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Macromolecular Crowding Meets Tissue Engineering By Self-Assembly

A thesis submitted to the National University of Ireland Galway for the degree of
Doctor of Philosophy

By

Abhigyan Satyam

Research Supervisor: Dr Dimitrios I Zeugolis

January 2015

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Network of Excellence for Functional Biomaterials (NFB)
Centre for Research in Medical Devices (CÚRAM)
National University of Ireland Galway (NUI Galway), Ireland
Table of Contents

Plagiarism Statement ........................................................................................................ i

Acknowledgments ............................................................................................................... ii

List of Abbreviations .......................................................................................................... iv

Abstract ............................................................................................................................... vi

Chapter 1 – Introduction ..................................................................................................... 1

1.1 Introduction ..................................................................................................................... 2

1.2. Scaffold-Based Cell Therapy ......................................................................................... 2

1.3. Scaffold-Free Cell Therapy ......................................................................................... 3

1.4. Surface Topography .................................................................................................... 4

1.5. Substrate Stiffness .................................................................................................... 8

1.6. Oxygen Tension ......................................................................................................... 12

1.7. Mechanical Stimulation ............................................................................................ 19

1.8. Animal / Human Sera and Serum-Free Media ......................................................... 22

1.9. Ascorbic Acid Supplementation ............................................................................. 26

1.10. Macromolecular Crowding ..................................................................................... 30

1.10.1 Current Cell Culture Practice: Dilute and not Crowded ...................................... 30

1.10.2. Macromolecular Crowding: A Biophysical Approach ........................................... 32

1.10.3. The Ideal Crowding Agent .................................................................................... 35

1.10.4. Size, Charge and Density of Macromolecular Crowder ..................................... 35

1.10.5. Dispersity of Macromolecular Crowder ............................................................. 36
1.10.6. Potential Macromolecular Crowder and Rationale.................................38
1.10.7. Evaluation of Macromolecular Crowding on DNA and Nucleus........41
1.10.8. Evaluation of Macromolecular Crowding on Protein Folding,
Aggregation and Stability ........................................................................41
1.10.9. Evaluation of Macromolecular Crowding on Intra-cellular Trafficking
and Cellular Homeostasis .........................................................................42
1.10.10. Macromolecular Crowding Accelerate Procollagen Conversion ........43

1.11. Project Rationale and Hypothesis ..........................................................52
1.12. Aims and Objectives .............................................................................53
1.12. References ............................................................................................54

Chapter 2 – Modulation of In Vitro Microenvironment Using Macromolecular
Crowding.......................................................................................................97

2.1. Introduction..............................................................................................98

2.2. Materials and Methods ..........................................................................101

2.2.1. Cell Culture.........................................................................................101

2.2.2. Phase Contrast Microscopy .................................................................102

2.2.3. Cell Metabolic Activity (alamarBlue®) ...............................................102

2.2.4. Cell Viability (Live/Dead® assay) .........................................................102

2.2.5. Collagen Extraction and Pepsin Digestion ..........................................103

2.2.6. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-
PAGE)............................................................................................................104

2.2.7. Immunocytochemistry ........................................................................105

2.2.8. Gelatin Zymography ..........................................................................106
2.2.9. Dynamic Light Scattering (DLS) Measurements ........................................107
2.2.10. Nanoparticle Tracking Analysis (NTA) .................................................108
2.2.11. Total Protein Extraction........................................................................109
2.2.12. SDS-PAGE and Gel Band Excision for Proteomic Analysis ............109
2.2.13. Trypsin Digestion ................................................................................110
2.2.14. Mass Spectrometry ................................................................................110
2.2.15. Proteomics Data Analysis .....................................................................111
2.2.16. Proteomic Validation by Immunocytochemistry .................................112
2.2.17. Preparation of Cell-Sheets using Thermo-Responsive Culture Surfaces ...........................................................................................................112
2.2.18. Scanning Electron Microscopy (SEM)..................................................112
2.2.19. Atomic Force Microscopy (AFM)..........................................................113
2.2.20. Hematoxylin and Eosin (H & E) Staining ..........................................114
2.2.21. Masson’s Trichrome Staining .................................................................114
2.2.22. Picro-Sirius Red Staining ......................................................................115
2.2.23. Statistical Analyses ................................................................................115

