribution, two different seeding methods were tested. The cells were seeded either through (a) in-plate seeding or (b) in-tube seeding. The final cell seeding density for both methods was 1 million cells per ml.

3.2.4. Optimised cell viability assay

Cell viability protocol as described in Section 2.2.5 was optimised by varying the reagents’ concentration according to the manufacturer’s instruction, and washing steps were added to eliminate the background staining.

Live and dead cells acquired from these images were quantified using particle analysis on ImageJ (for samples at day 0 and day 7) and manual cell counting and marking on ImageJ (for samples at day 14 and day 21). Three hundred cells from four different regions of each peptide hydrogel sample were counted (Wang *et al.* 2010). Cell viability was calculated by dividing the number of viable cells by the total number of counted cells times 100 (to give a percentage).
3.2.5. Rheological measurement of SAPH

See Section 2.2.6.

3.2.6. Immunofluorescence

At each time point, hydrogel samples containing bovine chondrocytes were washed with DPBS 3 times (20 mins each). Samples were incubated in a humidified 37°C, 5% CO₂ incubator throughout the washing process to ensure cell viability (due to the absence of serum and other nutrients). After washing, samples were fixed with 2.5% (w/v) paraformaldehyde (PFA) for 1 hour at room temperature. Longer fixation time was carried out to ensure cells throughout the hydrogel thickness were properly fixed. After fixation, the PFA solution was replaced with blocking buffer after washing with DPBS three times. The blocking solution was composed of 1% (v/v) goat serum, 0.1% (v/v) Triton X-100 and 1% bovine serum albumin (BSA) in 1X DPBS. Goat serum was used as the secondary antibodies were raised in goat. Blocking with serum from the animal in which the secondary antibody was raised helped to reduce the background staining from non-specific binding. Fixed and blocked samples were then stored in the fridge until the end of time point, to ensure all samples were stained at the same time.

3.2.6.1. Aggrecan

Upon blocking, samples were incubated at 4°C for 16 hours with primary antibody- mouse monoclonal which binds to aggrecan (ma316888, Fisher Scientific, Loughborough UK). Primary antibody was diluted at 1:200 (v/v) in dilution buffer which was composed of 1% BSA and 0.1% Triton X-100 in 1X DPBS. The next day, samples were incubated in secondary AlexaFluor 647 goat anti-mouse IgG (a21235, H&L, Abcam, Cambridge, UK) at 1:500 dilution. In order to retain the staining until imaging, samples were counterstained with ProLong™ Gold Anti-fade reagent with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen, UK). Prior to counterstaining, the secondary antibody solution was first removed, and samples were rinsed with DPBS. To ensure the effectiveness of the anti-fade reagent, samples were air-dried for 5 mins on the bench.

3.2.6.2. Collagen type II and I

Prior to incubation of antibody solution, rabbit polyclonal collagen type II (ab34712, Abcam, Cambridge, UK) and mouse polyclonal antibody collagen type I (ab6308, H&L, Abcam, Cambridge, UK) were diluted at 1:500 ratio in dilution buffer. Dilution buffer contained 1% BSA and 0.1% (v/v) Triton X-100 mixed in DPBS. Samples were incubated with these antibodies at 4°C for at least 16 hours. The solution containing primary antibodies was then removed, and samples were washed with DPBS to remove unbound antibodies. Subsequently, samples were incubated in secondary antibody solution for an hour (at RT). Goat anti-rabbit IgG Fc with AlexaFluor 594 and goat anti-mouse IgG (ab150092, H&L, Abcam, Cambridge, UK) with AlexaFluor 488 (a11029, H&L, Abcam, Cambridge, UK) were diluted 1:500 (v/v) in dilution buffer as described in Section 3.2.6. The gels were mounted onto glass slides with Prolong™ Gold anti-fade reagent with DAPI.
(Invitrogen, UK). Samples were kept in a humidifying chamber until images were taken. Images were captured using a fluorescence microscope (X63 magnification).

3.2.7. Actin staining

Actin fibres were stained using phalloidin dye reagent tagged with Oregon Green 488. Oregon Green 488 phalloidin (Life Technologies Ltd, Paisley, UK) was diluted at 1:300 ratio (v/v) in 1X DPBS. After overnight incubation (at 4°C), dye solution was removed, and samples were rinsed with DPBS. Samples were then counterstained with ProLong™ Gold Anti-fade reagent with 4′-6-diamidino-2-phenylindole (DAPI, Invitrogen, UK).

3.2.8. GAG assay

At 7, 14 and 21 days after chondrocytes were encapsulated in gel, the biochemical composition of the samples were examined to determine proteoglycan production by the cells. The level of production was compared to the proteoglycan (PG) level at day 0, and at every time point control samples containing no cells were used as background measurements.

After dissolving hydrogels in 1ml DMEM, a 100µl aliquot of this cell-hydrogel solution was digested in papain digest solution (2.5-unit papain/ml, 5mM cysteine HCl, 5mM EDTA) at 60°C for 3 hours. Digested samples were then centrifuged (10000G, 5 mins), and the supernatant was incubated with Blyscan dye reagent for 1 hour at RT. Then, samples were centrifuged at 10000G for 10 mins, and the supernatant was removed and replaced with dye dissociation reagent. 200µl of this solution was transferred to a 96 well plate and read for absorbance at wavelength 656nm (Tecan, Dorsett, UK). A standard curve was generated according to the manufacturer’s instructions with bovine trachea chondroitin sulphate. This standard was supplied by the supplier in the Blyscan GAG assay kit (Biocolor, Northern Ireland, UK).

3.2.9. Quantifying cell number

3.2.9.1. ATP assay

ATP standard curve

A serial dilution of cells was prepared in 50µl media. This was then added to 350µl peptide hydrogel in 12-well inserts. After dissolving each hydrogel in 5ml of DMEM, 100µl of each sample was added to the same amount of ATP reagent in 1.5ml microtubes. After incubation for 25 mins at room temperature (RT), luminescence was measured using the GloMax®-Multi Jr single tube detector (Promega, Southampton, UK).

A linear fit was produced based on this serial dilution, and linear regression ($R^2$) value of 0.9995 was obtained. The standard curve was produced from cells encapsulated within FK and FR (6 samples) in three independent replicates. Relative light unit (RLU) measured from each sample was plotted against known cell density seeded within hydrogels.
**ATP measurement**

CellTiter- Glo 3D cell viability assay (Promega, Southampton, UK) was used to determine the amount of ATP produced by cells in samples. After dissolving hydrogels in 5ml DMEM, 100µl from the samples were added to the same amount of reagent. The sample mixtures were vortexed to assist cell lysis and incubated for 25 mins in the dark at room temperature. Measurements were then taken using the GloMax®-Multi Jr, a single-tube luminometer (Promega, Southampton, UK).

Relative light unit (RLU) reading of control samples was used as the background measurement and subtracted from the readings obtained from samples. Background subtraction was carried out to reduce potential differences in environmental conditions, as well as to address the sensitivity of the assay which is reduced by the condition of assay storage and thawing frequencies. Batch-to-batch variation in this study was estimated from repeats that were carried out using different batches of cells and hydrogels.

3.2.9.2. PicoGreen® DNA assay

Cell proliferation was quantified through the measurement of DNA content, which was determined using the Quant-iT PicoGreen dsDNA Assay kit (Thermo Fisher, Loughborough, UK). Upon washing the samples with Dulbecco’s PBS, samples were incubated in 1ml of cold lysis buffer (200mM Tris-HCl, 20mM EDTA/ddH$_2$O/1% Triton X-100) for 15 mins at room temperature. Cell lysis was expedited by vortexing and three freeze-thaw cycles. For analysis, 100µl of cell/gel mixture was added with an equal amount of dye reagent in black 96 well plates. The fluorescence was then determined by a plate reader (Tecan, Dorset, UK) at the excitation wavelength of 435nm and emission wavelength of 529nm. A blank gel was used to obtain a background reading and subtracted from the measured value to give the corrected value. An absolute amount of DNA was calculated based on a standard curve of a serial dilution of calf thymus DNA in 1% (v/v) Triton X.

3.2.10. Statistical analyses

See Section 2.2.8.

3.3. RESULTS AND DISCUSSION

3.3.1. Cell encapsulation method affects the early spatial distribution of cells throughout hydrogels

Inhomogeneous spatial distribution of cells was observed in initial studies. Homogeneity of cells throughout the scaffold would lead to the maintenance of cell phenotype. Due to the issue of inhomogeneity in cell density, two different encapsulation methods, in-plate and in-tube seeding methods, were assessed. Samples were incubated with Live/Dead Viability Kit (see Section 3.2.4) and were imaged using a fluorescent confocal microscope using a x20 objective. Three different areas of each sample were imaged to visualise the distribution of cells upon seeding.
The samples were first imaged from the bottom of the hydrogel (see Figure 3-2(a) and Figure 3-3(a)) and then were imaged from the top (see Figure 3-2(b) and Figure 3-3(b)). When comparing cells seeded in-plate (Figure 3-2) to seeded in-tube (Figure 3-3), cells were observed to be more homogeneously distributed when seeded in-tube. This may result from the physical bulk mixing of cells within the hydrogel. These images were taken in the early part of the study, where cells were only seeded for an hour. More homogeneous distribution of cells throughout hydrogels is expected to result in a higher proliferation rate and ECM production (Weinand et al. 2009).

The difference in the effect of encapsulation method was clearly shown in Figure 3-4. This figure shows representative horizontal reconstructions of the confocal images. The images show that cells were seen to be denser at the top than the bottom of the gel when mixed in-tube (see Figure 3-4(c)) than in-plate (see Figure 3-4(a)). For in-plate seeding, 50µl of cell suspension was pipetted into a single plated gel (350µl) with the tip in a vertical circular motion while slowly releasing the pipette plunger to draw the suspension. Inhomogeneity may be due to 1) limited mixing motion, 2) minimal volume of cells suspension or 3) the gel started to harden when media was introduced. When in-plate seeding applied, the speed of mixing was limited compared to the Falcon tube for in-tube seeding. Vigorous mixing in insert would increase the chance of breaking the thin membrane of the insert.

This study also showed that lasers did not penetrate throughout the whole thickness of the samples due to limited working distance. Towards the middle part of the samples, less intensity of fluorescent light was observed, suggesting limited laser penetration (see Figure 3-4). Cells were detected in both top and bottom views. This observation proves that cells were distributed throughout the thickness of the hydrogel sample, but it was not possible to visualise this from only one side. Thinner samples by cutting, slicing or dissecting could improve the laser penetration distance in confocal microscopy. This was successfully applied in another study, where samples were cross-sectioned using a scalpel blade and were then viewed with a confocal microscope (Erickson et al. 2009).
Upon encapsulating bovine chondrocytes in FK peptide hydrogels, samples stained with Live/Dead stain were imaged to visualise initial cell distribution throughout samples. Green dye labels the live cells, while the dead cells were stained red. Samples were viewed from the bottom (a) and were then inverted to image from the top (b). For these samples, cells were encapsulated into hydrogel that was pipetted in a 12 well insert. The three images for each side were taken from three different areas of the hydrogel, first column from the left region, second column from the middle region and the third column from the right region. Images shown that more cells could be seen in the left region than other regions. Images are representative of 3 replicates. Scale bar: 500µm.

**Figure 3-2 In-plate cell seeding.**

Upon encapsulating bovine chondrocytes in FK peptide hydrogels, samples stained with Live/Dead stain were imaged to visualise initial cell distribution throughout samples. Green dye labels the live cells, while the dead cells were stained red. Samples were viewed from the bottom (a) and were then inverted to image from the top (b). For these samples, cells were encapsulated into hydrogel that was pipetted in a 12 well insert. The three images for each side were taken from three different areas of the hydrogel, first column from the left region, second column from the middle region and the third column from the right region. Images shown that more cells could be seen in the left region than other regions. Images are representative of 3 replicates. Scale bar: 500µm.

**Figure 3-3 In-tube cell seeding.**

Upon encapsulating bovine chondrocytes in FK peptide hydrogels, samples stained with Live/Dead stain were imaged to visualise initial cell distribution throughout samples. Green dye labels the live cells, while the dead cells were stained red. Samples were viewed from the bottom (a) and were then inverted to image from the top (b). The three images were taken from three different areas of the hydrogel. Images are representative of 3 replicates. Cell distribution was assessed immediately after seeding bovine chondrocytes into FK hydrogels. Cells were mixed in 15ml Falcon tube, and the hydrogel-cell mixture was then aliquoted into 12-well inserts (in-tube seeding). Compared to Figure 3-2, this cell seeding method led to more homogeneous cell distribution. Scale bar: 500µm.
Figure 3-4 Horizontal representative of confocal images of the distribution of cells throughout hydrogel.
Horizontal representative of confocal images of the distribution of cells throughout FK hydrogels immediately after seeding. Cells were either mixed in-plate (a & b) or in-tube (c & d). Images in (a) and (c) were viewed from the bottom, and in (b) and (d) were viewed from the top. These images show that cells are distributed throughout the depth of hydrogel; however, because of limited working distance, lasers could not travel through the whole depth of hydrogel. ‘B’ as in bottom and ‘T’ as in the top of the samples. It is also noted from the images in (d) that many cells were dead. This could be due to the thickness of the gel which perturbs the diffusivity of nutrient through the depth of the samples. Scale bar: 500µm.

The inevitable loss in fluorescence intensity is due to the limited penetration depth of the laser. This penetration depth is made worse by the aberration due to absorption and scattering of the laser energy. Laser light scattering in this phenomenon can be explained by the increased density of the cells and SAPH fibres. Absorption of laser light also increased with the laser travelling through different media of different refractive indices. Scattering of laser light can be improved by reducing the size of the pinhole. In confocal laser scanning microscopy, a pinhole excludes out-of-focus signal from the specimen. Reducing the size of the pinhole will, however, reduce the amount of signal encountering the detector. According to Semwogerere and Weeks (2005), this reduced light can be resolved by either intensifying the excitation light or by increasing the concentration of fluorophore molecules.

Looking at the images, this would suggest that it would be beneficial to use different intensity of laser at different depth, or that laser intensity has to be increased as we go deeper into the sample. However, this would cause us to use different laser intensity across different samples. Thus results may not be reproducible. So, the application of fluorescently tagged spherical beads located at a specified depth/location will be integrated in the future. The bead will tell us exactly/relatively at which depth we are at and laser intensity for certain depths can then be determined. The integration of beads can be applied to measure the loss of fluorescent intensity and to determine the right intensity corresponding to the focal plane. At each focal point, a 2D
image plane is obtained one pixel at a time, with specific laser intensity determined from the tagged fluorescent beads. A 3D reconstruction of the whole scaffold can then be achieved by combining the series of images of focal plane obtained at different depths, with compensation for intensity loss.

Another good alternative to resolve this matter is by the application of multiphoton fluorescence microscopy. Due to the application of near-infrared excitation light, improved depth penetration with efficient excitation was observed as reported by Combs et al. (2011). As the name suggests, multiphoton microscopy has more than one photon excited at one time, and its design omits the use of a pinhole which provides better-quality imaging of a 3D construct (König 2000, Dickinson et al. 2003).

It was also noticed that many cells were found dead after an hour of encapsulation. However, this is a typical situation for cells being initially seeded in a 3D environment (Meade et al. 2015). It may be explained by stress that cells experience when adapting to the surroundings, from being cultured in monolayer to 3D. It may also come from the shear stress cells were exposed to when they were physically mixed within the hydrogels. Other studies have also seen this phenomenon. However, cells recovered once they adjusted to the new environment (Sinthuvanich et al. 2012).

From this study, it was decided to use the in-tube seeding method for the rest of the study as it led to a more homogeneous distribution of cells throughout the scaffold. The cell suspension was also mixed into the gel in two-step addition as this was also observed to increase cell homogeneity.

3.3.2. Cell viability assay

Samples were stained with Live/Dead Viability assay (see Section 3.2.4) and imaged with an inverted confocal microscope (Leica, Milton Keynes, UK). Figure 3-5 shows representative images of cells in F9, FK and FR after culturing for 21 days. For these images, cells were encapsulated within hydrogels after being plated in 12-well inserts (in-plate seeding). In all samples, cells were seen to be alive indicating these hydrogels were not cytotoxic to the cells. Also, cells encapsulated in F9 formed small clusters which were sparsely distributed, while those in FK and FR clumped as a large colony and were close to each other. It was also seen from these images that the spatial distribution of cells throughout the hydrogels was inhomogeneous. This may result from the charge of different peptide hydrogel or cell seeding method, as discussed in detail in Section 3.3.1
Figure 3-5 Cells encapsulated in F9, FK and FR stained with Live/Dead viability assay. Representative images (two of each) of cells stained with Live/Dead viability kit after being cultured in different peptide hydrogels (F9, FK and FR) for 21 days. Green represents live cells, and red represents dead cells. However, it was observed that the red stain was also picked up by the peptide fibre. Each image was obtained using an inverted fluorescence confocal microscope. Distribution of cells throughout the hydrogel can also be observed. Orange circles highlight dead cells. Scale bar: 250µm.

Rounded highly intense red staining in the images (highlighted by orange circles) are dead cells, and few dead cells were seen in the images. It was also noticed that the Ethidium Homodimer-1 (red stain), also stained some hydrogel fibres. Figure 3-5 demonstrates that the intensity of the red stain on hydrogel fibre was high, which made it almost impossible to differentiate between dead cells and hydrogel fibre. Commonly, live/dead images have been used to quantify cell viability by automated cell counting using PointPicker or cell counter on ImageJ (Grishagin 2015). This automated cell counting is based on the intensity. For this reason, quantification of live cells was not carried out where the red stain on hydrogels could be miscounted as dead cells. To solve the problem of the stain being picked up by SAPH, especially charged ones, the Live/Dead assay was optimised in terms of its concentration, based on the protocol recommended by the manufacturer, and an additional washing step was applied.

3.3.2.1. Effect of charge and arginine on cell distribution and viability

Samples were stained with an optimised protocol of Live/Dead Viability assay (as described in Section 3.2.4) and imaged with an inverted confocal microscope (Leica, Milton Keynes, UK). Figure 3-6 shows representative images of cells in F9, FK and FR after culturing for 21 days. For these images, cells were encapsulated within hydrogels after being plated in 12-well inserts (in-tube seeding). In all samples, cells were seen to be alive indicating these hydrogels were not cytotoxic to the cells. However, from these images, the morphology of the cells encapsulated in F9 was more rounded than those in FK and FR. Elongated morphology of cells upon encapsulation (Day 0) suggests that the positive charge of hydrogels may interact with the cells membrane. Cells encapsulated in F9 formed small clusters which were sparse to each other, while those in FK and FR clumped as a large colony and were close to each other. It was also
seen from these images that initial spatial distribution of cells throughout the hydrogels was homogeneous. Even though cells were homogeneous at day 0 (Live/Dead was carried out 1 hour after seeding), cells formed clusters after 14 days of culture and the effect of charge was more apparent at this time point.

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<thead>
<tr>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
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Figure 3-6 Live/Dead assay on bovine chondrocytes upon encapsulation in F9, FK and FR. After the Live/Dead staining protocol was optimised, samples were stained using this optimised protocol at day 0, 7, 14 and 21 and images were viewed using confocal microscopy. Scale bar = 250µm. Green represents live cells, and red represents dead cells.

Three hundred cells were counted as explained in Section 3.2.4 in three separate regions of each hydrogel (3 replicates). Viability was assessed by dividing the number of viable cells by the total number of counted cells. Encapsulated cells were evaluated for cell viability (Figure 3-7) over 21 days of culturing within F9, FK and FR.
Figure 3-7 Quantification of viable cells based on the images of cells being stained with Live/Dead viability kit. Values were calculated by counting 300 cells in three separate regions of each hydrogel. Replicates ($n \geq 6$) were prepared separately using a different vial of cryopreserved bovine chondrocytes. The quantification was carried out by ImageJ through particle analysis (day 0 and 7) and manual counting and marking (day 14 and 21). (*) shows the significantly different value of F9 and FR at day 0. At other time points, no significant difference in cell viability was seen for all samples.

Figure 3-7 is a quantification of cell viability obtained from the cells encapsulated within F9, FK and FR hydrogels for up to 21 days. Cells in F9 hydrogels were observed to have significantly higher viability ($p<0.05$) compared to those seeded in FR hydrogels after being encapsulated for one hour (denoted as day 0). At other time points, the percentage viability of cells was not significantly different across the type of hydrogels. At day 21, the viability of cells in F9 samples was $87.4 \pm 6.6\%$ viable.

At day 0, the viability of cells in FK hydrogels was measured as $72.2 \pm 13.4\%$. This value remained constant until day 14 and increased non-significantly to $95.0 \pm 4.7\%$ at day 21. Figure 3-7 also shows that the viability of cells in FR hydrogels was not significantly different to those in FK hydrogels at day 0. Cell viability in FR hydrogels also did not change significantly with time up to 21 days of culture, where the cells were counted to have $90.3 \pm 1.8\%$ viability.

It was seen from Figure 3-6 that cells encapsulated in F9 hydrogel samples retained rounded morphology longer than cells encapsulated in FK and FR. Differentiation of bovine chondrocytes towards fibroblastic-like morphology after a more extended culture (more than 7 days), shows the need for either physical or chemical stimulation (Villanueva et al. 2009a). This result is parallel to the image obtained from samples of bovine chondrocytes seeded within F9, as reported by Szkolar (2015). Based on this result, F9 is the best gel for cartilage regeneration, compared to FK and FR. It is also noted that the optimisation of this assay protocol has reduced the red background staining of the hydrogel. However, it is also a concern that the protocol has also reduced the staining of the EthD.

It has previously been reported by another group that cell viability increased when bovine chondrocytes were encapsulated within less charged (HLT2, +5 charge) hydrogel, compared to
MAX8 (+7 charge) (Sinthuvanich et al. 2012). In this study, as shown in Figure 3-7, after being encapsulated for an hour in F9 (+1 charge), FK and FR (both +2 charge) hydrogels, the viability of cells was maintained at the post-seeding level ~80%. This suggests that the decrease in viability is not linked to charge or other properties of this gel such as the presence of arginine, mesh size (which affects nutrient infiltration) or mechanical properties.

3.3.3. Rheological properties of SAPHs culturing bovine chondrocytes

As stated earlier, F9, FK and FR used in this study were prepared at a concentration where they exhibited similar initial storage modulus of 10kPa. However, as shown in the previous chapter, SAPH's mechanical properties were compromised by the presence of cells and serum after one week in culture. Due to this reason, samples with bovine chondrocytes cultured at 37°C, 5% CO₂ were assessed using the rheometer at day 0, 3, 7, 14 and 21.

As shown in Figure 3-8, after three hours of encapsulation, the storage modulus (G') of F9, FK and FR hydrogel samples were measured to be 8900 ± 1200Pa, 7800 ± 2100Pa and 8000 ± 1700Pa, respectively. This showed that three hours after encapsulation with chondrocytes, the storage modulus of each sample was not significantly different to each other (G' of hydrogels before encapsulation was 10kPa, p>0.05).

F9 samples were observed to have the same value of G' at day 0 = day 21. It is also noted from the graph that there was a significant increment of G' in F9 samples from day 3 to day 7 (p<0.05), followed by a significant decrease at day 14 (p<0.05). Having said that, F9 samples were seen to increase its G' significantly at day 21 when this study discontinued. FK samples, however, showed a linear and gradual decrease of G' from day 0 to day 7. FK samples were observed to lose their integrity (G' of 1000 ± 350Pa) at day 14, even though a non-significant increase was observed a week later at 21 days. G' for FR samples remained the same after 3 days of culture and significantly decreased at day 7 (p<0.05). This value stayed the same up to day 21 of culture.

When comparing the samples, only F9 samples were stable at day 7, G' for FK and FR samples decreased by half of their G' values measured at day 0. Samples measured at day 14 showed that FK samples continuously decreased, while FR samples remained at the same value compared to day 7. At day 21 when the study ended, G' for F9 samples were measured to be 8000 ± 1900Pa, while the other samples were measured to be 2300 ± 1100Pa and 1800 ± 1170Pa, for FK and FR, respectively.
The storage modulus of all SAPH upon encapsulation with 1 million cells per ml of bovine chondrocytes was measured. Rheological properties of samples were analysed at day 0, 3, 7, 14 and 21. Error bars define mean ± standard deviation. n=6. (*) shows that the measurements for F9 and FR reduced significantly from day 3 to 7 (p<0.05). (**) shows value reduced significantly for F9 and FK from day 7 to day 14 (p<0.05). (***) shows that for F9, the reading increased significantly from day 14 to 21. At day 7 and day 21, F9 was significantly higher compared to the other samples at both time point, denoted by (#, p<0.05). At day 14, (##) shows FK had significantly lower value than other samples (p<0.05).

3.3.4. Cell morphology

Samples were stained with Oregon Green 488 Phalloidin which stains actin, counterstained with Prolong Gold Antifade reagent with DAPI. Figure 3-9 shows representative images of cells in F9, FK and FR hydrogels after culturing for 3, 14 and 21 days, showing that the morphology of the cells was affected by the peptide hydrogel. At day 3, chondrocytes encapsulated in F9 regained their characteristic shape by showing spherical morphology, while FK supported a mix of spherical and stretched cells. In FK, the formations of lamellipodia were also seen in most cells. However, chondrocytes encapsulated within FR were observed to be aligned in different directions at different depth.

Changes in cell morphology in FK and FR were seen to be unchanged with time. Cells were observed to have more spreading morphology towards 21 days of culture. In F9 nonetheless, a high number of cells were distributed in big spherical clusters, some cells were also observed to change their morphology towards a fibroblastic-like shape. Changes in actin morphology indicate cells behaviour, in terms of cytoskeletal strain, focal adhesion and cell migration (Mullen et al. 2014).

In addition, strong intensity of actin fibre of cells within FK and FR suggests reorganisation of the actin cytoskeleton bundles (Chang and Kumar 2013). Increased intensity of actin cytoskeleton staining indicates a higher amount of focal adhesions (Elosegui-Artola et al. 2014). Also at day 3, cells in FK were observed to form filopodia. Filopodia (marked by an arrow, Figure 3-9, actin) are dynamic structures that are part of pseudopodia. Often found protruding
from a lamellipodia actin network, filopodia act as sites for signal transduction and integrins and cadherins. Due to the finger-like structures of tight parallel bundles of filamentous actin, filopodia were reported to be associated with cell migration and adhesion to the ECM proteins (Mattila and Lappalainen 2008). Thus, as shown in Figure 3-9, actin stain may have suggested cell-SAPH interaction and cell migration.

The response of filopodia formation in different conditions was studied by Husainy et al. (2010). The environment was altered either genetically, chemically or physically. It was found that having poly-D-lysine to increase positive charge density not only enhanced cells adhesion but also significantly changed the behaviour of filopodia in terms of their lengths and separation distances (Husainy et al. 2010).

Reports also state that chondrocytes tend to change their phenotype when cultured without any biochemical or physical stimulation (Shahin and Doran 2011b, Meretoja et al. 2013), however not as early as day 3. Higher charge and presence of arginine may explain this modified morphology at the early time point. As reported by Mujeeb (2013), chondrocytes retained their spherical shape for up to 14 days when cultured in non-charged SAPH.
### Figure 3-9

Samples were stained for aggrecan and actin.

In F9, chondrocytes retained their spherical shape at earlier time points and became clustered towards the end of the study, while cells have adopted elongated morphology when cultured in FK and FR as early as day 3. Aggrecan was observed in F9 and FK samples at day 3. However, only F9 showed positive staining for aggrecan at day 14. Loss of aggrecan at day 21 may have suggested that the cells have dedifferentiated. The change of morphology as seen in these images also supported the sign of dedifferentiation of chondrocytes towards fibroblastic-like phenotype. The red arrow indicates filopodia extended out of lamellipodia. Scale bar: 75µm.

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 Merge of figures of samples stained with aggrecan and phalloidin staining.

Figures of samples stained with aggrecan and phalloidin in Figure 3-9 are merged. To confirm the presence of aggrecan within SAPH-cell system, fixed samples were first incubated with mouse monoclonal aggrecan (red stain) for 16 hours. Later, the solution was replaced with secondary antibody, AlexaFluor 647 goat anti-mouse IgG. Concurrently, samples were also stained with Oregon Green 488 phalloidin (green). Finally, samples were counterstained with ProLong Gold Anti-fade reagent with DAPI (blue). Scale bar: 75µm.

Other reports have also shown the effect of charged substrate/hydrogel on the morphology of cells (Chen et al. 2009, Dadsetan et al. 2011, Calabrese and Kaplan 2012, Nagayasu et al. 2012, Sinthuvanich et al. 2012, Sun et al. 2016). Dadsetan et al. (2011) incorporated small charged monomers sodium methacrylate (SMA, negative charged) or (2-(methacyloyloxy) ethyl)-trimethyl ammonium chloride (MAETAC, positive charged) to oligo(poly(ethylene glycol) fumarate) (OPF). Both gels were at the same compressive moduli. It was found that at day 7, chondrocytes had aggregated. At days 1 and 3, cells cultured in OPF-SMA and OPF-MAETAC were observed to have rounded morphology and have changed to fibroblastic-spindled shaped, respectively. In another report, when bovine chondrocytes were cultured in MAX8 (+7 charge) versus in HLT2 (+5 charge), not only did it change the morphology, but the viability of cells in the higher charged hydrogel was also compromised (Sinthuvanich et al. 2012). Cells deformation upon culturing in positively charged hydrogels is not isolated to chondrocytes (Chen et al. 2009, Nagayasu et al. 2012). Work involving self-assembled peptide hydrogels RADA16 (neutral charge), incorporated with either KASEA16(−) or KASEA16(+) has shown that NIH 3T3 cells formed spindle shape in neutral and positive charged hydrogels, whereas cells formed a rounded shape in negatively charged hydrogels (Nagayasu et al. 2012).
formation as shown in Figure 3-10, is parallel to other studies by Dadsetan and Nagayasu (Dadsetan et al. 2011, Nagayasu et al. 2012).

Due to the previous studies which showed the effect of charge as described above, the efficacy of using charge in hydrogels to direct differentiation of human mesenchymal stem cells (hMSC) was investigated (Calabrese and Kaplan 2012). It was found that hMSCs could differentiate to osteogenic phenotype after being cultured for 8 weeks in silk fibroin conjugated with poly-L-lysine (positive charge, without osteogenic media). The report also states that this differentiation was induced by the polarisation of hMSCs transmembrane potential ($V_{\text{mem}}$). This modified cells' morphology by charged hydrogels can also be explained by the charged phospholipid of the cell membrane (Klausen et al. 2016). It was reported that 20% of the phospholipids were negatively charged (Sackmann 2015). All of these reports have used hydrogels prepared at the same mechanical properties at the start of the study. However, neither of them evaluated the changes in the mechanical properties of hydrogels used over time.

Cells sense mechanical cues from their surroundings and translate these cues to the activation of specific channels to produce biochemical signals (Murphy et al. 2012, Paluch et al. 2015). This is known as mechanotransduction. Due to this, it is essential to be aware of the mechanical properties of hydrogels used to regenerate tissue, both spatially and temporally. Directly after encapsulation in F9, embedded chondrocytes regained their spheroidal shape. The alteration of cells morphology starting at day 14 (F9) and day 3 for FK and FR, respectively, may also be explained by the change of mechanical properties of each hydrogel as described in the previous chapter. Even though cells were encapsulated within SAPH with similar initial storage moduli (10kPa), each type of SAPH went through dynamic changes in terms of mechanical properties and biodegradation behaviour. As shown in Figure 3-8, FK and FR started to degrade and lose their mechanical integrity as early as day 7. Quick degradation rate in these SAPHs may not have provided adequate support for the cells.

Mechanotransduction has been widely studied, and it was found that chondrocytes prefer to be in contact with hydrogel prepared at lower mechanical properties ($\leq$ 5 kPa). Chondrocytes were observed to have more rounded morphology and enhanced chondrogenic markers (such as sGAG and collagen type II) when cultured in $\leq$ 5 kPa hydrogels (Subramanian and Lin 2005, Chen et al. 2012, Schuh et al. 2012b). Increasing the stiffness of the hydrogels not only led to elongated cell morphology, but also to an increase in cells adhesion (marked by vinculin staining) and migration (formation of lamellipodia and disorganised actin) (Murphy et al. 2012, Chen et al. 2014a, Zhang et al. 2016a).

Besides its effect on cell morphology, mechanical cues such as stiffness and viscoelasticity were seen to modulate stem cells in terms of cell proliferation and differentiation (Vining and Mooney 2017). MSCs were observed to have increased production of collagen type II and became less spreading when seeded on soft 1kPa polyacrylamine. In addition, two separate studies using collagen-GAG and PVA/HA scaffolds tuned to a range of storage moduli have
shown that seeding mesenchymal stem cells (MSCs) in a rigid hydrogel would lead to bone cells. Seeding human bone marrow stem cells (hBMSCs) in softer ranges of scaffold would lead to cartilage, followed by muscle and nerve cells (Murphy et al. 2012, Oh et al. 2016).

Our study shows that chondrocytes changed to elongated morphology when the hydrogels’ storage modulus (FK and FR) is 2000Pa, meanwhile cells retained their rounded morphology in mechanically stable F9 (at G’ of 8000 ± 1700Pa). This shows that in order for the cells to retain their phenotype, scaffold’s mechano-stability is essential to support the cells’ growth in long-term culture.

3.3.5. Immunofluorescence

3.3.5.1. Aggrecan

Cells morphology was reported to be closely related to the expression of chondrogenesis markers (Nicodemus et al. 2011, Chen et al. 2012, Sinthuvanich et al. 2012). The higher the number of cells with spherical morphology the higher the production of chondrogenesis proteins such as aggrecan and collagen type II. Protein expression of aggrecan, in response to different charge density and the presence of arginine, was determined using immunofluorescent staining (see Figure 3-9). Production of aggrecan was detected from aggrecan antibody attached to AlexaFluor 647 secondary antibody. Figure 3-9 shows that aggrecan was detected in samples F9 and FK. However, aggrecan was observed only on day 3 for both SAPHs, and a small amount of aggrecan was seen in F9 at day 14. At the earlier time point, aggrecan was found to be homogeneously distributed and diffused out of the vicinity of the cell in F9 and FK throughout the hydrogels. No aggrecan was observed in any of the samples at the later time point (day 21) which suggests cell dedifferentiation.

Aggrecan is one of the proteoglycans found in cartilage tissue, besides biglycan, versican and decorin. As described in Chapter 1, aggrecan is made up of over one hundred glycosaminoglycans (GAG) chains, making it one of the largest molecules in the cartilage ECM which is around 100MDa (Dhote et al. 2013, Sivan et al. 2014). These GAG chains trap water making aggrecan swell, which enables aggrecan to resist compressive loads (Sivan et al. 2014). A study found that Poly(ethylene glycol) dimethacrylate (PEGDM) with the lowest crosslinking density allowed the highest diffusion capability, due to large mesh size (Dhote et al. 2013). Being a large molecule raised the concern of its restrictive diffusion through hydrogels. Hence, in the future, it is essential to measure initial hydrogel mesh size to see the effect of diffusion of unbound ECM molecules throughout the gel.

Besides the aggrecan’s molecular size, the issue behind the charge of the molecules also needs to be considered. The anionic groups of the GAG chains in aggrecan have negative charges (Kiani et al. 2002). Lim and Temenoff (2013) reported the interaction between the negatively charged GAG chain (chondroitin sulphate, CS) and positively charged growth factors.
For this reason, the possibility of electrostatic interaction between newly produced aggrecan and positive charge within F9, FK and FR shall not be dismissed.

3.3.5.2. Collagen type II and I

To further analyse the feasibility of SAPH as cartilage regeneration scaffold, the presence of specific collagen (type II and type I) was evaluated. Samples were fixed and incubated with collagen type II (red) and collagen type I (green) antibodies (see Section 3.2.6.2). In all F9, FK and FR samples, it was observed that cells produced more collagen type I in comparison to collagen type II (see Figure 3-11). Immunostaining on all samples did not show positive staining for type II collagen.

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<tr>
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**Figure 3-11 Collagen type II and type I staining on cells encapsulated in F9, FK and FR at 21 days.**  
These are representative images taken from samples that were incubated with rabbit polyclonal collagen type II (1:500) and mouse polyclonal antibody collagen type I. Subsequently; samples were incubated with secondary antibodies goat anti-rabbit IgG with AlexaFluor 594 (red) and AlexaFluor 488 goat anti-mouse IgG (green). After, samples were counterstained with Diamond Prolong antifade reagent with DAPI (blue staining). Stained images were acquired using a fluorescent confocal microscope at x63 magnification. After 21 days of culture, collagen type II was only detected in F9. However, the ratio of collagen type II over type I was <1, which indicates dedifferentiation of chondrocytes. Scale bar: 75µm.

In terms of immunolocalisation, type I collagen staining was evident in all F9, FK and FR samples at day 21 as indicated by the green staining. Type I collagen was detected to be confined intracellularly in F9 hydrogels. On the other hand, a substantial amount of type I collagen was seen to be spread throughout the FK and FR hydrogels, but the highest intensity of the staining can be found adjacent to the clusters of cells. The difference in terms of collagen
type I immunolocalisation in F9 hydrogels compared to FK and FR hydrogels may be explained by the mechanical properties of these hydrogels. As shown in Figure 3-8, the G’ for FK and FR samples were 2000±1390Pa in average, while F9 samples were at more than four times higher than FK and FR (8000±1700Pa).

In articular cartilage, collagen fibres work as a pillar for ECM as they provide mechanical strength (Arnold and Fertala 2013). Collagen fibres also provide support for cartilage tensile behaviour (Schulz and Bader 2007). This is possible as collagen fibres immobilise proteoglycans within the ECM, and enable water to be trapped within (Yang and Elisseeff 2006).

Other reports have stated that besides collagen type II, which indicates redifferentiation of chondrocytes, chondrocytes also synthesised collagen type I and X (Levett et al. 2014, Pappa et al. 2014, Wang et al. 2014b). However, production of collagen type I and X would suggest dedifferentiation and hypertrophic condition of chondrocytes. Results shown in Figure 3-11 are parallel to other reports that have shown differentiation of chondrocytes towards fibroblastic-like morphology.

Figure 3-11 also showed that collagen type II was inhibited in all samples. This may be explained by the presence of fixed positive charge (both +1 and +2 charges) which probably interferes with the aggrecan-collagen fibre interaction. In healthy cartilage, negatively charged sGAG chains interact with the negative amino acids on collagen molecules which result in electrostatic repulsion (Rojas et al. 2014). Additional charge redistribution, as seen in this study, may alter the interaction between aggrecan and collagen fibres. Tanska et al. (2013) reported that reduced fixed charge density increased cell volume which may be attributed to the modification of the network of collagen fibres (Korhonen et al. 2008). Vice versa, this may be the explanation behind the accumulation of collagen type I and dedifferentiated morphology (see Section 3.3.4) in this study.

As stated in the previous section, besides the effect of charge, chondrocytes’ behaviour may be explained by the changes in the mechanical properties of each SAPH with time. The effect of stiffness on the accumulation of a specific type of collagen was widely reported (Schuh et al. 2012b, Sanz-Ramos et al. 2013, Wang et al. 2014b). Sanz-Ramos et al. (2013) found that sheep cartilage produced the highest levels of type II collagen when 3D cultured within collagen hydrogels with the stiffest properties (20kPa), compared to 2 and 10kPa hydrogels, in hypoxia. In contrast to this finding, others found chondrocytes perform best in a softer hydrogel. When comparing between two different storage moduli of agarose, porcine chondrocytes had the highest synthesis of type II collagen in 4kPa, as opposed to 53kPa agarose (Schuh et al. 2012b). In another study, the stiffness of gelatin-hydroxyphenylpropionic acid (Gtn-HPA) was varied, and rabbit chondrocytes produced the highest ratio of coll II/coll I in 1kPa hydrogel. The production of collagen type II by regenerated chondrocytes is influenced by the stiffness of the hydrogels, and once it is produced, collagen will then contribute towards the increase of
mechanical properties of the newly synthesised ECM upon 21 days of culturing (Silverberg et al. 2014).

Meanwhile, higher collagen type I over type II was also observed by others, besides the increased production of collagen type I and reduced amount of collagen type II and aggrecan, these other studies also found a high amount of matrix metalloproteinase (MMP) (Levett et al. 2014, Rojas et al. 2014). Hence, to further validate this finding, q-PCR of COL2A1, COL1, MMP and SOX-9 will need to be determined in the future. SOX-9 was reported as one of the earlier chondrogenic markers, that regulates collagen II expression (Pappa et al. 2014).

In summary, after 21 days of culture, chondrocytes in F9, FK and FR hydrogels exhibited production of collagen type I and stretched morphology. Thus, there is a need to incorporate other stimulation (physical/chemical) to maintain chondrocyte phenotype in these SAPHs.

3.3.6. Quantifying GAG production

Glycosaminoglycan synthesis was measured using Blyscan assay, with bovine tracheal chondroitin sulphate as standard (see Figure 3-12).

![Figure 3-12 GAG standard curve.](image)

GAG standard curve was generated using serial dilution of chondroitin sulphate supplied in Blyscan GAG assay kit. The curve was based on 2 samples run on three separate repeats (total n=6). The slope of this linear regression curve was used to convert absorbance measurement to the weight of GAG.

Figure 3-13 shows GAG production by cells encapsulated within different peptide hydrogel (F9, FK and FR). As expected, no GAG was detected after 6 hours of encapsulation. Cells will only start producing GAG after 3 days of culturing; especially when cells needed the time to adapt to the new 3D environment. A small increment of GAG was produced by cells encapsulated within F9 which then decreased at day 21. However, these changes are not statistically significant. There is, however, a significant increase in the value of GAG measured in FK samples from day 14 to day 21 (p<0.05). In cells cultured within FR, there was an insignificant decrease in GAG production at day 7 followed by an increase after day 14 (p>0.05).
However, in general, the increment of 1µg of GAG per gel is considered a non-significant (seen in all samples, regardless of the sequence). This can either be explained by 1) there was a high production of GAG, but undetectable due to inadequate cell extraction or hydrogel digestion, 2) low number of cells, which led to lack of cell-cell interaction thus unable to produce GAG, or 3) cells were not capable of producing GAG due to an unsuitable environment. GAG measurement as shown in Figure 3-13 supported the finding of aggrecan immunostaining as shown in Figure 3-9, which did not show any positive staining of aggrecan, especially at later time points. Concerns 2) and 3) will be further discussed at the end of this chapter and in Chapter 5.

Figure 3-13 GAG quantification using Blyscan assay.
At day 0, 7, 14 and 21 samples of peptide hydrogels containing bovine chondrocytes were analysed for GAG after papain digestion for 3 hours, as recommended by the supplier. At all time points, two separate repeats with 3 samples (total n=6) for each repeat were analysed. Error bars represent the standard deviation of different peptide hydrogel samples measured at a certain time point. (*) shows a significant decrease in GAG value in FK from day 14 to day 21 (p<0.05). At day 7, FR was significantly lower than FK (denoted by #, p<0.05). At day 14, F9 was significantly higher than FK (indicated by ##, p<0.01)

To address the issue of being unable to detect GAG, the GAG standard curve was re-generated from GAG standard encapsulated within hydrogels (see Figure 3-14), instead of measuring serial dilution of GAG suspension (see Figure 3-12). The value of linear fit regression ($R^2$) of this GAG standard curve would indicate how close the data is to linear regression fit. $R^2$ value of ~1 indicates linearity which suggests the accuracy of the standard curve which later is reliable to be applied in predicting or calculating the absolute value. Any $R^2$ value of <0.95 which shows non-linearity implies that this Blyscan dye protocol may need further optimisation. Figure 3-14 shows that among the SAPH investigated; only FR produced a linear correlation value $R^2$ of 0.97, and 0.93 and 0.94 for F9 and FK, respectively. It was also observed from the graphs that the absorbance value given by the FR sample was only 0.69±0.21, while F9 and FK samples gave readings of 0.92±0.23 and 0.90±0.32, respectively, even though these samples had the same concentration of GAG standards (0.4µg). This shows that the assay was not sensitive enough to detect the GAG seeded within these hydrogels.

As can be seen from Figure 3-14, the absorbance values for GAG standard concentration of 4.10µg seemed to be outliers for all samples F9, FK and FR. However, removing the outliers from the graph did not increase the value of $R^2$ (improve the linear correlation). In addition, the
linear curves for all samples had a non-zero intercept, despite that these values were background subtracted. This could be caused by the high fluorescence reading of the blank samples.

Figure 3-14 Standard curves of GAG standard encapsulated within different SAPH. The fluorescence level measured from Blyscan assay was converted to GAG concentration based on the standard curve produced from a serial dilution of GAG standard in solution. To further investigate the accuracy of the Blyscan assay in detecting GAG produced by cells encapsulated within these SAPH, serially diluted GAG standard was encapsulated within F9, FK and FR hydrogels and analysed using the Blyscan assay. It was found that each curve gave a different absorbance level, even though all SAPHs were encapsulated with similar GAG standard concentration.