2.3. Results ..........................................................................................................116

2.3.1. Identification of Optimal Culture Period, Serum Origin and Serum Concentration for Maximum ECM Deposition ..................................................116
2.3.2. Identification of Optimal MMC for Maximum ECM Deposition ........133
2.3.3. Production and Characterisation of ECM-Rich Cell-Assembled Constructs ........................................................................................................149
2.3.4. Proteomics Analysis of ECM-Rich Cell-Assembled Constructs ..........154
2.3.5. Production and Characterisation of Bone and Tendon ECM-Rich Cell-Assembled Constructs ................................................................. 159

2.4. Discussions........................................................................................................... 193

2.5. References ............................................................................................................ 198

Chapter 3 – Conclusions and Future Directions ...................................................... 210

3.1. Conclusions ........................................................................................................... 211

3.2. Future Directions ................................................................................................ 212

3.2.1. Evaluation of Presence of Macromolecular Crowder in Cell Layer.....212

3.2.2. Designing of Polydispersed and Variable Shape Crowding Models.....212

3.2.3. Identifying Carrageenan Alternatives ......................................................... 212

3.2.4. Oxygen Tension and Macromolecular Crowding....................................... 213

3.3. References ............................................................................................................ 214

Chapter 4 – Appendices............................................................................................ 216

4.1. List of Reagents, Chemicals, Kits and Antibody Used.................................. 217

4.1.1. List of Reagents, Chemicals and Kits Used.............................................. 217

4.1.2. List of Primary and Secondary Antibody Used....................................... 221

4.2. List of Protocols .................................................................................................. 223

4.2.1. Cell Culture Media Preparation .............................................................. 223

4.2.2. Cell Thawing and Culturing ................................................................. 224

4.2.3. Cell Counting .............................................................................................. 225

4.2.4. Cell Freezing .............................................................................................. 226

4.2.5. Cell Seeding for In Vitro Study ............................................................... 227
4.3. Research Outputs ................................................................. 254

4.3.1. Patent .............................................................................. 254

4.3.2. Awards and Honours ....................................................... 254

4.3.3. Publications ..................................................................... 254

4.3.3. Conferences Presentations ............................................... 255

4.4. Copyright License Agreements ............................................ 261
Plagiarism Statement

I certify that the thesis is all my own work and I have not obtained a degree in this University, or elsewhere, on the basis of this work.

Abhigyan Satyam
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Abhigyan Satyam
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>µl</td>
<td>Micro litre</td>
</tr>
<tr>
<td>µM</td>
<td>Micro Molar</td>
</tr>
<tr>
<td>ADSCs</td>
<td>Adipose-Derived Stem Cells</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>C</td>
<td>Centigrade</td>
</tr>
<tr>
<td>CR</td>
<td>Carrageenan</td>
</tr>
<tr>
<td>Cr</td>
<td>Crowder</td>
</tr>
<tr>
<td>CSTE</td>
<td>Cell Sheet Tissue Engineering</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's medium (DMEM)</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DPX</td>
<td>Di-n-butyl phthalate in Xylene</td>
</tr>
<tr>
<td>Dxs</td>
<td>Dextran Sulphate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic</td>
</tr>
<tr>
<td>ESCs</td>
<td>Embryonic Stem Cells</td>
</tr>
<tr>
<td>EVE</td>
<td>Excluded volume effect</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FC</td>
<td>Ficoll®</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>GAPD</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>hESCs</td>
<td>human embryonic stem cells</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia Inducible Factor</td>
</tr>
<tr>
<td>HS</td>
<td>Human serum</td>
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<tr>
<td>HTC</td>
<td>Human Tenocytes</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>MMC</td>
<td>Macromolecular Crowding</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Matrix metalloproteinase-2</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>NH0st</td>
<td>Human Osteoblasts</td>
</tr>
<tr>
<td>pNIPAAm</td>
<td>N-isopropyl acrylamide</td>
</tr>
<tr>
<td>pNTBA</td>
<td>N-tert-butyl acrylamide</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>NSCs</td>
<td>Neuronal Stem Cells</td>
</tr>
<tr>
<td>P4Hs</td>
<td>prolyl 4-hydroxylases</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>Pen/strep</td>
<td>Penicillin/streptomycin</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl Hydroxylases</td>
</tr>
<tr>
<td>PSS</td>
<td>Poly (Sodium 4-Styrenesulfonate)</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium do-decyl sulfate</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TESA</td>
<td>Tissue Engineering by Self Assembly</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel-Lindau</td>
</tr>
<tr>
<td>WI-38</td>
<td>Human Lung fibroblast</td>
</tr>
<tr>
<td>WS-1</td>
<td>Human Skin fibroblast</td>
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Abstract