This study showed the need for optimisation of the Blyscan protocol. It was also found in this study, that the background measurement of peptide hydrogel without assay gives a high reading (data not shown), which suggests that these hydrogels are auto-fluorescent. This also suggests that Blyscan dye reagent may not be suitable for cells seeded in charged peptide hydrogels. In addition, Blyscan assay, which is based on precipitation and resuspension of bound dye-sGAG, may result in under-estimation of GAG due to GAG loss from multiple step processing (Zheng and Levenston 2015). The Blyscan assay is based on dimethyl methylene blue (DMMB). DMMB assay works when cationic DMMB dye binds to polyanionic substrates (e.g. GAG), the dye changes its colour from blue to violet and can be detected at OD$_{525}$-OD$_{650}$. Some parameters that can be looked into are the duration of the reaction, pH, salt content in digested solution and presence of interfering polyanions (Green et al. 2015). In this current study, the Blyscan dye reagent used was at pH 3.0. The application of DMMB at pH 1.5 was observed in other studies to minimise the interference from contaminants such as DNA and hyaluronic acid (HA). At pH 3.0, DNA and HA are deprotonated as their acid dissociation constant is <3.00 (Wishart et al. 2013, Green et al. 2015). As suggested by Farndale et al. (1986), when polyanionic hydrogel with negatively charged carbonyl such as alginate is used,
DMMB at lower pH (1.5) should be used. In alginate, DMMB is prepared at pH 1.5 to avoid anionic interference from the alginate carboxyl group (Enobakhare et al. 1996). Thus, this may also be the case with charged SAPHs.

Moreover, for this study, samples were digested with papain digestion reagent for 3 hours at 60°C (according to the protocol provided by Blyscan assay’s manufacturer). As suggested by other literature, prolonged exposure (more than 16 hours) of samples to other digestive enzymes such as proteinase K or papain may help to degrade the matrix faster and a higher amount of sGAG shall bind to DMMB dye (Dadsetan et al. 2011, Sinthuvanich et al. 2012, Parmar et al. 2015). Other parameters that can be modified are the absorbance wavelength and the equipment used to read DMMB assay. More recent work from our group has shown that each peptide sequence reacted differently to different types and concentrations of enzymes. Burgess et al. (2018) demonstrated that F8, which has neutral charge, degrades most compared to other SAPH which are positively charged.

In addition, chondroitin sulphate (CS) was found to interact with positively charged growth factors (Lim and Temenoff 2013). This may also suggest that CS may have also interacted with our positively charged SAPH. This issue was discussed in detail in Section 3.3.5.1.

3.3.7. Cell number quantification

Up to this point, quantification of cell numbers was only performed semi-quantitatively through Live/Dead images. As stated earlier, automated counting using ImageJ could only be done for images taken at earlier time points. At later time points, this automated counting was not applicable due to the clustering of cells. For this reason, samples were analysed using ATP assay or PicoGreen® DNA assay to measure cell numbers.

3.3.7.1. ATP assay

Adenosine Triphosphate (ATP), as the name suggests, has three moieties of phosphate. Whenever cells need energy, the third phosphate is hydrolysed and used as a source of energy, leaving ADP (adenosine diphosphate) behind. ADP will then be recharged by mitochondria which convert it into ATP again (Hanson 1989). For this study, the ATP production of cells encapsulated within different peptide hydrogels was assessed using CellTiter-Glo 3D Cell Viability kit (Promega, Southampton, UK) at 0, 7, 14 and 21 days after encapsulation in different peptide hydrogels.

The amount of ATP in a cell specimen is directly correlated to metabolism and cell number. The ATP standard curve generated (described in Section 3.2.9.1) was used to calculate cell number contained in each hydrogel based on the ATP measurement. At pre-determined time points, three samples were measured for ATP with one additional control sample that was mixed with media instead of cells (see Section 3.2.9.1). The first measurement was taken after cells were encapsulated in hydrogels for 6 hours. Subsequently, this first measurement will be identified as day 0.
In Figure 3-15, it was observed that cell number in F9 hydrogels was constant from day 0 to day 7. At day 21, twice the number of cells were observed in F9 hydrogels compared to day 0. There was a slight reduction in cell number in FK hydrogels from day 0 to day 7, which continued to decrease non-significantly until day 21. However, the cell number in FK samples were found to not significantly change in time. In contrast, the quantification of cells in FR samples at t= day 0 was significantly higher than the same samples found in each gel at day 7, 14 and 21.

After 21 days of culturing cells in different hydrogels, F9 had a significantly higher number of cells compared to FK and FR. FR hydrogels contained the least number of cells (p<0.05) at day 21, despite having the highest cell number at day 0 (6 hr post-encapsulation) compared to F9 and FK (p<0.05).

The ATP assay of all samples of FK and FR hydrogels has shown reduced cell numbers at day 7 and 14 which may be explained by necrotic cells. Figure 3-6, however, does not support this as live/dead images for cells encapsulated within FK and FR hydrogels were alive and abundant at day 14, even though reduced viability and cell number were observed at day 7.

![Figure 3-15 ATP production in different peptide hydrogels was measured at day 0, 7, 14 and 21.](image)

ATP-cell number standard curve obtained previously was used to calculate cell number in each peptide hydrogel at each time point. At every time point, 3 samples were measured in 2 repeats (total n=6). Error bars represent the standard deviation of different peptide hydrogels at the specific time point. (*) denotes significantly higher (p<0.01) cell number in F9 in comparison to the same SAPH at other time points. (**) denotes significantly higher cell number in FR hydrogel compared to same samples at other time points. At day 14 and day 21, F9 was significantly higher than FK and FR at the specific time point (#). At day 21, FK was significantly different to FR (##).

Also, each gel was encapsulated with 0.4 million cells (400µl volume, 1 million cells per ml) at day 0. Figure 3-15 shows on day 0, 0.58±0.16 million cells were detected per gel on average.

The values for cell number at day 0 in all samples were less varied compared to those samples measured on day 14 and 21. A high variant of cell number in different hydrogels, especially at day 21, may be caused by different degradation rates of each SAPH. As depicted in Figure 3-8, at day 21, only F9 samples retained their storage modulus from day 0, while both FK and FR hydrogels had a significantly lower storage modulus compared to those measured at day 0. Decreased value of storage modulus may suggest that these hydrogels have fallen apart, and
the remaining volume of FK and FR may be less than F9 hydrogels. Lower volume of hydrogel would lead to decreased number of cells contained within the hydrogels. Thus, ATP measurement of FK and FR samples (per gel) may not accurately represent a whole intact hydrogel, especially at the later time points.

For this study, 5 million cells were suspended in 500µl medium and mixed into 5ml of the hydrogel, then aliquoted into the inserts (in-tube seeding). Looking at the pattern and high variability of cell number in each hydrogel, this may suggest that cell mixing within the Falcon tube needs to be improved. Lack of homogeneity of cells within the gel in the tube would lead to differences in cell number in each aliquot. It was hypothesised that mixing cells in a smaller portion of hydrogel (1 million cells suspended in 100µl and mixed to 1ml of hydrogel) or mixing in partial stage (50µl media containing 0.5 million cells was added twice to the hydrogel) would contribute to more homogenous and well-distributed cells throughout the hydrogel.

CellTiter-Glo luminescent viability assay has been used by many researchers and was reported to be feasible to quantify cells in 2D (Chen and Thibeault 2012, Vinci et al. 2012). However, looking at Figure 3-15, it is noted that the cell numbers estimated using ATP measurement do not concur to the number of live cells seen in confocal images of cells stained with Live/Dead assay within hydrogels (see Figure 3-6). This could be explained by the fact that cells are growing in clusters which decreases the efficiency of the enzyme detergent supplied by the manufacturer to lyse the cell membrane. It could also be that the gel interferes with CellTiter-Glo 3D assay due to the charge.

In addition, it is also possible that the longer the hydrogels are immersed in culture media, the harder it is to degrade the hydrogels. Hence, it is hypothesised that dissolving hydrogels in excess DMEM was not sufficient to degrade the hydrogels and to detach the cells from the fibre of hydrogels. Further optimisation is required to investigate the effect of including an additional enzymatic degradation step before running any analysis. Enzymes such as trypsin, papain, proteinase K and pronase are some of the enzymes that potentially degrade peptide fibres and consequently extract all cells from peptide hydrogels. As reported by a recent study in our group, the highly charged hydrogels need to be homogenised prior to exposure to the enzyme to enhance SAPHs’ degradation (Burgess et al. 2018).

To further investigate the feasibility of using CellTiter 3D ATP Assay, cells were encapsulated in F9 using two different seeding densities a) 1 million cells per ml (0.4 million cells per gel) and b) 0.5 million cells per ml (0.2 million cells per gel). Reduced seeding density (by half) was chosen to see if this would decrease the formation of clusters and increase the accuracy of the assay. The proliferation rate of chondrocytes embedded within F9 was evaluated by using metabolic ATP assay.

Figure 3-16 shows that the number of cells measured in samples reached the same value after being cultured for 21 days, regardless of the initial seeding density. Values shown in this graph again are not parallel to Figure 3-6 where a higher number of cells were observed in the F9 at
day 21 compared to day 0. The graph also raised doubts about the efficiency of the assay for these hydrogels.

Higher growth rate yielded by a lower cell seeding density was also observed by other researchers (Bernstein et al. 2009, Levorson et al. 2013). It was found that co-culture of rabbit bone marrow mesenchymal stem cells (MSCs) and bovine chondrocytes at 5 million cells per ml produced more glycosaminoglycan and collagen compared to 10 million cells per ml. The paper also reported that at 10 million cells per ml density, scaffolds were seen to be obstructed even at day 5 (Levorson et al. 2013). It was also found that matrix synthesis was inhibited when cells encapsulated at 10 million cells per ml were crowded within the scaffold. Increased production of SOX9 gene expression (chondrogenic transcription factor) was also seen when alginate was seeded with lower cell density (4 million cells per ml) compared to 7 million cells per ml (Bernstein et al. 2009).

It is also noted that there are reports that support higher seeding density in terms of de novo ECM production (Mauck et al. 2003, Buckley et al. 2009). Both of these reports stated that cells seeded at higher density (20 million cells per ml) increased production of ECM and agarose dynamic modulus. Despite these findings, there are also other benefits of seeding cells at low density into the scaffold. Clinicians are looking at the benefit of having a smaller volume of tissue extraction, which would reduce post-operative complications and infection site. Low seeding density also would shorten the passaging time, which is beneficial for clinical-translated tissue regeneration and limit the possibility of dedifferentiation of chondrocytes (Foldager et al. 2012).

Interestingly, as mentioned earlier, each gel was expected to have either 0.4 million cells or 0.2 million cells per gel. As can be seen from Figure 3-16, cell number quantified at day 3 was equivalent to expected value. Despite this finding, an analysis of hydrogel encapsulated with a known serial number of cells did not show a linear correlation between cell number and relative light unit (RLU) (data not shown). RLU is the measurement obtained from the plate reader indicating ATP level of cells.
Cells encapsulated at different seeding density quantified with ATP assay.

Cel number estimated using ATP assay of F9 encapsulated with bovine chondrocytes at a) 1 million cells per ml (0.4 million cells per gel) and b) 0.5 million cells per ml (0.2 million cells per gel). n=9 for samples at day 3 and 7, and n=6 for samples at day 14 and 21. The ATP value detected from samples seeded at both seeding density did not change significantly with time (p>0.05). Also, the value at day 21 was similar for both despite being seeded at different density.

Unlike other metabolic-based proliferation or viability assays that apply standard colourimetric methods such as AlamarBlue assay (based on resazurin reduction) and MTT assay (based on tetrazolium reduction), that are optimised for the use in the 2D system, bioluminescent CellTiter 3D ATP assay is developed to be used for 3D spheroids or hydrogel-based systems. This reagent quantifies luminescent signal produced as luciferin being converted to luciferase as a function of cytoplasmic ATP concentration. The ATP assay is integrated with a detergent composition and lysis buffer that would help the reagent to penetrate through the cellular membrane.

For our SAPH-based system, sole use of detergent and lysis buffer as supplied by the manufacturer has shown to be inadequate to facilitate the diffusion kinetics of the reagent. Additional dissolution step of the hydrogel-cell mix in media has shown little improvement in this assay, especially for these charged SAPH systems. Another report states longer incubation (40 mins) with vigorous agitation of samples within the lysis buffer, followed by 10 times of pipetting, and another incubation under strong agitation for an additional 40 mins seems to be useful to digest alginate, Matrigel and Collagen type I hydrogel (Stock et al. 2016).

### 3.3.7.2. PicoGreen® DNA assay

An alternative method to measure cell number via quantification of DNA using Quant-IT PicoGreen® assay was also carried out. PicoGreen® was used to quantify DNA using a fluorescent plate reader. Using a known concentration of DNA standard, as supplied by the manufacturer, a standard curve of DNA amount versus fluorescence was produced. Typically, this estimated amount of DNA was then converted to cell number based on 7.7pg DNA per cell.
Samples, containing SAPH and cells at 1 million cells per ml, were measured for DNA amount at day 0, 7, 14 and 21. PicoGreen quantification has shown that there is a significant increase in cell number in F9 samples from day 0 to day 7 (see Figure 3-17, p<0.05). Even though quantification of DNA was observed to decrease significantly at day 14, the number seems to significantly increase again after being cultured for an additional week. FK also had the same trend as the F9 samples. FR, on the other hand, only increased significantly from day 14 to day 21. The sudden increase in cell numbers in all samples at day 21 after a slump at day 14 may be explained by the initiation of SAPH degradation due to the presence of proteolytic enzyme as shown in Figure 3-8. The production of such enzyme may be parallel with other ECM protein synthesis as shown earlier in this chapter (see Sections 3.3.5.1 and 3.3.5.2). It could also be explained by the increased level of proliferation, as the cells have differentiated into fibroblastic-like phenotype. The cells' turning into fibroblasts is also supported by the stretched morphology (see Figure 3-10) and high production of collagen type I (see Figure 3-11).

At day 14, F9 had a significantly higher amount of DNA compared to FK and FR. At 21 days, DNA amount quantified from F9 was significantly higher than FR (p<0.05). Even though there is no significant difference in cell number in all samples at day 0, FR had the lowest number of cells compared to both F9 and FK at day 7.

Similar to the finding in Figure 3-15, at day 0, all samples were observed to have different cell number: 0.21 ± 0.01 million, 0.08 ± 0.004 million and 0.15 ± 0.11 million of cells per gel for F9, FK and FR, respectively (see Figure 3-17). For this study, each sample was expected to contain at least 0.4 million cells. Each sample underwent dilution in 5ml of media prior to mixing with an equal volume of cell lysis, buffer and dye solution. For the fluorometric based assay, it was observed that even SAPH containing no cells yielded high fluorescent readings. Data presented in Figure 3-17 were corrected to the blank sample, however, due to the high background reading of the blank these values may not be reliable. As can be seen in Section 3.3.2.1, the fluorescent dye may have been taken up by the charged SAPH. Thus, even blank samples give a high reading. Given that the fluorescent dye may produce spurious results with charged SAPH, in future it would be worth using a NanoDrop™ which quantifies nucleic acid concentration at a lower absorbance level (260nm) (Gurusinghe et al. 2015).

Also, there is a possibility of fluorescence distraction by the hydrogel, so less fluorescence would be detected and thus would underestimate cell number. Due to this reason, it is suggested to use the DNA-cell number standard curve instead of direct conversion in the future. It is also noteworthy to state that the PicoGreen assay is more sensitive than ethidium bromide and Hoechst 33258 (McGowan et al. 2002, Rengarajan et al. 2002).

Failing to produce a linear correlation of a standard curve using encapsulated DNA standard in hydrogel (data not shown), instead of DNA solution, has shown that there is a detection problem with this assay specifically with these SAPHs. Despite the inaccurate cell number at day 0, cell
number quantified through PicoGreen DNA assay shows the same trend as cell number measured through ATP production as shown in Figure 3-15.

![Figure 3-17 Bovine chondrocytes were cultured in different peptide hydrogels and assessed using PicoGreen. Samples were measured at day 0, 7, 14 and 21. PicoGreen DNA assay was used to quantify DNA. The DNA-fluorescent standard curve was used to reflect the DNA concentration in each sample. At every time point, 3 samples were measured in two repeats (total n=6). Number of cells were calculated based on each cell containing 7.7pg DNA. Error bars represent the standard deviation of different peptide hydrogels at the specific time point. (*) shows that the number of cells in F9 and FK was significantly different to the similar samples at other time points. As denoted by (**), number of cells in FR significantly increased from day 14 to day 21. Number of cells in FR was the lowest at day 7 (marked by #), and highest in F9 at day 14. At day 21, the value quantified from F9 samples was significantly different to those in FR samples (##). The efforts to quantify cell number from F9, FK and FR samples revealed that due to the SAPHs being auto-fluorescent, PicoGreen (which is based on fluorescence), was shown to be an unreliable assay for these hydrogels. Even though ATP was measured via luminescence, further work needs to be done to optimise the assay and reduce the background reading.

3.3.8. Combining peptide hydrogels

The idea of combining two different peptide hydrogels was adapted from Szkolar (2015) who studied the practicality of combining two different self-assembling peptide sequences in a 50:50 ratio before seeding chondrocytes in the hydrogel. This current study investigated another method of combining two different SAPH. In Szkolar (2015), the two peptide powders were mixed prior to being dissolved into solution and addition of NaOH to form a hydrogel. In this current study each hydrogel was prepared separately (one type of peptide powder dissolved, and pH modulated with NaOH to form the gel) and then physically mixed with another type of peptide hydrogel. In the case here, F9 hydrogel was seeded with cells in complete media (1 million cells per ml) and subsequently mixed with either FK or FR. These hybrid hydrogels are denoted as F9+FK or F9+FR, respectively. Besides looking at the efficacy of reducing the effect of peptide charge on chondrocytes, combining two different peptide hydrogels may increase hydrogels’ stability (see Section 2.3.4).

Results shown in the previous section depicted that cells adopt spherical morphology when encapsulated within the F9 hydrogel. This current study was carried out to see if the overall charges of SAPH play an essential role in the cells’ behaviour. It was hypothesised that when cells were enclosed within F9, before mixing into a higher charged hydrogel, this rounded
morphology would be retained. Besides, as shown in Figure 3-8 FK and FR hydrogels had a faster degradation rate. The degradable properties of either FK and FR within F9 may provide space for newly-synthesised ECM protein. To evaluate the success of combining peptide hydrogels, samples were assessed for cell viability and morphology and tagged with immunofluorescent antibodies to visualise the production of aggrecan and collagen type I and II.

3.3.8.1. Cell viability

Figure 3-18 shows Live/Dead viability assay carried out on samples up to 21 days in culture. First of all, cells were seen to be homogeneously distributed throughout the hydrogels regardless of the SAPH system. It was also noted that this encapsulation method (in comparison to when only one SAPH type was used) produced a better distribution of cells, without jeopardising cell viability, despite multiple physical mixings (see Section 3.3.2.1). Due to the initial homogeneous cell distribution, cells were seen to be more uniformly distributed than in single SAPH throughout the experiment.

Gross evaluation of the images has shown that the number of viable cells in F9 was constant until day 7. Even though there was a slight reduction of cell number at day 14, cells were seen to recover at day 21. There was also a slump in terms of the cell number for F9+FR sample, which can be noticeably seen at day 7, yet cells recover a week later, up to 21 days of culture. This was also observed when cells were seeded in FR only (see Figure 3-6). Figure 3-6 also shows a distinct decrease in number of cells in FK samples at day 7. However, by combining F9 and FK hydrogels, cells were seen to be continuously amplified up to 21 days of culture. Overall, there are higher numbers of cells within these hybrid hydrogels compared to single type of hydrogel, especially from day 14 onwards. Higher cell number can be explained either by a) having hybrid SAPH may have improved the cell distribution or b) encapsulation of cells in F9 before mixing into another SAPH may have improved the cell viability.

As the culture time increased, cells were seen to form sheet-like morphology, especially in F9+FK at day 14 onwards. This sheet-like morphology made the quantification of cells from Live/Dead images impossible. This same morphology was also observed in FK samples at a later time point (see Figure 3-6).

On the other hand, cells were seen to retain their morphology when encapsulated within F9, up to day 7 and mixed morphology of spherical and stretched at day 14. However, when the cells encapsulated in F9, and later mixed with FK or FR, cells changed their morphology as soon as day 7. This morphology alteration potentially had been caused by variation in terms of a) charge density, b) fibre density or morphology or c) mechanical properties.

As stated in Section 3.3.2.1, charge affects cell viability. In the case of hybrid SAPH, cells may have experienced a mixture of charge distributed throughout the hydrogel. Like the non-hybrid SAPH discussed earlier, cell viability was not compromised when two SAPHs with different charge were mixed.
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Figure 3-18 Viability assay on hybrid hydrogels.
Viability assay was carried out for samples containing bovine chondrocytes at day 0, day 7, day 14 and day 21. Bovine chondrocytes were either encapsulated in F9 only, in F9 + FK or F9+FR. Besides viable cells which indicate non-toxicity of the gel, the morphology of the cells was seen to be more spherical in F9 only up to day 14 and a mix of spherical and elongated cell shape in F9+FK up to day 7. Scale bar: 250µm.

3.3.8.2. Cell morphology

From gross evaluations of images of cells stained by viability dye, cells were seen to be spherical in both F9+FK and F9+FR samples at day 0 (after 3 hours of encapsulation, see Figure 3-18). This is also evident in Figure 3-19 which shows that bovine chondrocytes encapsulated in F9+FK (after 3 days) retained their spherical morphology and were not in direct contact with neighbouring cells. However, cells in F9+FR changed their morphology as early as day 3. Cells in F9+FR started to elongate, and some of the cells were reaching to the neighbouring cells. Extension of actin fibre towards other cells may also suggest cell migration which may be enhanced by the presence of arginine in FR. Different intensity of actin fibre between the two ends of cells indicates lamellipodium extension (bright) and tail retraction (dim, see Figure 3-19) and also supports the possibility of cell migration as the actin would become less intense when the fibres were not strongly attached to the scaffold fibres (Elosegui-Artola et al. 2014).

Another significant detail from the image is that at the same time point (day 3), cells encapsulated within F9+FR had a higher rate of mitosis where the division of the nucleus was seen (see Figure 3-19 and Figure 3-20). Faster proliferation may also suggest that these cells
have differentiated into a fibroblast phenotype where their proliferation rate is higher compared to chondrocytes. Future work measuring Ki67 cell proliferation marker be considered. Ki67 is an endogenous protein that is expressed during proliferation cycle of cells (Scholzen and Gerdes 2000). According to protocols described by Kim and Sederstrom (2015), Ki67 proliferation marker can be detected through immunostaining or flow cytometry. However, the later procedure would need an additional step to fully digest SAPH encapsulating the cells, which as discussed earlier, is yet to be developed. Thus, a protocol to stain Ki67 in paraffin-embedded tissue as described by Eminaga et al. (2016) and Wu et al. (2015) may be adapted for 3D cultured cells. Besides proliferation rate, a higher number of cells may also be explained by less cell death in F9+FR compared to F9+FK.

As explained earlier, without the presence of any chemical or physical stimulation, chondrocytes will change their phenotype which can be seen from the stretched elongated morphology of cells in both conditions at day 21. To date, there is no study on the presence of arginine on cell behaviour. However, there are reports on utilising peptide sequence arginine-glycine-aspartate (RGD) to improve cell adhesion. RGD was shown to increase the amount of vinculin produced by chondrocytes (Smith Callahan et al. 2013b, Jingjing et al. 2015). Due to this enhanced cell attachment, the presence of RGD was seen to increase the sensitivity of cells towards dynamic perfusion flow and be beneficial to mechanically loaded chondrocytes encapsulated within PEG hydrogels (Villanueva et al. 2009b, Degala et al. 2011). However, studies have also revealed contradicting effects of RGD on chondrocytes. When hydrogels were immobilised with RGD, cells were seen to have spreading morphology and reduced production of collagen type II and GAG (Moshaverinia et al. 2013, Smith Callahan et al. 2013b, Jingjing et al. 2015). As stated earlier, cell geometry is closely related to the maintenance of its phenotype (Cao et al. 2014).
Figure 3-19 Aggrecan and actin staining of chondrocytes cultured in hybrid SAPH.

It can be seen that at day 3 and 14, cells encapsulated within F9+FK had stretched morphology and clumped together, while those cells in F9+FR had differentiated into elongated shapes (actin, green). Aggrecan (red) was only produced by cells encapsulated within F9+FK at day 3. Images also show that no aggrecan was detected in F9+FR samples at any time point. Stretched morphology of cells and no aggrecan production may suggest cell dedifferentiation. Red arrows show the bright lamellipodia and dim tail retraction indication of cell migration. Scale bar=75µm.
3.3.8.3. Immunofluorescence
3.3.8.3.1. Aggrecan

In the extracellular matrix of cartilage, aggrecan is one of the major macromolecules that belongs to the proteoglycan’s family. Owing to the glycosaminoglycans that are covalently attached to the aggrecan, cartilage tissue is able to bear load (Bayliss et al. 2000, Kiani et al. 2002, Sivan et al. 2014). Thus, aggrecan is an excellent indicator of chondrogenesis. Figure 3-19 indicates at day 21 samples were shown to have no aggrecan in either system. Lack of aggrecan could be due to hypertrophic differentiation of chondrocytes.

It is also noteworthy that the structure of aggrecan domain differs between species (Rodriguez et al. 2006). The use of human chondrocytes instead of bovine chondrocytes may lead to a contrasting result.

3.3.8.3.2. Collagen type II and I

Chondrocytes producing collagen type II indicates that cells have retained their phenotype. Immunofluorescence stain to detect type I and type II collagen shows that bovine chondrocytes encapsulated within F9+FK and F9+FR produced type I collagen (see Figure 3-21) indicates hyperthrophy and is supported by the low production of aggrecan. However, collagen type II was observed to be minimal in F9+FK samples.
Samples were only assessed at day 21 as earlier time points showed no positive staining for collagen (data not shown). In terms of immunolocalisation, it was also observed that type I collagen was found to be abundant either in the cytoplasm or near vicinity.

<table>
<thead>
<tr>
<th>Type II collagen</th>
<th>Type I collagen</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td>F9 + FK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F9 + FR</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3-21 Samples were incubated with antibodies to type II (red) and type I (green) collagen. The samples were also counterstained with ProLong™ Gold Anti-fade with DAPI. A higher ratio of collagen type II:collagen type I indicates that cells retained their chondrogenic phenotypes, while lower ratio shows that cells have dedifferentiated into fibroblastic phenotype. This image has suggested hypertrophy differentiation for cells in both samples. Scale bar=75µm.

Combining two different SAPH has shown that the presence of F9 in FK seemed to not be beneficial in terms of aggrecan and collagen type II production. Cells encapsulated within F9+FR did not exhibit any positive staining of aggrecan or collagen type II. Cells in F9+FK were observed to have mixed morphology compared to being rounded when encapsulated in F9 on its own. This suggests that charge affects the cells morphology which also governs the production of aggrecan or collagen type II. This, as explained in detailed in Chapter 1, may be supported by the relation between actin cytoskeletal activities and gene expression, followed by protein production. It is also concluded that the presence of arginine affects chondrocyte behaviour, which may be due to mechanotransduction.

Other groups have studied effects of seeding cells in charged hydrogels by attaching fixed negative and positive charge on polymers (Villanueva et al. 2009a) and positively charged peptide hydrogels (Schneider et al. 2004, Sinthuvanich et al. 2012). Fixed negative charge on the scaffold was found to benefit chondrocytes where it led to higher production of GAG by chondrocytes (Dadsetan et al. 2011, Farnsworth et al. 2014). The fixed negative charge may mimic the negatively charged aggrecan in cartilage ECM. In contrast, dynamic mechanical loading on chondrocytes seeded in hydrogels with fixed negative charge led to an increase of nitric oxide level, reduced cell proliferation and collagen production (Villanueva et al. 2009a). Production of nitric oxide signified the increase of catabolic activities which leads to cartilage degradation (Villanueva et al. 2008).
On the other hand, fixed positive charge was seen to give mixed effects on cells seeded in 3D hydrogels. While the fixed positive charge of hydrogel increased chondrocytes’ production of collagen type I instead of type II, which agrees with the finding in this current study, it has beneficial effects on osteoblast and fibroblast (Schneider et al. 2004, Dadsetan et al. 2011). Cell attachment and morphology of osteoblast and fibroblast was reported to be better in fixed positive charged than RGD-ligand hydrogels (Schneider et al. 2004). Moreover, the effect of more positively charged peptide hydrogel (MAX8) has shown higher cell death than HLT2 immediately after encapsulation of bovine chondrocytes. However, after 8 weeks of culturing, cell number in MAX8 (carry +8 charge monomer) samples increased and produced more GAG and collagen than those in HLT2 (carry +5 charge monomer) (Sinthuvanich et al. 2012).

Besides the change in morphology of cells in charged SAPH, low GAG production was probably due to low cell density. Low cell density might lead to less cell-cell contact within the peptide hydrogel. Increasing cell number will hypothetically increase GAG production. According to Ivan Martin, initial high cell seeding and uniform cell distribution increased the rate of cartilage matrix formation (Martin et al. 2004). Studies have found that moderate cell seeding density within 3D hydrogel is preferable in terms of nutrient transfer and extracellular matrix (ECM) production (Heywood et al. 2004, Kobayashi et al. 2008, Hadidi et al. 2015). A study found that when chondrocytes were seeded at 12.5 million cells per ml, earlier production of GAGs, higher production of collagen and higher tensile modulus of the tissue produced were observed, than cells which were seeded at 3 or 50 million cells per ml (Hadidi et al. 2015). The same finding was reported Concaro et al. (2008), using human chondrocytes seeded in chitosan scaffold. Talukdar et al. (2011) found that 25 million bovine chondrocytes per ml seeded in silk fibroin was shown to contribute to faster proliferation rate compared to 50 and 100 million cells per ml.

Moderate over high cell density is preferable because of the possibility of necrosis. Higher cell density has been reported to cause necrosis. Earlier detection of cell death at the centre of the scaffold was detected when higher cell number was seeded. While alginate scaffold being seeded at higher (40 million cells per ml) cell density showed central lost viability after 1-3 days, lower (5 million cells per ml) cell density would only demonstrate central lost viability after 12-15 days (Heywood et al. 2004). The same phenomenon was also seen by Kobayashi and his group. It was noticed in their study that after five days of culture, cells cultured at high density decreased their ability to produce GAG, and GAG was only found at the edge of the alginate scaffold. This might suggest limited diffusivity towards the middle, which also leads to apoptosis (Kobayashi et al. 2008). Besides seeding density, seeding methods, cell migration speeds and hydrodynamic factor were also seen to be essential elements as shown in a computational model that related cell population to mass transport dynamics (Cheng et al. 2009). This study was carried out using 1 million cells per ml. Based on the literature discussed above, higher seeding density (5 to 10 million cells per ml) will enhance the production of GAG by chondrocytes in peptide hydrogels. Thus, continuing this study with higher number of cells should be considered in the future.
Different cell seeding methods shall be carried out in future investigations. The seeding method is expected to contribute to homogeneous cell distribution upon encapsulation. Initial homogeneous distribution of cells is essential to preserve cells’ phenotype. Studies to investigate the effect of cell seeding method on cell number and chondrogenesis have been reported (Weinand et al. 2009, Shahin and Doran 2011a). Shahin and Doran (2011a) seeded human chondrocytes into polyglycolic acid (PGA) using different seeding techniques: static seeding, semi-static seeding, dynamic seeding and PGA-alginate seeding. Cell seeding using the dynamic motion of a spinner flask was also studied and was found to affect the cells negatively. These cells were shown to be in a high state of stress and produced less biochemical content than other seeding methods (Shahin and Doran 2011a). Even though static cell seeding produces minimal stress in cells, fewer cells were found to attach to scaffolds seeded in this way (Weinand et al. 2009, Shahin and Doran 2011a). Weinand et al. (2009) investigated dynamic oscillating seeding where a Falcon tube containing scaffold and the cell suspension was rotated at 40Hz horizontally at 37°C overnight. This dynamic seeding technique gave a more homogeneous cell distribution throughout the scaffold. It was shown that dynamic oscillating seeding was more beneficial than static seeding and centrifugal cell immobilisation technique when low cell seeding density was used. This dynamic seeding gives more homogenous cell distribution throughout the scaffold. Theoretically, with a modified seeding technique, initial homogeneous cell distribution would be achieved, which would lead to improved cell-cell interaction and consequently increased synthesis of ECM molecules.

3.4. CONCLUSION

The efficacy of utilising SAPH in cartilage regeneration has been studied in this chapter. Bovine chondrocytes were encapsulated in 3D and cultured for 21 days. Samples were analysed for cell viability, morphology and expression of the chondrogenic markers, aggrecan and collagen type II. Cells were found to behave differently throughout the study, depending on the peptide hydrogel. It was also found that cells morphology correlated with the production of chondrogenic markers. This finding may be due to the differences in charge, fibre morphology or hydrogel stiffness. These effects were also seen in F9+FK and F9+FR hybrid systems, which reiterate the chondrocytes mechano-transduction mechanism, and the influence of the content of hydrogels on chondrocytes morphology and ECM production. One of the main findings of this study is F9 at initial storage modulus of 10kPa retained chondrocytes’ morphology and was able to support the cells to produce neocartilage protein. However, the structural integrity of the hydrogel is a crucial factor to consider especially when physical stimulation is applied. Chondrocytes encapsulated in positively charged SAPH were seen to dedifferentiate based on stretched morphology and production of collagen type I. In the future, an ideal approach would be to incorporate chemical stimulation which will help the cells to produce ECM protein to replace the degraded hydrogels.
CHAPTER FOUR
EARLY INTERACTION BETWEEN HUMAN IMMORTALISED CHONDROCYTES AND SELF-ASSEMBLED PEPTIDE HYDROGELS

4.1. INTRODUCTION

Cell-cell and cell-material interactions have been shown to play a vital role in cartilage engineering. Different biomaterial properties can significantly influence the initial cell attachment to the substrate, which is crucial for chondrogenesis. Studies have been carried out to enhance the chondrocytes interaction with biomaterial scaffold. The interaction between cells and the scaffold is essential as it serves as a communication means that would contribute towards cells distribution, morphology, cytoskeletal organisation as well as matrix production (Zhang, Biomedical Mat, 2014). One prominent strategy is to modify the biomaterials to create cell binding sites, either by tethering binding sequences such as arginine-glycine-aspartate (RGD) or increasing the surface roughness (Salinas and Anseth 2008b, Jingjing et al. 2015). RGD is a sequence that is recognisable by the integrins (see Table 1-1). Ingavle et al. (2014) had to incorporate cell binding peptide RGD to the construct as the interpenetrating network PEGDA-agarose hydrogel lacked bioactive binding site. It is also noted, however, in a study by Smith Callahan et al. (2013b) that the higher the concentration of RGD peptide in the scaffold, the less its ability to maintain chondrocyte spherical morphology, phenotype and production of chondrogenic ECM contents. Besides incorporating RGD, other groups have been designing composite hydrogel where the synthetic polymeric hydrogel would provide the mechanical support, and natural hydrogel such as fibrin and HA would offer biological benefits (Hwang et al. 2014, Lee et al. 2015). Roberts et al. (2014) developed PEG hydrogel that has HA-binding affinity peptide sequence.

As stated in Section 1.5.2.2, collagen hydrogel has been utilised in regenerating both auricular and articular cartilage (Cohen et al. 2016, Ren et al. 2016). The suitability of collagen hydrogel as cartilage scaffold may be due to the cartilage ECM which mainly consists of collagen fibres. Krouwels et al. (2018) found that cells interact with collagen hydrogel through integrin aggregation, via vinculin, which leads to the activation of focal adhesion kinase (FAK). In a separate study, cells were observed to interact with the injectable chitosan when collagen was incorporated into the hybrid hydrogels (Choi et al. 2014).

Self-assembling peptide hydrogels (SAPH) are seen to have high potential in tissue engineering applications, as well as for drug delivery, as the properties of peptide fibrils can be controlled by changing peptide sequences, concentration and pH. Our novel-designed peptides self-
assemble forming stable β-sheet structures when a low concentration of peptide is dissolved in deionised water (Saiani et al. 2009, Boothroyd et al. 2014). The SAPH was designed to turn to a gel using pH switch. Previous works have shown that our novel designed self-assembled peptide hydrogels (SAPHs) are capable of encapsulating cells and facilitate tissue regeneration (Mujeeb et al. 2013, Diaz et al. 2016, Wan et al. 2016). However, little is known about initial cell-SAPH interactions.

In the previous chapter, cells were observed to respond differently to different SAPH based on its charge and mechanical properties. So, the purpose of this study was to investigate the early interaction of chondrocytes with the SAPH. This current study investigates the effect of different peptide sequences based on the repeating of phenylalanine, glutamic acid, arginine and lysine amino acids. These peptide sequences were either +2 charged, +1 or +0 charged at neutral pH. We also show the effects of using different peptide hydrogels on the attachment, proliferation and spreading of T/C-28a2 immortalised human chondrocytes.

The presence of arginine was seen to affect cell behaviour; in terms of the cells’ morphology and protein production. Stretched and networked morphology of cells have also been reported in other studies when another SAPH, RAD16-I (containing arginine), was utilised (Shamsi 2016). Due to their finding, and enhanced cell attachment due to RGD, we hypothesised that substituting lysine (K) with arginine (R) in our novel-designed peptide sequences would enhance cell interaction with the SAPH. In addition, atomic force microscopic (AFM) images were used to provide microscale surface characterisation, which permits a better understanding of cell-material interactions.

4.2. MATERIALS AND METHODS

FEFKFEFK, FEFRFEFK, FEFKFEFKK, FKFEFKFK and FEFKFRFK peptide powder (>95% purity) were purchased from Biomatik Corporation (Cambridge, Canada). All supplements for cell culture work were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. Trypan blue solution (Hyclone; Thermo Fisher Scientific, Loughborough, UK) and Live-Dead Assay (Life-Technologies, Paisley, UK) were used for analytical work. For hydrogel preparation, sodium hydroxide (NaOH; Thermo Fisher Scientific, Loughborough, UK) was used, and hydrogel was pipetted into 12-well culture plate cell culture inserts with a pore size of 1.0µm (Greiner, Stonehouse, UK). CellTiter-Glo 3D cell viability assay (Promega, Southampton, UK) was used to assess ATP produced by cells cultured in peptide hydrogels. Agarose hydrogel was purchased from Sigma-Aldrich (Dorset, UK). Collagen I, rat tail (Gibco®) was acquired from Thermo Fisher Scientific, Loughborough, UK.

4.2.1. Hydrogel preparation

4.2.1.1. Self-assembled peptide hydrogels

Peptide solutions were prepared by suspending the solid phase peptide in distilled water (see Section 2.2.1). FEFKFEFK (F8), FEFRFEFK (F8R), FEFKFEFKK (F9), FKFEFKFK (FK) and
FEFKFRFK (FR) were prepared at specific concentrations which produced a storage modulus of 10kPa. The pH of the peptide solutions was increased using 0.5M NaOH to form hydrogels. Table 4-1 outlines the peptide sequences tested in this study. The table also shows the net charge of the peptide upon reaching pH 7.

<table>
<thead>
<tr>
<th>ID</th>
<th>Peptide sequence</th>
<th>Net charge at pH 7</th>
<th>Forms gel at pH</th>
<th>Concentration at 10kPa (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F8</td>
<td>FEFKFEFK</td>
<td>0</td>
<td>3.8 ± 0.2</td>
<td>3%</td>
</tr>
<tr>
<td>F8R</td>
<td>FEFRFEFK</td>
<td>0</td>
<td>4.4 ± 0.3</td>
<td>3%</td>
</tr>
<tr>
<td>F9</td>
<td>FEFKFEFKK</td>
<td>+1</td>
<td>5.4 ± 0.4</td>
<td>1%</td>
</tr>
<tr>
<td>FK</td>
<td>FKFEFKFK</td>
<td>+2</td>
<td>6.8 ± 0.5</td>
<td>2%</td>
</tr>
<tr>
<td>FR</td>
<td>FEFKFRFK</td>
<td>+2</td>
<td>6.6 ± 0.6</td>
<td>2%</td>
</tr>
</tbody>
</table>

Table 4-1 Self-assembled peptide hydrogels used in this study with abbreviated ID used throughout this chapter. The net charge on each peptide sequence at neutral pH is also shown. Each SAPH was prepared at a storage modulus of 10kPa and NaOH was used to modulate the pH of peptide solution until it formed a hydrogel.

4.2.1.2. Collagen

As suggested by the literature, 2 mg/ml final concentration of collagen hydrogel was used in this study (Macaya et al. 2013, Lund et al. 2014, Li et al. 2015b). Reagents were kept on ice throughout preparation, and 2.5% (v/v) of 1N NaOH and 10% (v/v) 10XPBS (Fisher Scientific, Loughborough, UK) were mixed in a sterile tube. Distilled water was added to top up to final volume. Once vortexed, collagen was mixed to the tube by pipetting the solution up and down. Upon pipetting the hydrogel into the 8-well chambered slide (polymer, uncoated, Ibidi®, Thistle Scientific, Glasgow, UK) using a positive displacement pipette (Microman®, Gilson, Bedfordshire, UK), the collagen hydrogel was kept at 37°C until gelation and was rinsed with serum-free media prior to cell seeding.

4.2.1.3. Agarose

Agarose hydrogel was prepared according to the procedure suggested by the manufacturer, 2% (w/v) of agarose was prepared by adding Dulbecco’s phosphate buffered saline (DPBS) to the agarose low gelling powder. This agarose solution was vortexed to ensure proper mixing and autoclaved at 126°C. Agarose gel was kept in the 37°C incubator until usage and was pipetted into the 8-well chambered slide using a positive displacement pipette while it was still malleable.

This concentration of agarose hydrogel was chosen based on rheological measurement at 37°C. It was found that 2% (w/v) agarose produced a storage modulus of 10kPa, which is similar to the SAPHs. This concentration of agarose has also been used by others (Khoshgoftar et al. 2014, Nims et al. 2014, Cigan et al. 2016).
4.2.2. AFM- surface topography

The principle of AFM can be described by referring to Figure 4-1. At the end of the cantilever probe, there is a tip which can be selected from a range of different shape and material. The accuracy of the measurement can be increased by choosing the right tip and cantilever, which offer differences in terms of its spring constants (Cubillas and Anderson 2013). Prior to the measurement, the tip was slowly lowered to merely touching the sample, which was identified by sensing a low force between the sample and the tip. This force is estimated according to Hooke’s Law. To measure the surface roughness, the tip runs over the substrate, and the weak interactions between the sample and the tip deflect the cantilever. The deflection is then recorded by the movement of the laser beam which is reflected from the back of the cantilever to the quadruple photo-detector (Gao et al. 2018). The laser collected by the detector is then converted to electrical signal interpreted as topographic height. This information is sent through a feedback control mechanism to the piezo scanner (Cubillas and Anderson 2013, Shan and Wang 2015).

Figure 4-1 The fundamental of atomic force microscopy.  
Schematic drawing of how the AFM works with the main component of AFM used in measuring the hydrogels’ surface roughness. The main components are the laser beam, photo-diode detector, cantilever probe and the sample stage.

SAPH was prepared as described in Section 4.2.1.1. SAPH was pipetted into a 35mm plastic disposable Petri dish (Nunc, Thermo Fisher Scientific, Loughborough, UK). The volume of the hydrogel for each Petri dish was set to be 600µl which produced ~0.68mm thickness of hydrogel. Samples were then immersed in complete media (DMEM+10%FBS) and kept at 37°C, 5% CO₂ for 24 hours. Subsequently, samples were rinsed with DPBS, followed by dH₂O twice. Samples were then fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, Dorset, UK). After rinsing the samples with dH₂O, samples were air-dried at room temperature (RT). Images were taken using Nanoscope Multimode V (Bruker, France) mounted on Nikon Eclipse Ti inverted light microscope (Surrey, UK). The surface roughness of the SAPHs was measured in the ScanAsyst mode with silicon nitride cantilever (ScanAsyst Air, Bruker, France). The cantilever had 0.4Nm⁻¹ nominal spring constant. The surface was imaged, and overview scans (20x20µm) and detailed scans (2x2µm) were acquired from 3 randomly chosen positions.
Samples were tested at ambient temperature and images were obtained at a scan rate of 0.5Hz with a resolution of 512x512 pixel per line. Topographic images were captured, and a 3D perspective of the surface plots was generated by the Nanoscope Analysis software (Bruker, France). Average roughness ($R_a$) and root-mean-square ($R_q$) were calculated from the images acquired from duplicate samples (Torrent-Burgüés and Sanz 2014).

4.2.3. Human immortalised chondrocyte cell line (T/C-28a2)

Cell-based therapy in cartilage regeneration has made use of numerous types of cells - primary chondrocytes, growth plate culture, clonal cell lines and immortalised cell lines (Phull et al. 2016). Human immortalised chondrocytes cell line (T/C-28a2) was studied for its phenotypic expression. It was found that the cell line produced similar gene expression as the primary chondrocytes, even though the level of expression was different (Finger et al. 2003). T/C-28a2 was supplied by Dr Stephen Richardson University of Manchester. T/C-28a2 was dissected from rib cartilage of a 15 year old female, transfected with SV40 T antigen (Goldring et al. 1994). Human chondrocyte media consisted of Dulbecco’s Modified Eagles Medium (DMEM, Sigma, D4591 – 4500mg glucose without phenol red), 2% L-glutamine, 1% sodium pyruvate, 1% penicillin-streptomycin fungizone (PSF), ascorbate-2-phosphate (0.086mM) and 10% (v/v) fetal bovine serum (FBS) was used. DMEM without phenol red was used to enhance visibility when samples were stained fluorescently and viewed using a fluorescent microscope.