Introduction: Advancements in molecular and cell biology have led to the development of tissue engineering by self-assembly. The driving hypothesis of this concept is that replacement, repair and restoration of lost tissue function can be accomplished best by using the cells’ inherent capacity to create highly sophisticated structures with precision and efficiency still unmatched by human-made devices. However, the prolonged culture time required to develop an implantable device jeopardises clinical translation and commercialisation. It has been demonstrated that macromolecular crowding enhances the deposition of extracellular matrix. Herein, the influence of crowding molecules on matrix deposition and the potential of this technology in tissue engineering by self-assembly was investigated.

Materials and Methods: Human fibroblasts (lung and skin), tenocytes and osteoblasts were cultured under various MMC conditions (dextran sulphate, Ficoll™ & carrageenan) in a range of fetal bovine serum (FBS) and human serum (HS) concentrations (0.0-10%). ECM deposition was verified by SDS-PAGE, immunocytochemistry (ICC), atomic force microscopy (AFM), scanning electron microscopy (SEM) and mass-spectrometry (MS). The MMC molecules were characterized by dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA). The influence of crowders on cell morphology, cell viability and metabolic activity were evaluated using phase-contrast microscopy, Live/Dead® and AlamarBlue® assays respectively. pNIPAAm and pNTBA based thermo-responsive copolymers were developed to facilitate detachment of ECM-rich cell-sheets.

Results: The SDS-PAGE and densitometry demonstrated that MMC significantly increases the collagen type-I deposition (p<0.0001) at all tested serum concentrations (maximum deposition was in 2 days & 0.5% FBS or HS). ICC, AFM and SEM further confirmed enhanced deposition of fibrillar ECM in presence of MMC. DLS and NTA demonstrated that CR has highest polydispersity among all tested crowders. Phase-contrast microscopy, Live/Dead® and AlamarBlue® assays confirmed that cellular morphology, viability and metabolic activity respectively were not affected by MMC. Thermo-responsive coating with 65% pNIPAAm: 35% pNTBA facilitated detachment of ECM rich cell-sheet from culture. Complementary ICC for MS validation confirmed the enhanced deposition of collagens (III, IV, V, VI) and other ECM molecules (laminin, fibronectin, hyaluronic acid, decorin, lysyl oxidase), without changing collagen-VII, elastin, fibrillin-1, transglutaminase-2, α-
smooth muscle actin, epithelial keratin, tubulin, chondroitin sulphate, keratin sulphate, heparin sulphate, aggrecan, biglycan, CD248 and IL-10.

**Discussion and conclusions:** This work reports that the efficacy of macromolecular crowding in enhancing matrix deposition is amplified in human fibroblast, tenocyte and osteoblast cultures in the presence of low serum concentration, due to the low proteolytic activity of serum; in fact, an over 80-fold increase in extracellular matrix deposition is documented within 48 hours. It further identifies that macromolecular polydispersity is key modulator of extracellular matrix deposition, due to the generation of effective volume exclusion effect. Using a custom-made thermal responsive polymer, living substitutes with tissue-specific protein composition and structure were attained. This approach enables modulation of the *in vitro* microenvironment, without negatively impacting on cellular functions, and therefore opens new avenues for a more rational design in engineering cohesive tissue modules.
Chapter 1 – Introduction

Parts of this chapter have been published at:

*Engineering in vitro microenvironments for cell based therapies and drug discovery.*
1.1 Introduction

The age of the Earth is 4.54 billion years [1, 2] and we, as human beings (homo sapiens), have been living on Earth for about 250,000 years [3]. Throughout this period, we have faced many unmitigated problems, such as pollution, deforestation, war and famine. In the last 100 years, improved life quality and expectancy have been achieved due to advancements in medicine, biology and engineering to diagnose and treat diseases. Nevertheless, injuries and degenerative conditions remain major setback [4], imposing the need for new therapeutic strategies.