4.2.3.1. Monolayer passaging

A vial of cryopreserved T/C-28a2 was resuscitated in 10ml complete media. After centrifugation (300G, 5 mins, RT), the supernatant containing dimethyl sulfoxide (DMSO, Sigma-Aldrich, Dorset, UK) was replaced with fresh media. Resuspended cells were cultured in a T175 flask (Greiner, Stonehouse, UK) at 0.1 million cells per cm$^2$ (Lin et al. 2006, Claassen et al. 2011). When cells reached 90% confluence (generally after culturing for 4 days) they were split in 1:12 ratio. Upon detaching using trypsin, media containing FBS was then used to stop trypsinisation and centrifuged (300G, 5 mins, RT) to wash away trypsin prior to cell counting. Cell count was ascertained using a haemocytometer and trypan blue dye exclusion, where 10µl samples of the cell suspension were pipetted and mixed with trypan blue solution at a ratio of 1:1 (v/v). Automated cell counter (TC10, Bio-rad, Hertfordshire, UK) was used to determine cell number and cell viability.

4.2.3.2. Cryopreservation method

After expansion, cells were detached from the T flask using trypsin (2.5ml to cover a T175 flask) for less than 5 mins. Detached cells were re-suspended in complete media. Cells were counted using a cell counter and aliquoted into cryogenic storage vials in 10% dimethyl sulfoxide (DMSO) and complete media at 1x10$^6$ cells per ml. Vials were transferred into a Mr Frosty™ Freezing container (Thermo Fisher Scientific, Loughborough, UK) and left overnight at -80°C, before being transferred to liquid nitrogen storage.
4.2.4. Cell seeding methods

4.2.4.1. For 2D seeding

Using a 1000µl positive displacement pipette (Microman®, Gilson, Bedfordshire, UK), 150µl of hydrogel was transferred into an 8-well µ-slide (polymer, uncoated, IBIDI®, Thistle Scientific, Glasgow, UK). Hydrogels were first immersed in serum-free media and kept at 5% CO₂. Cells were detached from the T flasks and re-suspended in fresh medium at the desired stock cell concentration. From this stock, 150µl was pipetted onto each hydrogel giving a final seeding density of 0.5 million cells per cm².

4.2.4.2. For 3D encapsulation

T/C-28a2 human immortalised chondrocytes were physically mixed in the prepared hydrogel, giving a final cell density of 4 million cells per ml. After counting cells using Trypan blue exclusion, 8 million chondrocytes were resuspended in 200µl media. This cell suspension was then physically mixed into 1.8ml of the prepared hydrogel. Subsequently, 400µl cell-hydrogel mixture was pipetted into each 12-well insert (ThinCert™, Greiner Bio-one, Fisher Scientific, Loughborough, UK) using a positive displacement pipette, giving a final concentration of 4 million cells per ml.

Prepared samples were then treated as follows: immersed in 1.5ml media, cultured within a 12-well plate, kept within a humidified 37°C, 5% CO₂ environment with media changed twice a week.

4.2.5. Cell viability

The samples were tested for cell viability according to Section 3.2.4. However, the incubation of live/dead assay on 2D samples was shortened to 15 mins. The images were attained from z-stack images of a hydrogel thickness of 20µm.

At pre-determined time points, samples were washed at least 3 times with DPBS (5 mins for each wash). Samples were then incubated with Live/Dead viability kit (5µM EthD-1 and 2µM of Calcein AM) for 30 mins at room temperature. Images were then obtained using a confocal microscope (Leica, Milton Keynes, UK). Calcein fluorescent signal was observed at 525nm EX, 580-640nm EM and EthD-1 at 625nm EX, 660-725nm EM wavelength. Presented figures were attained from z-stack images of a hydrogel thickness of around 500µm (3D samples) and 20µm (for 2D samples), and these images were then stacked together into a maximum projection.

This protocol was optimised by varying the reagents’ concentration according to the manufacturer’s instruction, and washing steps were added to eliminate the background staining.

Live and dead cells acquired from these images were quantified using particle analysis on ImageJ. Total of live and dead cells was taken as number of cells. Cell viability was calculated by dividing the number of viable cells by the total number of counted cells times 100 (to give a percentage).
4.2.6. Cell adhesion assay

An established cell adhesion protocol was modified in this study, where cells were first pre-stained with PKH26 (Sigma-Aldrich, Dorset, UK). T/C-28a2 were first stained with PKH26 according to the manufacturer’s instruction. Upon detaching the cells from T flasks, cells were pooled to obtain at least 10 times more cells than the number of cells required in the end. In a sterile tube, cells were rinsed with serum-free media twice. After centrifuging at 350G for 5 mins, cells were re-suspended in 1ml Diluent C, provided by the manufacturer. PKH26 dye solution was diluted in 1ml Diluent C in a separate Falcon tube. The cell suspension was mixed into the dye solution, giving a final concentration of the dye of 4x10^{-6} M PKH26 (Blewiss et al. 2007, Pabbruwe et al. 2009, Song et al. 2015). The cells were incubated in the dye for less than 5 mins at RT with periodic pipetting. An equal volume of FBS was added (1 min) to stop the reaction of PKH26. Upon centrifugation at 400G for 10 mins, the cell pellet was rinsed twice with complete media (DMEM+10%FBS).

Hydrogels were plated in black 96 well plates (Cellstar, Greiner Bio-One, Stonehouse, UK). The volume for each well was 50µl hydrogel. Cells were then seeded on top of the hydrogel at 5000 cells/well. After culturing for three hours, non-adhered cells were removed. Adhered and non-adhered cells were measured, using a fluorescence microplate reader (Fluostar Optima, BMG Labtech, Bucks, UK) at (Ex/Em: 544/590nm).

4.2.7. Immunofluorescence

Samples were prepared according to Section 3.2.6. Samples were stained overnight with phalloidin, vinculin antibody and Prolong Gold Antifade Reagent with 4’-6-diamidino-2-phenylindole (DAPI, Invitrogen, UK).

4.2.7.1. Vinculin staining

Immunofluorescent staining was performed according to the following procedures. Briefly, T/C-28a2 were cultured on the agarose, collagen, SAPHs and glass. After 3, 6 and 24 hours, samples were washed with sterile DPBS twice before fixing with 4% paraformaldehyde (PFA) for 30 mins at room temperature (RT). After washing with DPBS twice, samples were incubated with blocking buffer containing 1% (v/v) goat serum with 0.1%(v/v) Triton X-100 in 1X DPBS solution for 30 mins at RT. Triton X-100 was required to permeabilise the cell membranes. Vinculin antibody (mouse monoclonal to vinculin, ab18058, Abcam, Cambridge, UK) was diluted at 1:100 dilution in dilution buffer (1% (v/v) BSA, 0.1% (v/v) Triton X-100 and 1X DPBS). Following that, samples were incubated with the antibody solution overnight at 4°C. BSA was used to block non-specific binding sites on the cells.

Subsequently, secondary antibody goat anti-mouse IgG H&L AlexaFluor® 568 (ab175473, Abcam, Cambridge, UK) was diluted to 1:200 in dilution buffer. Upon washing the samples with DPBS, samples were incubated with secondary antibody solution for 2 hours at RT. Samples were then counterstained with ProLong™ Diamond Anti-fade reagent with DAPI for 24 hours in
a dark environment (at RT). Samples were stored in a sealed humidified box at 4°C until imaging.

The study was carried out in an 8-well chamber to enable imaging of the samples directly without having to transfer samples to glass cover slides. All fluorescence images were acquired with a laser scanning confocal microscope (Leica, Milton Keynes, UK) with a X20 eyepiece and an X63 oil immersion lens.

4.2.8. Morphology

Actin fibres were stained using phalloidin dye reagent tagged with CytoPainter 488 phalloidin (ab176753, Abcam, Cambridge, UK). Dye was diluted at 1:1000 ratio (v/v) in 1 X DPBS. After 90 mins incubation (at RT, dark), dye solution was removed, and samples were rinsed with DPBS. Samples were then counterstained with ProLong™ Diamond Anti-fade reagent with DAPI for 24 hours in a dark environment (at RT). Samples were stored in a sealed humidified box at 4°C until imaging.

4.2.9. Encapsulation of T/C-28a2 in 3D

4.2.9.1. Cell viability assay

The protocol to assess cell viability when encapsulated in peptide hydrogels was adopted from Section 3.2.4.

4.2.9.2. Histology

For histological analysis, samples were fixed with 4% paraformaldehyde (PFA, Sigma Aldrich, Dorset, UK) at RT for 1 hour. Upon washing with PBS and processing of samples, all samples were embedded in paraffin and sections of 5µm thickness were cut using a microtome (RM2145, Leica). After overnight drying, these sections were then deparaffinized and stained with hematoxylin and eosin (H&E) to allow visualization of cell distribution and morphology. The histology staining was developed by the School of Material’s (University of Manchester) histologist, Ms Marie O’Brien.

4.2.10. Statistical analyses

See Section 2.2.8.

4.3. RESULTS AND DISCUSSION

4.3.1. 2D cell interaction

4.3.1.1. Surface roughness of SAPH

AFM topographical images of the surfaces of SAPHs were used to determine the surface roughness of each SAPH. The topography of hydrogels has been shown to have promoted cell attachment to substrates (Zheng et al. 2009, Olivares-Navarrete et al. 2015, Hu et al. 2016). The surface roughness was measured to eliminate the fact that if more cells adhered to the
surface of the hydrogels (if any), it was not due to the difference in surface roughness. Scan size of 20x20µm was obtained from a randomly chosen position. For each sample, three different positions were assessed. Figure 4-2(a) illustrates the representative images of the 3D perspective of the surface plots of F8, F8R, F9, FK and FR. These images (replicates from 2 different samples for each type of SAPH) were further analysed to obtain the average roughness (Ra) and root-mean-square (RMS) roughness (Rq). The Ra is the average deviation of the height values from the mean line, while the Rq is the root-mean-square deviation from the mean (standard deviation from the mean).

The surface roughness characteristics of the SAPHs are displayed in Figure 4-2(b). These data, as shown in this figure, were calculated from 2x2µm scan area (n=50 for each SAPH), due to the fact that this is the size of area sensed through a single focal adhesion. As stated in Section 1.4.4, focal adhesion comprised of the protein within the extracellular matrix (ECM), such as fibronectin and laminin to name a few; and transmembrane proteins such as integrins and intracellular proteins such as actin and vinculin. Adherence of cells to the surface or substrate is governed by these proteins and complexes of proteins, which are measured to be in the range of 10nm to 10µm in sizes.

As shown in Figure 4-2(b), values of Ra (mean surface roughness) for the neutral charged SAPHs F8 and F8R, were not significantly different. This shows that substituting lysine (K) with arginine (R) did not change the topography of the hydrogel. Ra for +1 charged SAPH F9 was also comparable to F8. Ra of +2 charged FK, on the other hand, was calculated as twice the value of Ra of F9 (p<0.05), suggesting the repulsion of charge within the peptide may have potentially increased the Ra. The effect of charge was, however, inconclusive due to Ra of FR (which is also +2 charged) not being significantly different from the neutrally charged version of SAPH containing arginine, F8R. Substitution of lysine (K) with arginine (R) in FR decreased the value of Ra in the scan size of 2x2µm, compared to FK (both has +2 charge). From this graph, only FK has higher Ra and Rq than F8, suggesting the effect of higher charge, but when arginine is present, no difference was observed (FR8 vs FR). Thus, from this graph, we learnt that arginine did not change the surface roughness.
Atomic force microscopy (AFM) was used to measure surface roughness of self-assembled peptide hydrogels (SAPH).

a) Images were generated from 20x20µm scan size and projected into 3D images. b) Average roughness (Ra) and root-mean-square deviation from the mean (Rq) are calculated from 2x2µm size as this is the area experienced by cell focal adhesion. (*) and (**) show significantly higher than all other samples for Rq and Ra, respectively (p<0.01).

The measurement of the deviation of surface roughness from their mean value shows that Rq (root-mean-square of roughness) values for F8, F8R, F9 and FR were not significantly different, and both Ra and Rq of FK was found to be significantly higher than other SAPHs (Kruskal Wallis test, p<0.01).
These AFM topographical images were acquired in a dry condition at ambient temperature as described in Section 0. Earlier attempts to obtain images in wet condition were found to be impossible, especially when the cantilever was in contact with the charged SAPHs. However, the measurements of surface roughness for these SAPHs are comparative to each other since all images were taken in the same condition/environment.

4.3.1.2. Effect of seeding density on the efficacy of cell adhesion

The aim of this study is to choose the seeding density for further studies. The effect of seeding density on cell adhesion was studied using Live/Dead assay. For this experiment, two different seeding densities were studied, 0.5 million cells per cm$^2$ or 1.0 million cells per cm$^2$. F8, F8R, F9, FK and FR were pipetted (150µl) into 8-well glass chambered slides (Ibidi®, Germany). Collagen and agarose hydrogels were included as positive and negative control hydrogels, respectively, and the glass surface was included as a non-hydrogel control. Samples were kept incubated at 37°C, 5% CO$_2$ throughout the study.

At pre-determined time points, media was removed from the top of the samples and samples were then washed with sterile DPBS twice. Cell viability was assessed using a confocal fluorescent microscope, and representative images are shown in Figure 4-3. Cells were observed to be alive in all samples, indicating that none of these hydrogels was cytotoxic to the cells.

At 3 hours, number of cells adhered to FR hydrogels was observed to be comparable to those on collagen hydrogel at its respective time point for 0.5 million cells per cm$^2$. Number of cells adhered on the other SAPHs were found to be on the lower side compared to FR and collagen hydrogels. It was also observed from Figure 4-4 that seeding density affected cell adhesion density on collagen, F8, F9 and FR hydrogels after being cultured for 3 hours (p<0.01). Upon culturing for 3 hours, a higher number of cells were observed to adhere to these four hydrogels when seeded at 1.0 million cells per cm$^2$ in comparison to 0.5 million cells per cm$^2$. It was also noticed that a higher number of cells adhered to FR upon culturing for 3 hours, regardless of the seeding density compared to other SAPH. Lower adhesion rate on F8 and F8R (after 3 and 24 hours) may be explained by the effect of charge on cell adhesion rate, for both seeding densities.
Seeding density | 0.5 million cells per cm$^2$ | 1.0 million cells per cm$^2$
---|---|---
Time (Hours) | 3 | 24 | 3 | 24
Coll | ![Coll 3](image) | ![Coll 24](image) | ![Coll 3](image) | ![Coll 24](image)
Glass | ![Glass 3](image) | ![Glass 24](image) | ![Glass 3](image) | ![Glass 24](image)
Agar | ![Agar 3](image) | ![Agar 24](image) | ![Agar 3](image) | ![Agar 24](image)
F8 | ![F8 3](image) | ![F8 24](image) | ![F8 3](image) | ![F8 24](image)
F8R | ![F8R 3](image) | ![F8R 24](image) | ![F8R 3](image) | ![F8R 24](image)
F9 | ![F9 3](image) | ![F9 24](image) | ![F9 3](image) | ![F9 24](image)
FK | ![FK 3](image) | ![FK 24](image) | ![FK 3](image) | ![FK 24](image)
FR | ![FR 3](image) | ![FR 24](image) | ![FR 3](image) | ![FR 24](image)

Figure 4-3 Cell viability for T/C-28a2 seeded on hydrogels with different seeding density. Cells were seeded on different SAPHs, agarose, collagen and glass at a seeding density of 0.5 or 1.0 million cells per cm$^2$. This study was carried out with the presence of fetal bovine serum (10%(v/v)). Upon staining with the Live/Dead assay, images were acquired at magnification x20. Z-stack images were then projected into a single image. Green shows live cells, and red shows dead cells. It was seen from the images that cells adhered on different SAPH at different density. Scale bar = 250µm.
Figure 4-4 Number of cells adhered at 3 and 24 hours at different seeding density. The graph shows that when seeded at a higher density, significantly higher number of cells adhered to collagen, F9 and FR, at both time points (*, p<0.01). When samples were imaged after 3 hours, a significantly higher number of cells were observed on F8 (##, p<0.01). However, this was not observed on F8 at the other time point. At 24 hours, FK had a significantly higher number of cells adhered to its surface (#, p<0.01). Increasing the duration of culture only affected cell adherence on glass, agarose and F8, but not to the other samples. These quantifications were based on six samples, prepared on two different occasions.

When media was removed 24 hours after seeding with 1.0 million cells per cm$^2$, a noticeably higher number of cells adhered to collagen, F9, FK and FR compared to F8, F8R and agarose. This also may potentially result from the effect of charge on cell adhesion rate. Effect of charge on cell adhesion has been widely studied (Chun et al. 2004, Schneider et al. 2004, Kim et al. 2009, Zheng et al. 2009, Dadsetan et al. 2011, Dadsetan et al. 2012, Renò et al. 2012). It was shown that when hydrogels were attached to a positive fixed charge, higher density of chondrocytes attached to the hydrogels. Bovine chondrocytes were cultured on the modified surface of poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres and cell attachment was reported as higher on positively charged microspheres (Chun et al. 2004). Dadsetan et al. (2012) incorporated either sodium methacrylate (SMA, negatively charged) or (2-(methacryloyloxy)ethyl)-trimethyl ammonium chloride (MAETAC, +vely charged) to oligo(poly(ethylene glycol) fumarate) (OPF) and found more chondrocytes attached on the positively charged hydrogels, even though cells in the negatively charged hydrogel produced more collagen type II and glycosaminoglycan (GAG) than those in the positive ones. Endothelial cells and osteoblasts were also found to adhere more to positively charged hydrogels (Schneider et al. 2004, Kim et al. 2009). However, extreme density of positive charge (>3 wt %) of poly-L-lysine on chitosan led to cell death of MC3T3-E1, an osteoblast-like cell line (Zheng et al. 2009). In addition, a separate study found that fetal osteoblast adhesion increased in relation to higher negative charge density of bis(2-(methacryloyloxy)ethyl)phosphate (BP) incorporated into oligo(polyethylene glycol) fumarate (OPF) hydrogel (Dadsetan et al. 2012). Another study by Renò et al. (2012) found that higher protein adsorption was observed on positively charged poly-l-lysine modified gelatin hydrogels and human keratinocytes (HaCat) were found to have
spreading morphology on polyglutamic acid (very charged) modified gelatin hydrogels, indicating cellular adhesion (Renò et al. 2012).

In this present study, very few cells adhered to agarose hydrogel or glass after being cultured for 3 hours. The glass slide was hydrophobic, and cells do not adhere to hydrophobic surfaces. Other groups have studied the effect of reducing contact angle through surface modification, to increase hydrophilicity and improve cell adhesion (Ino et al. 2013, Chuah et al. 2015). Increasing the seeding density did not improve adhesion rate for cells on agarose hydrogel, at any time point. Agarose is built by repeated disaccharide 3,6-anhydro-α-L-galactose and β-D-galactose. The low cell adhesion rate on agarose hydrogel is due to the unavailability of adhesion integrin binding sites in agarose (Luo and Shoichet 2004, Wong et al. 2004, Yixue et al. 2013, Krouwels et al. 2018). This was suggested in a study by Krouwels et al. (2018) which showed that no vinculin was detected in agarose seeded with human intervertebral disc cells (IVD).

When it comes to the viability of the cells upon seeding on the hydrogels, each sample behaved differently when the seeding density was varied. Figure 4-5 shows that when cells were seeded with 0.5 million cells per cm² (at 3 hours), the viability of cells seeded on all samples was not significantly different. At this time point, the cell viability was slightly lower for agarose, and the cell viability reduced even more after 24 hours (for both seeding densities). At 0.5 million cells per cm², cell viability went from 98.1±3.0% at 3 hours to 65.7±12.5% at 24 hours for agarose. This non-significant reduction in cell viability may be due to the unavailability of cell adhesion sites and low surface roughness on agarose. This may also explain why the viability of cells on glass was higher when seeded with higher seeding density.

No significant change in cell viability with time was observed with collagen, F8 and F8R, at each time point. However, cell viability of F9 decreased significantly when seeded with 1.0 million cells per cm² from 98.7±0.6% at 3 hours to 76.0±5.2% at 24 hours. While it was observed that cell viability on FK reduced with time when seeded with 0.5 million cells per cm², cell viability of FR reduced significantly for both seeding densities.

When it comes to the quantification of cells adhered on SAPH, based on the live/dead images, F9 (+1 charge), FK and FR (both +2 charges) had the highest number of cells adhered to it at both 3 and 24 hours.
Figure 4.5: Quantification of cell viability based on the Live/Dead images in Figure 4.3.

No significant changes in cell viability of cells seeded at a different density on all samples at 3 hours. Among others, lower seeding density was favourable for F9 and FR, while cell viability was significantly increased on FK when more cells were seeded. (*) signifies significantly different cell viability at different seeding density when assessed at 24 hours (p<0.01).

According to Wang and Kandel (2004), to enable investigation of integrin, cell density was reduced by ten times lower than the normal seeding density. Thus, 1.0 million cells per cm² density was initially investigated. It was observed that upon seeding at a higher density, cells were closer to each other and tended to form cell-cell adhesion which contributed to higher adhesion rate. This phenomenon was apparent for collagen, F8R, F9, FK and FR hydrogels. This study aimed to examine cell-SAPH interaction. Thus, lower cell seeding density (0.5 million cells per cm²) was chosen for further study in order to avoid cell-cell interaction which would prevent the aim of this study.

4.3.1.3. Effect of serum on cells viability

The purpose of this section was to assess the role of serum on cells adhesion. Cells were expanded in T-175 flasks as described in Section 4.2.3.1. Upon detaching the cells from the flasks, cells were washed twice with DMEM before re-suspending in media without FBS. For this current study, T/C-28a2 human immortalised chondrocytes were seeded at 0.5 million cells per cm². Samples were kept incubated at 37°C, 5% CO₂ throughout the study. At 3 and 24 hours post-culturing, media was removed, and samples were washed twice with DPBS. Samples were then immersed in Live/Dead stain as described in Section 4.2.5.

Images of samples stained with Live/Dead assay were acquired from confocal fluorescence microscopy (Leica, Milton Keynes, UK). Representative images of T/C-28a2 seeded on different SAPHs, and collagen, agarose and glass as controls are shown in Figure 4-6 and Figure 4-7.
<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>With serum</th>
<th>Without serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>24</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>3</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>24</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 4-6 The images show how depletion of FBS from media would affect cell viability. T/C-28a2 was seeded on different hydrogels/samples with and without serum at 0.5 million cells per cm². Upon staining with the Live/Dead assay, images were acquired at magnification x20. Z-stack images were then projected into a single image. The absence of serum did not affect the rate of cell attachment; however, cell viability was negatively affected. Green shows live cells and red shows dead cells. Scale bar = 250µm.
With serum | Without serum
---|---
Time (hr) | 3 | 24 | 3 | 24
Coll | ![Image](179x660 to 250x731) | ![Image](267x660 to 338x660) | ![Image](355x660 to 426x660) | ![Image](441x660 to 512x660)
Glass | ![Image](179x583 to 250x654) | ![Image](267x583 to 338x654) | ![Image](355x583 to 426x654) | ![Image](441x583 to 512x654)
Agar | ![Image](179x506 to 250x577) | ![Image](267x506 to 338x577) | ![Image](355x506 to 426x577) | ![Image](441x506 to 512x577)

Figure 4-7 Cell viability assay on cells seeded on control samples. Cells stained with cell viability assay upon being seeded on positive control collagen hydrogel and negative control hydrogel, agarose. It was also seen from these images that, at both 3 and 24 hours, cells seeded on agarose hydrogel had reduced viability. This, however, was not observed on glass non-hydrogel negative control.

Samples seeded with cells without the presence of serum were compared to those with serum. For comparison purposes, samples that were seeded with and without serum were prepared in the same setup on the same day. Half of these samples were tested after 3 hours, and the other half after 24 hours of culture, without media change between these time points. Upon being cultured for 3 hours, in comparison to samples seeded with the presence of serum, a lower number of cells adhered to all SAPH when serum was omitted. As this phenomenon was also observed in positive control collagen hydrogels, then the reduced number of adhered cells may be attributed to the cell itself and not due to the differences of SAPH. In addition, cell adhesion rate on glass and agarose hydrogel was observed to not be affected by the absence of serum. However, the absence of serum was found to affect the viability of cells seeded on glass (69.4±11.2%), agarose (58.0±9.5% viability), FK hydrogels (37.8±12.5% viability) and FR hydrogels (66.5±10.6% viability), but not on other samples (after 3 hours, see Figure 4-8).
Images in Figure 4-6 were analysed using ImageJ to assess the effect of serum on cell adhesion and cell viability. Cells were seeded on hydrogels with (+) or without (-) serum. The graph shows a significant reduction of cell viability after both 3 and 24 hours for agarose, glass, FK and FR samples (p<0.01). When cells were seeded in the absence of serum, cell viability on glass decreased significantly at 3 hours (p<0.01).

Samples of collagen hydrogel that were assessed after 24 hours of culturing had no significant changes in number of cells adhered to it without the presence of serum, in comparison to similar samples with serum at the same time point. This was also seen for the non-charged SAPH-F8 and F8R. This was also observed in F9 samples. Albeit, the effect of removing FBS was apparent on the viability of cells cultured on FK and FR without the presence of serum. At 24 hours, the viability of cells on all samples of FK and FR hydrogels reduced to 0% viability in the absence of serum.

The effect of the presence of serum on cell adhesion has been reported by Zwolanek et al. (2015). It was reported that when saline was replaced with serum, the rate of rat MSCs attachment to cartilage lesions increased. On the contrary, Schneider et al. (2004) who studied the effect of charge on cell attachment found that the presence of serum did not affect cell attachment rate. Serum-supplemented media was also used by other researchers who studied cell attachment on biomaterials scaffold (Dadsetan et al. 2011, Dadsetan et al. 2012, Liu et al. 2012, Krouwels et al. 2018).

Reduced cell viability signified the necessity of serum in this study. Thus, serum would be included for further studies.

4.3.1.4. Is FBS essential for cell adhesion or cell survival?

As stated earlier in Section 4.3.1.3, reports by other researchers suggest that the presence of serum is not essential for cell adhesion. The study in this current section was carried out to further investigate the role of serum in cell adhesion and cell survival. Figure 4-9 shows the images of cells seeded on different hydrogels and glass, with serum removed at different points during the experiment. Upon detaching T/C-28a2 from the flasks, cells were counted as per
protocol described in Section 4.2.4 and divided into two tubes. Cells in Tube 1 were washed twice with DMEM, prior to re-suspending in media without FBS, while cells in Tube 2 remained in complete media (DMEM+10% FBS). After plating the hydrogels in an 8-well glass chambered slide (labelled as ‘Prior to seeding’), cells from Tube 1 were seeded on each hydrogel. Cells in Tube 2 were seeded on another set of hydrogels plated in another chamber slide (labelled as ‘Upon seeding’) at the same seeding density (0.5 million cells per cm²). Both chamber slides were kept incubated at 37°C, 5% CO₂. Media from the ‘Upon seeding’ chamber slide was replaced with media without FBS after 6 hours of culturing. Samples from both conditions were tested using Live/Dead assay after culturing for 24 hours. Images acquired using a confocal fluorescent microscope (Leica, Milton Keynes, UK) are laid out as below in Figure 4-9.

Cells from Tube 1 (prior to seeding) were observed to be viable in all samples except for those on agarose, FK and FR hydrogels. Cell viability for both FK and FR was calculated to be 0% viable throughout all three repeats, using hydrogels which had been made independently. It was initially hypothesised that this was due to the role of serum in cell adhesion. However, cells were also found to be non-viable even when serum was present during the first 6 hours of culture where the initial interaction between cells and hydrogels takes place. Lower cell viability may be explained by the stress cells had to experience when cultured on high-charged SAPH. Figure 4-10 shows that there was also significant differences in cell viability for agarose. At 24 hours, significantly higher cell viability was observed when FBS was removed upon seeding the cells on agarose (p<0.01). This could be due to the availability of adhesion protein contained in FBS that helps the cells to adhere to agarose during the first 6 hours.

When samples were compared between the two conditions, removal of serum at an earlier point of the culture did not affect the number of cells adhered, suggesting that cell adherence is not regulated by serum. As depicted in Figure 4-11, the point of FBS removal did not affect cell adherence. When the number of cells adhered to the substrate or hydrogel were counted based on the live/dead images, no significant difference between those that serum was removed prior to seeding or upon seeding for all samples was observed. Based on this result from this study, it may suggest that serum was not required for cell adhesion on these samples.
Serum removal | Prior to seeding | Upon seeding
---|---|---
Agar | ![Image](image1.png) | ![Image](image2.png)
Coll | ![Image](image3.png) | ![Image](image4.png)
F8 | ![Image](image5.png) | ![Image](image6.png)
F8R | ![Image](image7.png) | ![Image](image8.png)
F9 | ![Image](image9.png) | ![Image](image10.png)
FK | ![Image](image11.png) | ![Image](image12.png)
FR | ![Image](image13.png) | ![Image](image14.png)
Gls | ![Image](image15.png) | ![Image](image16.png)

Figure 4-9 Study was carried out to see if removing fetal bovine serum (FBS) at different points of cell seeding affects cell viability. FBS was either removed from the point prior to seeding on samples, or 6 hours upon culturing of the samples. At the point of the assay, samples were deprived from serum for 24 hours (left), and 18 hours (right). Images were acquired at magnification x20. Z-stack images were then projected into a single image. In both conditions, few cells were seen on glass and agarose. In terms of cell viability, cells were dead in both conditions for FK and FR, suggesting cells could adhere to the surface even without serum, but it affected cell viability. Scale bar = 250µm.
The images in Figure 4-9 were analysed to assess the effect of removing FBS upon cell seeding and prior to cell seeding on cell viability. The effect of removing the FBS prior to seeding was apparent for cells seeded on agarose (p<0.01). Even though the point of serum removal does not change the cell viability on all other samples, it was seen that cell viability on FK and FR was 0% for all samples (n=6) when serum was removed prior to cell seeding.

Figure 4-11 Number of cells adhered when serum was removed upon seeding or prior to seeding. The number of cells adhered on all samples were not significantly different to similar sample which was treated differently (p>0.01).

It was learnt from the literature that FBS is responsible for the re-differentiation of chondrocytes. Encapsulation of bovine chondrocytes in 3D collagen sponges showed that matrix production was lower in the presence of 1% ITS (Insulin, transferrin, selenium) and 2% Nutridoma, compared to those samples cultured in 5% FBS (Yates et al. 2005). In a separate study, supplementation of 20% FBS to chondrocytes seeded within 3D agarose hydrogels increased the value of GAG/DNA but had no effect on collagen/DNA. Another study found that the effect of the absence of serum was only observed in the dynamic and not in the static cultivation (Yang and Barabino 2011). When samples were exposed to the hydrodynamic environment, accumulation of collagen type II was enhanced in the presence of FBS. Despite this, a...
conflicting finding was reported when cells were cultured in monolayer on type II collagen-coated inserts. In the presence of FBS, bovine chondrocytes lost their spherical morphology and when FBS was replaced with ITS, higher production of sulphated GAG and collagen type II was observed (Ahmed et al. 2014).

There was a concern that cells might be in shock upon removal of serum. Thus, a separate experiment was carried out to remove serum from expanded cells in phases. Cells were first cultured in monolayer in flasks with complete media and split into 1:12 ratio upon reaching 90% confluency. Triplicate flasks were cultured for at least 24 hours in complete media (DMEM+10% FBS) at 37°C, 5% CO₂. Media was then replaced with media containing a gradually reduced concentration of FBS, according to the protocol as described by van der Valk et al. (2010). Cells were cultured in DMEM+5% FBS, 2.5%, 1% and finally in DMEM+0% FBS over a period of 6 weeks. Triplicate flasks of cells were cultured in DMEM+10% FBS in parallel as controls. It was found that cells cultured in the reduced concentration of FBS lost their capability to proliferate, as well as failed to adhere to the flask and had reduced viability compared to the control flasks (data not shown). Cells cultured in reduced FBS concentration did not reach 50% confluency even when cultured for more than 2 weeks, compared to the controls which reached 90% confluency after 4 days of culture. This was also reported in a study by Kokenyesi et al. (2000) where T/C-28a2 cultured in serum-deprived monolayer condition resulted in a reduction of cell proliferation and survival rate.

As described in Section 4.2.3, T/C-28a2 comes from rib cartilage that was transfected with antigen SV 40. Another study found that the viability of other types of cells (immortalised murine endothelial cell lines SVEC 4-10 and 2H-11, and immortalised human umbilical vein endothelial cell line HUVEC) upon transfection increased proportionally with increasing incubation time within media + FBS (Bosnjak et al. 2014). The effect of FBS on cell survival was also observed by another study. Survival of MC3T3-E1 (osteoblastic cell line) cultured on Ti6Al4V decreased upon FBS deprivation (especially during the first 24 hours). However, the cell viability recovered after 3 days of culturing (Felgueiras and Migonney 2014). Given that this chapter focuses on early cell interaction, the culture duration that we are interested in looking at is ≤24 hours.

Serum contains multiple components as detected by proteomic analysis of FBS (Zheng et al. 2006). Among others, serum contains amino acids, proteins, vitamins, carbohydrates, lipids, hormones, growth factors, inorganic salts and trace elements (Yao and Asayama 2017). These abundant components of serum increase its viscosity, which helps to protect cells in suspension from shear damage due to pipetting and stirring (Price 2017, Yao and Asayama 2017). The free amino acids help the cell culture media to increase its buffering capacity. Serum also contains hormonal factor that stimulates cell growth and promoting differentiation (Gstraunthaler 2003). Franke et al. (2014) found that the phenotypic changes of primary equine fibroblasts were due to the absence of serum. Lipid, such as lipoprotein from the albumin free fatty acids, can also be found in serum. Lipid functions as one of the sources of energy for cell signalling and biosynthesis, besides being the main structural component of cell membrane (Price 2017).
As reported by other literature, FBS contains fibronectin and vitronectin, both of which promotes cell adherence (Oehlke et al. 2011, Ahmed et al. 2014). Due to this, it is highly possible that when the cell adhesion study is carried out in the presence of serum, the cell adhesion capacity may be attributed by the presence of these proteins in the serum. Fibronectin and vitronectin contain RGD sequences that serve as the binding sites for integrins (Almonte-Becerril et al. 2018).

Having said that, due to the findings in this section, serum was included in later studies throughout this chapter. However, in the future, the role of serum in cell adhesion may be further studied by manipulating the study- using cells in conditions as described for Tube 1, and complete media (DMEM+10% FBS) will be introduced after 6 hours of culture to ensure cell survival.

4.3.1.5. Cell adhesion assay

This section looked at the rate of cell adhesion on different SAPH. Cells behave differently, in terms of their adhesion, migration and differentiation, all of which are controlled by chemical and physical interaction with biomaterial scaffolds (Yang et al. 2010, Nagayasu et al. 2012, Bradshaw et al. 2014). The rate of cell adhesion on different sequences of SAPH was investigated through a modified protocol of the cell adhesion assay. Hydrogels were prepared as described in Section 4.2.1 and 50µl of each hydrogel were plated in a 96-well plate in triplicate. Since F8 and F8R were prepared at pH 4 (F8 and F8R precipitate at pH>4), samples were immersed in serum-free media and were incubated at 37°C, 5% CO₂ to modulate the pH. T/C-28a2 were trypsinised from the flask, and 10 times the required number of cells were stained with PKH26 as described in Section 4.2.6. Stained cells were seeded at 15000 cells per well (0.5 cells per cm²) and were kept at 37°C, 5% CO₂ throughout the study. After being cultured for 3 hours, media (containing the un-adhered cells) was transferred to an empty well and fresh media was flooded into the well with (the retained) adhered cells. Samples in the well plate were then measured using a fluorescence plate reader (Fluostar Optima, BMG Labtech, Bucks, UK) at the excitation of 544nm and emission of 590nm. Data in Figure 4-12 were pooled from n=6 samples, carried out in two independent preparations. The fluorescent measurement was background subtracted and converted to cell number from the standard curve of the known number of cells to raw fluorescent measurement. Total number of cells adhered and un-adhered were calculated to be 16000±3000 cells per well on average for all samples.

Percentage of cells adhered on collagen was measured to be 88.2 ± 11.4%. This value is comparable to all SAPH and treated TCP. Agarose hydrogel, however, had a significantly lower adhesion rate than collagen hydrogel. This finding suggests that SAPHs used in this study may have the same capability as the collagen hydrogel in terms of cell adhesion rate. The non-significant difference of cell adhesion percentage in all SAPH, regardless of the charge and presence of arginine suggests that these factors did not affect cell adherence of these SAPHs.
Figure 4.12 Graph shows the percentage of cells adhered on SAPH, with Agarose, TCP and collagen as the control samples.

Cell adhesion assay was carried out using cells stained with membrane dye PKH26. Cells at a density of 15000 per well were left incubated, and unadhered cells were removed to an empty well after 3 hours. Percentage of cell adhesion on TCP was significantly lower than F8, F8R, FK and FR (**, p<0.01, Kruskal-Wallis, n=10). Cell adhesion assay has also shown that agarose had the lowest number of cells adhered compared to other samples (#, p<0.01).

The role of positive charge on enhancing cell adhesion was however observed by others studies, as discussed in Section 4.3.1.2. Besides positive charge, cell adhesion percentage can also be increased by increasing the surface roughness. The strong influence of topography on cell adhesion is potentially caused by the initial protein adsorption from the media or protein secreted by the cells (Lee et al. 2004, Schulte et al. 2009). AFM topographical measurement of the surface of chitosan modified with poly-L-lysine (positively charged) showed an increment in terms of its surface roughness, which is responsible for improving cell adhesion (Zheng et al. 2009). Work by Schulte et al. (2009) which improved the topography of poly-ethylene-glycol (PEG) through the fabrication of substrate not only increased focal adhesion density but also affected the cytoskeletal arrangements. This study, however, found that increased surface roughness on FK did not lead to a higher percentage of cell adhesion.

To date, there have been limited studies on the effect of the presence of arginine amino acid on cell adhesion, except for studies on self-assembled peptide hydrogel (RADA16-I) and the binding site sequence arginine-glycine-aspartate (RGD) (Schuh et al. 2012a, Smith Callahan et al. 2013a, Bradshaw et al. 2014). It was reported that cells attached to RADA16-1 hydrogel through a non-integrin interaction, even in the presence of RGD (Shamsi 2016). In another study, cells adhesion density was observed to be higher on RAD-1 and RAD-II, in comparison to non-arginine containing SAPH KFE-8 and KLD-12 (Sieminski et al. 2008).

Besides RADA16-1 and RGD, another group coated the PLA surfaces with L-arginine and found that the cells interacted with the surfaces via β1 integrin receptor (Lee et al. 2004), while another researcher found cell adhesion rate decreased when L-arginine was removed from the
culture media (Pavlyk et al. 2015). Findings from this current study to assess the effect of the presence of arginine in the peptide sequence on cell adhesion are also inconclusive. Thus, further studies need to be carried out to confirm the effect of peptide charge and presence of arginine on cell adhesion.

There are multiple ways to carry out cell adhesion assays. In a study by Jeon et al. (2012), adhered cells (0.01 million cells per cm²) were quantified using a light microscope after cells were incubated on oxidised hydrogels for 4 hours, while Cutongco et al. (2016) used CyQuant® to count adhered cells upon culturing them for 24 hours. For this current study, an attempt was initially made to carry out cell adhesion assay adapted from a standard procedure as outlined by Humphries (2000). AlamarBlue® was used instead of crystal violet as suggested in this protocol. However, earlier work had shown that when AlamarBlue® was used, the reading for the background measurement was high even though no cells were present. Thus, cells were stained with membrane dye PKH26 prior to seeding to avoid a high background measurement reading.

This study has found that cell adhesion rate on SAPH was comparable to the positive control collagen hydrogel. The effect of the presence of arginine in the peptide sequence or the surface roughness of the hydrogels on cell adhesion was found to be inconclusive from this study. Thus, the next section will look into the vinculin staining.

4.3.1.6. Vinculin staining

This study looked into the vinculin staining that indicates the presence of focal adhesion. As stated in Section 1.4.5, vinculin bridges the actin and β-integrins to form focal adhesions. For this study, T/C-28a2 was seeded on F8, F8R, F9, FK and FR, while agarose, collagen and glass were treated as control samples. Cells were seeded at the same seeding density as determined in Section 4.3.1.2.

As can be seen in Figure 4-13, representative images of cells being stained with vinculin antibody and imaged using a fluorescence confocal microscope (Leica). It was observed from this figure that no vinculin was detected when samples were analysed for immunofluorescence tagging of vinculin antibody at 3 hours.

After being cultured for 24 hours, images showed a positive indication of vinculin on collagen, F8R and FR. Not only that, vinculin staining on these samples was intense. Interestingly, vinculin staining was detected in cells seeded on FR samples at 6 hours of culture, while this was not observed on collagen hydrogels at the same time point. However, Figure 4-13 also shows non-specific vinculin staining on some samples. No vinculin was detected in cells cultured on agarose hydrogel and glass, even after 24 hours culture. This was also observed by Krouwels et al. (2018). Due to the unavailability of integrin binding sites in agarose, no vinculin was detected when human intervertebral discs (IVD) were cultured in agarose in the presence of 10% FBS. Distribution of vinculin for collagen, F8R and FR was observed to be co-localised.
with actin which suggests the formation of focal adhesion between the cells and the hydrogels (see Figure 4-15). However, some of the vinculin staining on these samples diffused through the cell, which is not expected for vinculin, again suggesting non-specific staining.

Interestingly, vinculin, as was found by Thievessen and colleagues, inhibited cell migration in 2D cultivation, while in 3D it was observed to enhance cell migration, cell elongation and cell protrusion (Thievessen et al. 2015). Being a connector between the actin cytoskeleton and ECM, vinculin is also perceived as a mechanotransducer, where vinculin gene trapping within ATDC5 cells negatively impacted the gene expression of type II collagen and aggrecan (both are considered as chondrogenic markers) (Shen et al. 2011, Koshimizu et al. 2012).

Positive vinculin staining in SAPHs containing arginine (R) amino acid suggests that arginine potentially contributes towards the activation of integrin binding sites on the cell membrane. Even though images of vinculin staining have shown this, as stated in the previous section, there is no difference between all SAPHs in terms of cell adhesion. This suggests that cells may have attached to the peptide hydrogel surfaces through other cell-matrix adhesion molecules besides vinculin. It could either be through CD44 or laminin. However, due to the non-specificity in vinculin staining, this work may be repeated in the future.

4.3.1.7. Cell morphology

Spreading morphology of cells is another indication of cell adhesion. T/C-28a2 were seeded on hydrogels which were plated in an 8-well chambered slide at 0.5 million cells per cm² and stained for actin. After 3 hours of culturing, cells were observed to be elongated on F8R, F9, FK and collagen hydrogels. At this time point, cells on FR hydrogel were observed to be extended, while cells with rounded morphology were dominant on F8 hydrogels.

Another set of samples was stained with phalloidin after culturing for 6 hours. Cells were seen to have adopted spreading morphology after being seeded for 6 hours on F8R, F9 and collagen hydrogels. Cells on FK and FR, on the other hand, were observed to be elongated. The size of cells on F8 seemed to have increased after seeding for 3 hours, but the cells remained rounded. It was also noticed from Figure 4-14 that intensifying actin fibres across the nucleus for cells on F9, FK and collagen hydrogels, while punctate actin surrounding the nucleus was observed on F8R and FR at 6 hours of culture.
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Figure 4-13 Samples stained with vinculin. No positive staining of vinculin was observed until 24 hours on all samples including collagen hydrogel. After 24 hours of culturing, positive staining of vinculin was only detected on collagen and arginine containing (denoted by *) SAPH, F8R and FR. Diffusion of staining in cell and blue circle marks indicating non-specific staining would be disregarded.
Figure 4-14 T/C-28a2 being stained with phalloidin (green). The morphology of T/C-28a2 seeded on SAPH, collagen, agarose and glass was observed. Cells were seen to have stretched morphology especially for FK and FR (+2 charged) and rounded for those on agarose, glass and F8 (0 charged). It can, however, be seen that cells on F8R (0 charged, contains arginine*) also became elongated.
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Figure 4.15 Merged images of the previous two figures and nuclei are stained with DAPI (blue). Positive vinculin staining was observed in cells seeded on F8R and FR hydrogels. However, it is not possible to see from the images that these vinculin staining are co-localised at the actin as expected. Vinculin staining and cell morphology of T/C-28a2 supports each other in terms of cell adhesion behaviour. Scale bar =10µm.
After being cultured for 24 hours, cells on all SAPHs (except for F8) and collagen hydrogels were observed to have spreading morphology, signifying adhesion and migration for cells cultured on all hydrogels except for F8 (Connelly et al. 2008). These differences may be explained by either the presence of charge or arginine amino acid in certain SAPHs. No cell staining was found on agarose and glass at earlier time points, and cells on these samples were seen to have rounded morphology even at 24 hours of culture. Even though there were cells on agarose hydrogel and glass, as shown in Figure 4-3, cells on these samples in this current study may have been washed away due to multiple washing steps in the immunofluorescence dye staining protocol as described in Section 4.2.7.

Early interaction between cells and SAPH was studied via 2D seeding of cells on SAPH. It was learnt through vinculin staining and cell morphology, that interaction between the cells and arginine-containing SAPH exists even at an early time point, regardless of the charge of hydrogels, which was indicated by the rounded morphology and sign of vinculin.

Changes in the cytoskeleton due to adhesion affects cell chondrogenesis (An et al. 2001, Vinatier et al. 2009). As reported by other studies, cells would produce chondrogenic gene expression when cells are in spherical morphology (Goldring et al. 1986, Saadeh et al. 1999). However, since this interaction work was performed in 2D, this was not a concern. Hence, there is a need to carry out further work in the future to analyse cell interaction with these SAPHs in 3D.

4.3.2.3D encapsulation of T/C-28a2

4.3.2.1. Cell viability

In an effort to assess the feasibility of culturing human chondrocytes, human immortalised chondrocytes T/C-28a2 was first studied, as the source of cells is closer to the end application of this project. Cell viability of T/C-28a2 was investigated in this preliminary study. Upon encapsulating 4 million cells per ml in F8, F8R, F9, FK and FR hydrogels, the live/dead assay was carried out, and representative images are shown in Figure 4-16. In this study, cells were also encapsulated within agarose and collagen hydrogels, and on glass as controls (see Figure 4-17). However, for the control samples, cell viability was assessed at 24 hours culture only to show that cells were viable upon detaching from the flasks.

In comparison to other SAPHs, F8R and F9 were seen to contain the highest number of cells. From the Figure 4-16, it can be seen at 24 hours that very few cells were observed in the F8 hydrogel, and cell viability in FK and FR hydrogels was lower than other samples. Lower cell viability in FK and FR may be explained by the stress the cells experienced when mixed in high charged SAPH. Interestingly, cell viability in F8R was not affected by the pH of the hydrogel, even though this hydrogel was prepared at pH 4. A significant reduction of cell number was also observed in FK hydrogel at 3 days of culture, whilst the cell number in FR seemed to increase at this time point.
At 7 days of culture, cells were observed to recover in terms of cell viability and increased cell number was seen in all SAPHs. Despite reduced cell viability at the earlier time point, cell viability in all SAPHs increased upon being cultured for 21 days. Not only that, cells were also observed to form a sheet in all SAPHs at 21 days of culture. Formation of cell-sheet like at the end of the study may be due to the max projection of the images, cell migration to the surface, or to the weak spot/plane of the hydrogel where cells prefer to reside at, or cell differentiation towards fibroblastic phenotype. The sheet-like formation of cells was also observed in the previous chapter when FK and FR were encapsulated with bovine chondrocytes at day 21 of culture (see Section 3.3.2.1). This may be explained by the loss of mechanical integrity of these two SAPH as shown in Section 2.3.4. Interestingly, this was not observed in F9 samples, which as shown earlier, has superior stability compared to FK and FR.

It is shown in this study that T/C-28a2 human immortalised chondrocytes survived in all SAPHs up to 21 days. This indicates the high chance of survival of human chondrocytes upon being encapsulated within SAPHs.
Figure 4-16 Cell viability assay with green signifies live cells while red signifies dead cells. To see the feasibility of culturing human chondrocytes in 3D SAPH, 4 million cells per ml were encapsulated, and cell viability was imaged at 24 hours, 3, 7 and 21 days at x10 magnification. Cell number in F8, F8R, FK and FR were observed to be higher than F9. This could be due to differences in the dynamic mechanical stiffness of each SAPH. ▲ signifies that the SAPH contains arginine. Scale bar = 500µm.

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Figure 4-17 Cell viability assay on cells seeded on control samples. Cells were also encapsulated on agarose and collagen and cultured on glass and imaged at 24 hours of culturing, to observe cell viability upon 3D seeding in agarose and collagen. Moreover, cells were seeded on the glass as a 2D control.

4.3.2.2. Cell distribution through hematoxylin and eosin histology staining

Samples containing T/C-28a2 were processed and stained for H&E to see cell distribution. After culturing the human chondrocyte line within the different sequence of peptide hydrogels for 21
days, samples were processed for histology staining. Figure 4-18 shows H&E staining of cells within peptide hydrogels F9, FK and FR. These three peptide sequences were selected for the same justification stated in Section 2.3.3.1. DNA in the nuclei, which are acidic, binds to haematoxylin and are observed as a purple stain, while basic cytoplasm bound to eosinophilic eosin and were visualised as pink.

Figure 4-18 H&E staining of immortalised human chondrocytes T/C-28a2 encapsulated in F9, FK and FR. Human chondrocytes encapsulated within F9, FK and FR were cultured for 21 days. At the end of the study, samples were processed for H&E staining to see the human chondrocytes' morphology and cell distribution through the hydrogel. Two images from each type of SAPH are shown here as representative images. More cells were detected in F9 samples, and the cells were found to be distributed throughout the samples. In FK and FR samples, fewer cells were observed, and the cells were seen to be either on the edge or within the void (weakest point) of the sample. Nuclei were stained as purple, while cell cytoplasm was stained as pink. In this figure, the pink eosinophilic stain was also observed to be picked up by the peptide hydrogel fibres. Scale bar= 50µm.

A minimal amount of cells (stained purple) are seen in the images. The samples were sectioned vertically. There were slightly more cells in F9 than in FK and FR. Also, cells in F9 were more distributed, while those in FK and FR were seen to be in small clusters. These figures show that all cells were seen at the weak point of hydrogels and mostly at the edge of the sections. Again, this may be explained by the faster degradation rate of FK and FR as shown in Section 2.3.4. This also supports the finding in Section 4.3.2.1 and Section 3.3.2.1 where cells encapsulated in FK and FR hydrogels were seen in sheet-like formation, but not in F9 hydrogels.

In addition to this, H&E staining was also being picked up by the hydrogel fibres. Fibre stain by H&E is more apparent in FK and FR than in F9 hydrogel. Hydrogel fibre stain may be explained by charges on peptide hydrogels (Refer Section 3.3.2.1). These samples were also stained with Safranin O to see any production of GAG. However, no GAG was detected, and this result is not shown. Lack of GAG production may be explained by the absence of Nutridoma SP or other chondrogenic-supplemented media, which is an important supplement that will trigger this cell line to produce cartilage-specific biochemical molecules. In another study by Yates et al. (2005) reported that when FBS was replaced by Nutridoma, chondrocytes seeded within collagen sponges produced more chondrogenic gene expression.
4.4. CONCLUSION

In terms of 2D cell seeding, cell response to the surface of a tissue engineering scaffold is reliant on surface hydrophobicity, protein adsorption, surface charge, surface roughness and surface stiffness.

Higher charged SAPH was observed to affect cell behaviour in terms of their morphology. Even though it was seen in Section 4.3.1.5 the charged substrate was found not to affect cell adhesion rate, semi-quantification of live-dead images in Section 4.3.1.2 on the other hand, showed significantly higher number of cells adhered on higher charged SAPH, F9, FK and FR, especially at higher seeding density. Changes in morphology as seen in Section 4.3.1.7 may be explained by the electrostatic interaction between the substrates and the anionically charged cell membrane. As the membrane protein or phospholipids come in contact with these charged substrates, the charged macromolecules would have changed the conformation of absorbed protein such as the fibronectin. Stimulation of substance efflux from cells such as this was observed by Fischer et al. (2003) in the study of the presence of poly-l-lysine.

Besides charge, vinculin staining of T/C-28a2 seeded on F8, F8R, F9, FK and FR showed positive staining for those on arginine-containing SAPH, which suggests that the arginine may contribute towards focal adhesion of cells on F8R and FR. This again, however, was not observed in Section 4.3.1.5, justifying the need to look into other effective methods to quantify cell adhesion on SAPH, especially the charged ones, and optimisation of vinculin immunofluorescence staining.

Cellular adhesion to the scaffold is essential for cells’ ECM production, migration, differentiation and survival. In order to facilitate cell adhesion onto biomaterial scaffolds, researchers have been modifying the biomaterials to create cell binding sites, either by tethering binding sequences such as RGD or increasing the surface roughness. There have been attempts to enhance chondrocyte adhesion to biomaterials scaffold. Lin et al. (2014) embedded adhesion molecule anti-CD44 antibody-avidin to chitosan to improve cell adhesion. It was found that when this adhesion molecule was immobilised to the scaffold, cell detachment rate decreased and proliferation rate increased. However, from our results, we have shown that cells are already capable of readily adhering to these F8R and FR. This was shown by cell morphology and vinculin staining. On the other hand, in another study, adhesion of human umbilical vein endothelial cells (HUVECs) on the charged surface was triggered by the phosphorylation of the integrin binding site by the charged molecules (Arima and Iwata 2015). Reports have shown a beneficial effect of fixed positive charge hydrogel on osteoblast and fibroblast, even though there was a higher production of chondrocytes’ collagen type I rather than type II (Schneider et al. 2004, Dadsetan et al. 2011).

Key findings of this chapter include that cell attachment to the SAPHs was comparable to those on positive control hydrogel, collagen. This was observed from the cell adhesion assay, cell morphology and detection of vinculin which marked the formation of focal adhesion between the
cells and the SAPHs. It would also be beneficial for this study to continue with future work that would explore more of the cell interactions with SAPHs. Some of the future work includes:

- Physiologically, chondrocytes have been shown to attach to chondronectin and collagen type II, and this attachment is controlled by integrin (Hewitt et al. 1980, Loeser 1993, Loeser 2014). Further investigation of which α- and β-integrin subunit is responsible for mediating the adhesion of T/C-28a2 to self-assembled peptide hydrogel will be essential for this study. It would also be essential to assess different adhesive proteins that may be involved in this cell-SAPH interaction.

- Future work will also include the application of fluid shear bioreactor to observe cells adhesion properties to these peptide hydrogels under dynamic conditions.

- We know that cells behave differently in 2D than 3D. The interaction between cells and biomaterial scaffolds is not known, and low chondrocyte adhesion to biomaterial scaffolds has been reported in the past (Lin et al. 2014). Due to this, it would be beneficial to investigate cell interaction with biomaterial scaffold upon encapsulation in 3D. A study using another type of cell may also contribute more towards the knowledge of cell interaction with SAPHs.

- Migration behaviour of T/C-28a2 on different self-assembled peptide hydrogels would also be beneficial for this study.

In addition, encapsulation of human immortalised chondrocytes T/C-28a2 in F9, FK and FR has shown results which are parallel to findings in the previous two chapters. This emphasised the inferior mechanical stability of FK and FR, and the effect of scaffolds' stiffness or environment's mechanical properties to the cytoskeletal deformation and cells’ dedifferentiation.
CHAPTER FIVE
GENERAL DISCUSSION, FUTURE WORK AND CONCLUSION

5.1. GENERAL DISCUSSION

For the past 20 years, researchers have been putting in a lot of effort to attempt to regenerate cartilage tissue (Brittberg et al. 1994, Salinas and Anseth 2008a, Olvera et al. 2017, Zhao et al. 2017, Agrawal et al. 2018). As described in detail in Chapter 1, the triangle factor of tissue engineering comprises of cells, biomaterials and physical/chemical stimulation. Researchers have been investigating the feasibility of using hydrogel as the biomaterial scaffold for cartilage tissue engineering. However, the challenges come with 1) the dynamic changes of the physical and mechanical properties of the hydrogel in vitro and in vivo, 2) the phenotypic changes of chondrocytes and 3) the complex non-homogenous multi-layer of cartilage. Thus, this chapter will be covering these issues specifically on the method of measuring mechanical properties of hydrogels, spatially and temporally, preventing phenotypic changes of chondrocytes or directing cells towards chondrogenic properties and hybrid hydrogels.

5.1.1. Measurement of focal-local mechanical properties

The change of the mechanical properties of these hydrogels is due to the degradation of the hydrogel and the production of neo-matrix. The degradation of the scaffold, as described by Hutmacher (2000), can be categorised into biodegradation, resorption, bulk absorption and surface erosion. It is highly desirable to design a biomaterial scaffold that has a controlled degradation rate, where the degradation behaviour is mediated by the cells. Subsequently, the scaffold should ideally degrade leaving some space for the newly secreted protein.

As suggested by Zhu (2010) and Zhang et al. (2014a), the rate of biomaterial degradation should match the production of the de novo extracellular matrix (ECM). Some hydrogels, such as polyethylene glycol (PEG) and alginate, are either not biodegradable or have extremely slow degradation rates. Studies have shown the effect of slow degradation which inhibits cell proliferation and limits the diffusion and infiltration of nutrients throughout the hydrogels (Dhote et al. 2013). Researchers have integrated matrix metalloproteinase (MMP) sensitive peptides to PEG and alginate to increase the rate of degradation (Patterson and Hubbell 2010, Nguyen et al. 2011). Also, the degradation of alginate hydrogel was reported to accelerate when the hydrogel was oxidised (Jeon et al. 2012).

Some other hydrogels, on the other hand, have uncontrollably fast rates of degradation. Rapid degradation was reported to affect cell viability and inhibit cell migration (Sung et al. 2004, Pilipchuk et al. 2013). At the initial point of regenerating chondrocytes, a scaffold is expected to
provide support to the cells as the cells proliferate and secrete matrix protein (Xu et al. 2015). For this reason, it is essential to assess the degradation rate of a scaffold, in vitro and in vivo. Wet/dry mass, topology, geometry, mechanical and chemical properties are some of the methods used to assess hydrogels’ degradation (Zhang et al. 2014a, Zhang et al. 2016c). In Chapter 2, the degradation behaviour of self-assembled peptide hydrogel (SAPH) was measured by the hydrogel rheological properties and intact peptide monomer using high-performance liquid chromatography (HPLC). Both of these methods are destructive. Recently, other researchers have been looking into the initiative of applying non-destructive measurement of hydrogel’s degradation. This non-destructive method is beneficial as the measurement can be done in real-time which is deemed to be more accurate as the same hydrogel sample can be measured throughout the experiment rather than a different sample at each time point. As described in Chapter 2, all samples in this degradation study were prepared at day 0, and one sample was randomly chosen at each time point, assuming the integrity of all samples were similar. Another advantage of using the non-destructive measurement is that the stability of the hydrogels can be monitored in vivo.

Fluorescence multispectral imaging, μ-computed tomography, ultrasound elasticity imaging and MRI are some of the non-destructive techniques applied to assess biomaterials degradation properties (Zhang et al. 2016c). In a study by Walker and colleagues, the rheological properties of non-seeded agarose hydrogels correlated with the model calculated using the value measured using the ultrasound probe (Walker et al. 2011). In addition, Neumann et al. (2016) found that ultrasound measurement could measure the degradation rate of chondrocyte-encapsulated-modified PEG, as well as the alignment of hydrogel fibres and clusters of ECM protein. In another study, the feasibility of fluorescently labelled hyaluronan hydrogels was analysed in vivo, and it matched with the hydrogel’s mass loss (Zhang et al. 2016c).

5.1.2. Improving the chondrogenesis of cells seeded hydrogels

In cartilage regeneration, the majority of the literature to date have utilised primary cell chondrocytes in their studies (Chun et al. 2004, Yang and Barabino 2011, Jingjing et al. 2015). Other options of cell sources that have been reported include bone marrow-derived stem cells and adipose-derived stem cells (Mhanna et al. 2013, Kohli et al. 2015, Scioli et al. 2017, Zhao et al. 2017). Non-human mammalian chondrocytes such as bovine, ovine and mice have always been used for their availability and low-cost, especially for initial cell work in assessing the feasibility of a novel designed biomaterial. Having said that, the use of human sourced cells is essential due to the discrepancy in cell response across different species. As stated in Chapter 4, T/C-28a2 (human immortalised chondrocytes) were used as they were closer to the end use of this project, which is to regenerate human chondrocytes. To the knowledge of the author, to date, no study has been done to encapsulate human immortalised chondrocytes in biomaterial scaffold. Even though primary chondrocytes have always been the number one choice in cell source, the scarcity of autologous healthy chondrocytes to be seeded in biomaterial scaffold has urged researchers to increase the number of cells through monolayer expansion. It has
however been reported that prolonged monolayer expansion resulted in dedifferentiation of chondrocytes (Smeriglio et al. 2014, Miao et al. 2017). The next two sections will discuss in detail some of the options or efforts carried out by other researchers to improve the chondrogenesis of cell-seeded hydrogels.

5.1.2.1. Physical and chemical stimulation

A recent study which expanded chondrocytes on a collagen substrate was found to reduce the possibility of dedifferentiation of chondrocytes compared to using agarose, alginate or Matrigel® substrate (Miao et al. 2017). This could be due to the availability of native ligand on collagen substrate or the differences in stiffness between these substrates.

Besides 2D monolayer expansion on the modified substrate, researchers have been applying mechanical stimulation on chondrocytes seeded in hydrogels. Upon being cultured in tissue culture plastic (TCP) on which cells tend to lose their phenotype, mechanical stimulation has been used to enhance the chondrogenesis of/re-differentiate the cells. Mechanical stimulation has been proven to be essential in increasing ECM production and proliferation rate. Mechanical stimulus, such as fluid-flow induced shear, helps to enhance the transport of molecules and nutrients. Hypothetically, perfusion flow will increase the diffusivity rate of molecules through these charged SAPH.

The effect of perfusion fluid on improved mass transfer and uniform distribution of cells and ECM protein in chondrocyte-seeded hydrogels was observed by Dahlin et al. (2014b). At a flow rate of 3ml/min, it was seen that perfused samples had more homogeneous cell distribution and higher production of type II collagen, but lower GAG production, over static samples (Gemmiti and Guldberg 2006, Yu et al. 2015). This suggests that the presence of fluid flow-induced shear is beneficial for cell mechanotransduction; thus enhanced the nutrient and oxygen flow.

Besides, there were other mixed findings in studies applying perfusion fluid flow on 3D constructs encapsulating chondrocytes (Gemmiti and Guldberg 2006, Schulz et al. 2008, Grogan et al. 2012a). Even though cells and ECM were observed to be more homogeneous throughout the scaffold, perfused samples were measured as having reduced collagen accumulation compared to static ones (Dahlin et al. 2014b). This reduction may be due to the low perfusion flow rate applied in this study, which was 10µL/min.

It is also noted, continuous perfusion flow at 1.22ml/min (Kock et al. 2014) and 0.1ml/min (Grogan et al. 2012b) were reported to not give any beneficial effect to the cells. In this study, for example, a preliminary work putting SAPH on perfusion fluid using stirred flask was carried out earlier in this study. The peptide hydrogels without cell were injected into dialysis cassette (2kDa MWCO, Slide-A-Lyzer™, Thermo Fisher Scientific, Loughborough, UK) and cultured in DMEM in the stirred flask (see Supplements, Section 6.2). Despite embedded within the permeable membrane of the dialysis cassette, the peptide hydrogel rapidly degraded when cultured in stirred condition. However, given the finding as stated in Section 3.3.5,
implementation of either chemical or physical stimulation shall be considered in the future. Thus, optimisation on bioreactor work shall be carried out in the future. As stated in Section 1.6, other types of bioreactor might also be taken into consideration may be more beneficial for SAPH application.

Also, the detrimental effects of combining mechanical loading, growth factors and perfusion have been reported (Grogan et al. 2012a, Dahlin et al. 2014b). In a study, samples of human articular chondrocytes seeded in 2% alginate were exposed to load (20% strain) at 0.5Hz. At the same time, these samples were also exposed to perfusion flow at 0.1ml/min. This loading and perfusion combination showed a reduced level of biochemical molecules produced by seeded chondrocytes (Grogan et al. 2012a). Adverse effects reported in these studies may be explained by 1) rate of perfusion fluid used in this study 2) simultaneous mechanical load or 3) continuous fluid flow may not be beneficial for cell growth.

Continuous versus intermittent exposure of mechanical stimulation has been studied, and Schulz et al. (2008) found intermittent medium perfusion of 0.5ml/min (10min ON, 50min OFF) was advantageous for swine chondrocytes seeded in agarose than continuous flow at the same rate. In a different study by Shahin and Doran (2011b), polyglycolic acid (PGA) and hybrid PGA-alginate were seeded with human fetal epiphyseal cartilage. A positive effect on chondrogenesis was observed when these samples were exposed to a gradual increase of flow rate compared to samples that were exposed to the fixed high flow rate (0.2ml/min) or low flow rate (0.075ml/min) (Shahin and Doran 2011b).

Besides flow rate, another factor would potentially be a need for pre-culturing cells seeded in the scaffold in a static condition before exposing to any fluid shear. Human bone marrow MSCs seeded in chitosan-based scaffold were precultured in a static condition for a day before transferring to a flow perfusion bioreactor (Alves da Silva et al. 2011). It was seen that cells in these samples had rounded morphology and produced more collagen type II than non-perfused samples. In another study, five days of preculturing gave advantageous effects to the perfused samples at 0.2ml/min than those samples that were exposed to perfusion flow directly upon being encapsulated within hydrogels (Shahin and Doran 2011b).

Apart from flow perfusion, the hypoxic condition also has potential as another mechanical stimulation. Co-culture of chondrocytes and MSCs produced more GAG and a higher ratio of collagen type II: type I, when cultured in lower oxygen tension (20% versus 5% CO$_2$) (Meretoja et al. 2013). Chondrogenic effect of cells upon being exposed to the hypoxic condition can be explained by the low oxygen tension in native cartilage.

Besides physical stimulation, studies have shown that supply of growth factors (GFs) improves the chondrogenic properties of chondrocytes seeded within hydrogels (Miller et al. 2011, Florine et al. 2014, Kopesky et al. 2014). Different types of growth factors have been studied to see their effect on the regeneration of cartilage. These growth factors have shown to contribute to the remodelling of cartilage in terms of balanced synthesis and degradation of matrix
components. TGF-β1 increased the modulus of the cartilage explants, while IGF-1 and BMP-7 increased the production of GAG (Asanbaeva et al. 2008).

Modulating the supply or delivery of GFs was also studied, temporally and spatially (Miller et al. 2011, Ng et al. 2011, Balcom et al. 2012, Florine et al. 2014). The effect of supplying GFs in transient mode produced a doubling in the production of GAG when compared to a continuous supply of growth factors (Ng et al. 2011, Balcom et al. 2012). Throughout the years, GFs have been supplied through culture media using bioreactor (continuous supply) or added manually while changing media. Recently, researchers have attempted to bind GFs to biomaterial scaffolds. Besides being tailorable to mechanical properties, biodegradable and injectable, another advantage of using peptide hydrogels is their ability to be functionalised. Charged monomers on peptide hydrogels have also been utilised to tether to GFs. With regards to peptide hydrogels, researchers have attempted to bind GFs to self-assembly peptides. Miller et al. (2011) bound IGF-1 and TGF-β1 to peptides. Unlike IGF-1, TGF-β1 was successfully attached to (KLD)₂ SAPH (Miller et al. 2011). Positively charged heparin-binding IGF-1 (HB-IGF-1) bound to charged amino acids on RAD (Florine et al. 2014). HB-IGF-1 was seen to then interact with the negatively charged sulphated GAGs. Kopesky et al. (2014) studied different techniques to load GFs in self-assembly peptide. For agarose and peptide hydrogels, the growth factor’s uptake by hydrogels was higher when it was added before gelation. When comparing the absorption between these two hydrogels, there were more GF molecules found in peptide hydrogel than agarose (Kopesky et al. 2014).

Parmar et al. (2015) functionalised collagen mimetic peptide hydrogels with either hyaluronic acid or chondroitin sulphate. As stated in Section 1.4.6, matrix metalloproteinases (MMPs) were also bound to the peptide to control the degradation of peptide hydrogels.

5.1.2.2. Co-culture

The chondrogenic phenotype of engineered cartilage can also be enhanced via co-culturing of primary chondrocytes and adult stem cells. By combining another type of cell to the culture, the amount of cartilage to be extracted can be reduced, thus lowering the area of site morbidity. Also, the partial replacement of chondrocytes with stem cells will reduce the expansion passage, which will decrease the possibility of chondrocytes dedifferentiation.

According to literature, when it comes to co-culture, 1) cell mixing ratio, 2) direct or indirect culture and 3) environment are some of the factors that need to be considered. For instance, Ko et al. (2016) found that chondrocytes and bone marrow stem cells (BMSCs) co-cultured in 20:80 ratio in PCL-PEG-PCL hydrogel expressed higher collagen type II and GAG gene markers than other combination ratios. Due to the scarcity of healthy chondrocytes, researchers have always studied the possibility of co-culturing MSCs and chondrocytes at the lowest chondrocytes: MSCs ratio. Some researchers used 25:75 ratio (chondrocytes: MSCs) (Meretoja et al. 2012, Dahlin et al. 2014a, Critchley et al. 2017), while Sabatino et al. (2015) found a lower ratio of chondrocytes: MSCs, which was at 5:95.
Even though there was no study carried out on the cell-cell interaction between these two types of cells, most researchers found that co-culturing of stem cells and chondrocytes worked best when cultured at the closest proximity. Co-culturing of chondrocytes and human BMSCs in pellet produced higher collagen type II than in a transwell (Acharya et al. 2012). Interestingly, another study found there was no difference in chondrogenesis between cells co-cultured in pellet and collagen sponges (Sabatino et al. 2015). Recently, researchers have started to utilise the use of 3D hydrogel for co-culturing. Hydrogels were manipulated to encapsulate the different type of cells in the same hydrogel (direct contact) or separate hydrogels (indirect contact) and depend on the media to deliver the cytokine factor between the hydrogels. Bian et al. (2010b) studied human MSCs + chondrocytes co-cultured in single HA hydrogel. While Levorson et al. (2014) found rabbit MSCs + bovine chondrocytes had the highest collagen deposition when cultured in separate PCL/ECM hybrid hydrogel (indirect) than within the same gel (direct) in flow perfusion.

Researchers mainly agree that chondrogenicity of chondrocytes is enhanced when a higher ratio of chondrocytes: MSCs was used. However, it was found that a lower ratio of chondrocyte: MSCs can be used in the presence of growth factor such as TGF-β3 (Bian et al. 2010b, Meretoja et al. 2012, Ko et al. 2016). Even though the use of TGF-β3 was reported to enhance the chondrogenesis of monoculture chondrocytes, a separate study found that less concentrated TGF-β3 was needed in co-culture and that the cells were more stable phenotypically upon withdrawing the growth factor (Dahlin et al. 2014a). Despite this, Acharya et al. (2012) successfully co-cultured human chondrocytes and bone marrow MSCs without the presence of any growth factor.

5.1.3 Hybrid hydrogel

Section 3.3.8 investigated the feasibility of fabricating hybrid peptide hydrogels using two different charged hydrogels. Researchers have been putting a lot of effort in designing hybrid hydrogels in attempts to modulate the mechanical properties of the hydrogel, such as to increase the possibility of injecting the hydrogel, to improve the biodegradability and to strengthen the hydrogels’ stiffness.

Researchers used hybrid hydrogels to increase the hydrogels’ mechanical properties. The role of additive polycaprolactone (PCL) in between the layers of alginate has shown that this hybrid system can be a self-supported bio-printed construct (Kundu et al. 2015). Similarly, poly(glycolic acid) PLGA-collagen hybrid sponge was reported to improve in terms of the collagen sponge’s mechanical strength (Dai et al. 2010, Hongxu et al. 2011). On the other hand, there are also other studies which attempted to enhance hydrogels’ degradation behaviour. Poly(lactic acid) PLA has a slow degradation rate. However, as shown in one study, the presence of collagen hydrogel in hybrid collagen/PLA and chitosan/collagen/PLA was found to increase its degradation rate (Haaparanta et al. 2014). The benefit of improving the degradation rate was also reported to facilitate the production of ECM protein when chondrocytes were cultured within...
PEG-gelatin hydrogel (Sridhar et al. 2015b). PEG as itself, was reported to lack bioactive sites for cell adhesion. Besides increasing the degradation rate, this same study also found there were a higher number of cells encapsulated within the hybrid hydrogel in the presence of gelatin. Apart from this, other studies have looked into the hybridisation of hyaluronic acid (HA) and collagen to poly(ethylene glycol) PEG and alginate, and found that these hybrid hydrogels enhanced the chondrogenicity of the cells (Khanarian et al. 2011, Callahan et al. 2012).

Besides improving the mechanical properties and cell seeding propensity, the hybrid hydrogel was also manipulated to deliver dual growth factor and cell types in attempts towards osteochondral regeneration (Kim et al. 2013, Sheehy et al. 2013, Lam et al. 2014). Engineering osteochondral tissue has been challenging for researchers due to its biphasic layer. Each layer has varied mechanical properties and cell type and has to be cultured in different growth media.

5.2 Conclusion

There has been a high demand for research surrounding regenerative cartilage. This is due to the fact that cartilage has a limited capacity to self-repair, as it is avascular. Hydrogel scaffolds have been manipulated to provide a 3D chondro-inductive environment to support the growth and differentiation of cells. Recently, the rise of self-assembling peptide hydrogels has attracted researchers to explore the feasibility of using it as an injectable cell carrier.

Self-assembling peptide hydrogels have been designed by embracing the nature of self-assembly in protein folding and DNA double helix. Self-assembling peptide hydrogels (SAPH) are seen to have high potential in tissue engineering applications as well as drug delivery; as the properties of peptide fibrils can be controlled by changing peptide sequences, concentration and pH. Interestingly, these peptides can self-assemble into ordered nanostructures through the synergistic effects of intermolecular non-covalent interactions, which include hydrogen bonding, π–π stacking, electrostatic, hydrophobic and van der Waals.

The novel designed SAPH sequences studied in this thesis were inspired by an octapeptide hydrogel FEFEFKFK, a pioneering design of peptide sequence in Saiani’s group. Chapter 2 has outlined the characterisation of SAPHs that have the potential for cartilage engineering applications. Based on the pioneer sequence FEFEFKFK, the peptide sequences used in this study were designed by manipulating a single amino acid in the sequence to produce +0, +1 and +2 charged sequences. The variation of peptide design was created by replacing phenylalanine (F) with valine (V), substituting lysine (K) with arginine (R), switching the location of lysine and glutamic acid (E); and adding another lysine (K) at the end of the peptide sequence. In order to evaluate the effect of modulating these peptide sequences, their gelation properties, mechanical properties, and stability and degradation profiles were studied. Earlier in the chapter, it was shown that altering the overall charge and hydrophobicity of the peptide changed the hydrogel gelation behaviour and mechanical stability. The +0 SAPH sequences gelled at lower pH and precipitated at neutral pH, except for VEVKVEVK (V8). However, due to its reduced hydrophobicity, the substitution of phenylalanine with valine in V8 exhibited less
stable SAPH. Also, the effect of substitution of lysine with arginine depended on the presence of overall charge of the sequence. F9 (+1 charge) and FK (+2) gelled at pH 5.5 and remained a gel up to neutral pH. However, when one of the K in these sequences was replaced with R, the gelation behaviour of FR9 (+1 charge) changed. FR9 precipitated at neutral pH, while FR (+2 charge) behaved similarly to FK. Subsequently, F9, FK and FR were chosen for further analysis as these SAPH sequences gelled at neutral pH. We have also shown that these peptide hydrogels have a different storage modulus depending on the concentrations and the pH of hydrogels. Also, the degradation behaviour of these peptide hydrogels was assessed in the effect of the presence of serum and cells. It was revealed from the rheological study and HPLC analysis that while FK and FR degraded significantly with time, F9, however, was less prone to degradation.

The efficacy of utilising F9, FK and FR in cartilage regeneration has been further studied in Chapter 3. Bovine chondrocytes were encapsulated in 3D and cultured for 21 days. The experimental setup for this study was designed to not incorporate the use of growth factor at any point of the study. Samples were analysed for cell viability, morphology and expression of the chondrogenic markers, aggrecan and collagen type II. Cells were found to behave differently throughout the study, depending on the peptide hydrogel used. Immunofluorescence staining showed a positive indicator of aggrecan and collagen type II production in F9 samples, but only in the early time point. It was also found that cells morphology correlated with the production of chondrogenic markers. Cells encapsulated in the F9 hydrogel were observed to be spherical up to day 7, while the higher charged SAPH, FK and FR were found to affect cell morphology, where cells were seen to have elongated morphology as soon as day 3. This may be described by the electrostatic interaction between the scaffold and F-actin, which may also potentially be due to the differences in hydrogel stiffness. One of the main findings of this study is F9 with its initial storage modulus of 10kPa retained chondrocytes’ morphology and was able to support spherical chondrocytes in the first 2 weeks of culture. Even though FK and FR were also used at 10kPa, their storage moduli were measured to reduce substantially after culturing for seven days. This finding has emphasised that the real-time structural integrity of the hydrogel is an important factor to be considered. Besides charge, the presence of arginine was seen to affect cell behaviour; in terms of the cells’ morphology and protein production. Stretched and networked morphology of cells have also been reported in other studies when another SAPH, RAD16-I (containing arginine), was applied. The second section of Chapter 3 was a study looking into the possibility of fabricating a hybrid peptide hydrogel system. The effects of change of morphology and the absence of ECM protein were also observed in F9+FK and F9+FR hybrid systems, which reiterate chondrocytes mechano-transduction mechanism, and the influence of the content of hydrogels on chondrocytes morphology and ECM production. At the end of the 21 days of study, chondrocytes encapsulated in these SAPH were seen to undergo phenotypic changes towards hypertrophic dedifferentiation. Thus, in the future, an ideal approach would be to incorporate chemical/physical stimulation to enhance the production of
ECM chondrogenic protein to replace the degraded hydrogels and to look at the feasibility of negatively charged SAPH.

Even though as stated in Chapter 1, the project was aiming to regenerate both auricular and articular cartilage. However, throughout the study only articular chondrocytes was used. So, this work will be continued in the future to assess the feasibility of SAPH in regenerating elastic cartilage, with the presence of chemical/physical stimulation.

Previous works have shown that these novel designed SAPHs are capable of encapsulating cells and facilitating tissue regenerations. However, little is known about initial cell-SAPH interactions. Cell-cell and cell-material interactions have been shown to play a vital role in cartilage engineering. Different biomaterial properties can significantly influence the initial cell attachment to the substrate, which is crucial for chondrogenesis. Chapter 4 looked into the study of early cell interaction with SAPH. For this study, cells were cultured on SAPH in a 2D method for cell adhesion purposes. In terms of 2D cell seeding, cell response to the surface of a tissue engineering scaffold is reliant on surface hydrophobicity, protein adsorption, surface charge, surface roughness and surface stiffness. For this chapter, F8 and FR8 were also included in the study in addition to F9, FK and FR to see the effect of the presence of charge and arginine in cell adhesion. AFM measurement showed that out of these five peptide hydrogels, only FK had a significantly different mean surface roughness compared to other SAPH. This chapter also showed the effects of using various peptide hydrogels on the attachment and spreading of T/C-28a2 immortalised human chondrocytes. Key findings of the chapter include that the cell adhesion rate on SAPH was comparable to those on positive control collagen hydrogel. It was also observed stretched morphology of chondrocytes seeded on SAPH suggests cell adhesion behaviour is affected by the charge and arginine present in peptide hydrogels. Otherwise, no other effect of charge was observed throughout the study. From this study, the effect of surface roughness, charge and presence of arginine in the SAPH sequence on cell attachment to the SAPHs was however inconclusive. It would be beneficial for this study to continue with future work that would investigate which α and β integrin subunits are responsible for mediating the adhesion of T/C-28a2 to SAPH. It would also be essential to study other types of cell adhesion.

Generally, this study adds up to the knowledge of the effect of hydrophobic amino acid and charge of a peptide sequence on the SAPH gelation and biodegradation behaviour. The findings unlock a new branch of research on controlling the biodegradation of SAPH. It also emphasises the importance of assessing the mechanical properties of the scaffold with time. The discovery of dynamic changes of mechanical properties which affect cells behaviour raises questions about other previous studies which neglected the dynamic changes of hydrogels’ mechanical properties. Also, this current study reiterates the detrimental effect of using a positively charged hydrogel for cartilage regeneration. In addition, the study pioneers the research looking at the cell interaction with SAPH and creates another question- does the presence of arginine in SAPH facilitate cell adhesion?
5.3. FUTURE WORK AND RECOMMENDATIONS

Further studies in investigating the effect of charge on cells behaviour can be divided into 1) effect of fixed charge on the cell membrane ion channel’s activity and 2) to look in-depth at the effect of charge on cells through the application of electrical stimulation. Physiologically, hyperpolarisation of chondrocytes cell membrane activates stretch-activated ion channels that initiate the activation of Ca\textsuperscript{2+} -dependent K\textsuperscript{+} ion channels. In a scenario such as when cartilage is compressed, fluid will exit the ECM, leaving only negatively charged proteoglycans (PGs). This charge difference will attract positively charged ions such as Na\textsuperscript{+} and K\textsuperscript{+} to travel into the matrix and consequently induce chondrogenesis (Pingguan-Murphy and Knight 2008). This could also potentially explain the detrimental effect of utilisation of fixed charged peptide hydrogels as a scaffold for regenerating cartilage.

Besides in-depth studies on the effect of fixed charges on chondrocytes, it would be useful to study the possible mechanotransduction or biochemical pathways that are responsible in the differentiation of stem cells co-cultured with chondrocytes, in effect of exposure to either physical or chemical stimulation. Exploring the impact of the expansion of human cells could also be integrated into this project.

This present study has shown the effect of charged peptide hydrogels on cell viability, morphology and cell distribution. It also demonstrated that these peptide hydrogels were stable for more than 21 days. Possible future work will include an assessment of the effect of perfusion fluid flow on the stability of peptide hydrogels and its impact on cells viability and ECM production of chondrocytes seeded in peptide hydrogels. It is hypothesised that incorporating intermittent perfusion fluid shear will be beneficial for chondrocytes growth. Besides, incorporating growth factors may also be favourable to speed up the production of ECM proteins that would replace the void space of degraded SAPH and subsequently increase the storage modulus of the samples with time.

As suggested by Hunter et al. (2004), the mechanical signals transmitted to cells may vary substantially between different scaffold environments. Chondrocytes in native tissue bound to the ECM via cell adhesion can transfer matrix strains to mechanosensitive ion channels directly to the cytoskeleton. This present study has shown that cells formed focal adhesions when seeded on SAPH. Thus, further study needs to be carried out to investigate possible interaction pathways, in both static and dynamic environments, in 2D and 3D cultures. A study observing which α and β integrin subunit is responsible for mediating the adhesion of T/C-28a2 to SAPH would be highly recommended. This will potentially give a better view of the enzymatic degradation required to extract cells out of the scaffolds.

As explained earlier in this chapter, the capability in functionalising SAPH can be manipulated to control and monitor the degradation in spatial and temporal manners. However, all of this recommended future work will be more meaningful and quantifiable with optimisation of analytical protocols to assess the production of ECM protein in cells encapsulated in SAPH,
including the development of specific enzymes that can be used to extract cells and proteins from SAPH for *in vitro* analysis purposes. Perhaps, the application of the electric probe can be utilised to extract cells from the charged SAPH. Additionally, the hybrid peptide system can be used as a platform for designing a multiphasic scaffold for osteochondral regeneration. Co-culture of two different cells within two SAPH with different mechanical properties in a suitable environment can also be further investigated.
SUPPLEMENTS

6.1. THE PERMEABILITY OF DIFFERENT PEPTIDE HYDROGELS

A few attempts to measure the permeability of different peptide hydrogels were carried out earlier in this study. The first study used phenol red containing DMEM. UV absorbance of the phenol red intensity passed through each sample was measured at pre-determined time points. However, the study was discontinued as the gels absorbed the phenol red, especially the charged ones.

Another approach to assess the diffusivity of different SAPHs was carried out, adopted from Chiu et al. (2012). A standard curve of absorbance at 560nm to known concentrations of rhodamine (Sigma-Aldrich, Dorset, UK) in phenol-red free DMEM (Sigma-Aldrich, Dorset, UK) was generated (see Figure 6-1(a)). The DMEM containing gradient concentration of rhodamine (starting at 25µg/ml, total volume with DMEM is 1ml) was transferred into disposable plastic cuvettes. The solutions were measured using a UV spectrophotometer (6715 UV/VIS spectrophotometer, Jenway, Bibby Scientific, Staffs, UK).

Figure 6-1 Permeability studies of different SAPHs.
(a) Permeability standard curve was generated using serial dilution of rhodamine in phenol red-free DMEM (n=3). The absorbance level measured was converted to rhodamine concentration based on the standard curve (b). The graph was based on two separate repeats with 3 samples each (total n=6). The samples were analysed at 6 and 25 hours, and the value was corrected to the value obtained at 0 hours. (*) denotes that F8 and F9 were both significantly higher than other samples at the specific time point (Kruskal Wallis, p< 0.05). Error bars represent the standard deviation of different peptide hydrogel samples measured at a certain time point.
As shown in Figure 6-1 (a), the measured absorbance was plotted against known concentrations of rhodamine. The curve fit the polynomial trend, giving $R^2$ of 0.9799. In this study, F8, F9, FK and FR were used to see if the difference in charge and peptide sequence would affect the peptide hydrogels’ diffusivity. These SAPHs were prepared at the same concentration as stated in Table 4-1, using the protocol as explained in Section 2.2.1. SAPHs were pipetted into 12-well inserts using a 1000µl positive displacement pipette (500µl volume, Microman®, Gilson, Bedfordshire, UK). Phenol-red free DMEM with 12.5µg/ml of rhodamine was pipetted on top of each hydrogel at the volume of 1.5ml. Upon plating all hydrogels and DMEM+rhodamine, the intensity of the liquid in the well plate (contains DMEM only, 1.5ml volume) was analysed at 350nm to 650nm wavelength (considered as a baseline, t=0).

At 6 and 25 hours, the 1.5ml volume of DMEM in each well was measured at the same wavelengths as t=0. The value obtained was then converted into the concentration of rhodamine using the standard curve generated earlier. Figure 6-1 (b) shows that there was a significant increase of rhodamine concentration for all SAPH at different time points. It was, however, seen from the graph, that the rhodamine concentration measured from the F8 and F9 samples were significantly different to other samples ($p<0.01$). Even though the +2 charged hydrogels FK and FR had significantly lower diffusion rate compared to the other two sequences, F9 (+1 charged), on the other hand was significantly higher than the neutral charged SAPH, F8.

From this result, we have learnt that the effect of charge of peptide sequences on the diffusivity was rather inconclusive. Future work involving solutions with different molecular sizes and charges may be carried out. However, from this study, we have learnt that the diffusivity of hydrogels was affected by its thickness. Due to this, all subsequent work as stated in Section 3.2.3 used a lower volume of hydrogels.

### 6.2. Bioreactor – Stirred Flask

As stated earlier in Section 1.6, chondrocytes require either chemical or physical stimulation to maintain their phenotype. In this section, preliminary work exposing peptide hydrogel F8 to hydrodynamic force was carried out.

F8 hydrogel was prepared at a concentration 3% (w/v) according to the protocol mentioned in Section 2.2.1. F8 was injected into the disposable dialysis cassette (Product no.: 66380, Slide-A-Lyzer™, Thermo Fisher Scientific, Loughborough, UK). The cassette was filled with 1.5ml F8 hydrogel using a needle (20G, Terumo™, Surrey, UK). The F8-filled cassette was then attached to the floating buoy and placed in the 250ml plastic beaker, filled with non-sterile DMEM (Sigma Aldrich, Dorset, UK). The media in the beaker was stirred at 300 RPM using a magnetic stirrer (Big Squid, IKA™, Fisher Scientific, Loughborough, UK) (see Figure 6-2(a)).

This part of the study used the dialysis cassette with the molecular weight cut off (MWCO) of 10kDa and a volume capacity of 0.5-3.0 ml. Figure 6-2 (b) was taken after an hour of the F8-
filled cassette being in the stirred flask. As can be seen from the picture, the peptide hydrogel degraded into small pieces, even though it was only run for an hour. Thus, the study was modified using a dialysis cassette with a lower MWCO (2kDa, Product No.: 66205, Slide-A-Lyzer™, Thermo Fisher Scientific, Loughborough, UK).

Figure 6-2 Peptide hydrogel F8 cultured in a stirred flask. Images of two separate experimental setup (a & b) and (c & d). The first setup utilised the 10kDa MWCO dialysis cassette (a). The image in (b) was taken after an hour of the sample being in the stirred beaker. Initially, there was 1.5ml of peptide hydrogel in the cassette. Blue arrows show the fragments of peptide hydrogel within the membrane of the cassette upon removal from the beaker. The second experimental setup used a 2kDa MWCO dialysis cassette with 0.5ml of the F8 hydrogel at the start of experiment (c). A quarter of the hydrogel was observed in the membrane after 12 hours in the stirred flask (d).

For the second part of the study, the 2kDa MWCO dialysis cassette was filled with 0.5ml F8 hydrogel. Upon attaching the cassette to the floating buoy, the cassette was immersed in 250ml DMEM. This current study used a wide mouth GLS 80® laboratory glass bottle fitted with membrane vented screw cap (250ml, Duran®, VWR International, Lutterworth, UK) (see Figure 6-2(c)). The bottle was left on a magnetic stirrer at 200 RPM overnight.

Figure 6-2 (d) shows the cassette after being left in the stirred flask for 12 hours. Initially, the cassette was filled to the maximum capacity (cassette volume capacity:0.1-0.5 ml), as can be
seen from this figure, however after 12 hours in the stirred flask only a quarter of the whole volume was left in the cassette. Both of these studies were carried out at RT with only one sample each. These studies have shown that F8 hydrogel stability was compromised upon being cultured in a stirred flask, thus led to the studies in Chapter 2 to look at other possible peptide sequences with higher stability.

The study may be continued in the future, looking at different parameters to optimise the study (e.g. hydrodynamic flow rate, suitable membrane/cassette, environment temperature) and incorporating proper side-by-side controls in the same volume of media in a static condition.
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