Cell-based therapy has gained tremendous interest in the last decades and represents a new hope for the treatment of injuries and degenerative conditions. Indeed, cells have enormous therapeutic competence, as they can sense diverse signals, move to specific sites in the body and execute complex response behaviours, all in the context of a specific tissue environment. Further, clinical trials have highlighted the benefits of using cells as therapeutic agents [5-11]. The preference for cell-based therapy delivery format is dependent on the tissue and disease. In all cases, the aim is high cell retention, followed by good integration capacity and optimal survival rate, with marginal side effects and minimal stress for the patient. In recent years, two conceptually different strategies to deliver cells have been adopted: the first is based on combinatorial implants consisting of cells and an instructive scaffold [12-15], whilst the second is a fully biological approach, based on tissue engineered implants [16, 17].

1.2. Scaffold-Based Cell Therapy

Aiming to develop biological substitutes to restore, replace or regenerate defective tissues, scaffold-based tissue engineering has continued to evolve as an exciting and multidisciplinary field. Cells, scaffolds and biologics are the basic constituents [18], often referred as the tissue-engineering arpeggio. Scaffolds, the extracellular matrix (ECM) surrogate [19], are typically made of polymeric biomaterials and provide the structural support for cell attachment and subsequent tissue development. The use of a scaffold material also enables localised and sustained delivery of biological / therapeutic molecules [20-23]. Several degradable [24-30] and non-degradable [31],...
natural [26-28] or synthetic [24, 25, 31] in origin scaffolds have been evaluated over the years [32]. Despite advances in chemistry and engineering, scaffold-based tissue engineering is still far way from matching nature's ability to grow and repair tissues and organs [33], as numerous shortfalls have been documented over the years, including immunogenicity, toxicity of degradation products, inflammatory responses, fibrous tissue formation, mechanical properties mismatch and mismatch between degradation and neotissue formation [34]. These limitations triggered investigations into scaffold-free cell-based therapies.

1.3. Scaffold-Free Cell Therapy

Although direct cell injections demonstrated very promising preclinical and clinical outcomes [35-37], the site of injury does not offer control over the size, shape and locality of the injected cell suspensions [38, 39], leading to variable therapeutic efficiency. These limitations have led to the development of scaffold-free cell therapies [40] or cell-sheet tissue engineering [41, 42] or tissue engineering by self-assembly [43] to treat injured and degenerated tissues (e.g. blood vessel, [44, 45], heart [46], nerve [47] and immune system [48]). The rationale of this concept is that functional regeneration can be best achieved using the innate capacity of cells to create their own tissue-specific ECM avoiding shortfalls of man-made devices [49]. Scaffold-free approaches have also been integrated with nano- and micro-fabrication technologies, aiming to a better repair and regeneration outcome [16]. Despite the documented findings in preclinical and clinical setting outcomes for skin [50-52], blood vessel [16, 44, 45, 53, 54], cornea [55, 56], heart [38, 57, 58], lung [59], liver [60] and bone [61], only Epicel® (Genzyme, USA) for skin and LifeLine™ for blood vessel (Cytograft, USA) have been commercialised. This limited technology transfer from bench-top to clinic has been attributed to the long period required for ex vivo culture (e.g. 14-35 days for corneal epithelium) [56]; 84 days for corneal stromal [62, 63]; 28 days for corneal endothelium [64]; 70 days for lung cell-sheet [59]; and 196 days for blood vessel [44] that leads to loss of native phenotype and cell senescence [65, 66]. To this end, considerable efforts are focused on engineering more functional in vitro microenvironments, using surface topography; substrate stiffness; oxygen tension; mechanical stimulation; animal / human sera and/or
serum-free media; ascorbic acid supplementation; and localised density, in the form of macromolecular crowding, that would facilitate development of functional cell-assembled tissue equivalents.

1.4. Surface Topography

The cell-substratum interface presents primary signal for cellular adhesion and the subsequent induction of tissue integration [67]. An increased knowledge of the extracellular environment, the topographical cues present at the cellular level, and how cells react to these stimuli has resulted in the development of functionalized surfaces via topographical modification with means to control cellular growth, regulate cell adhesion, guide cell motility and direct stem cell differentiation [68, 69]. The rationale of this approach is based on the fact that the ECM is composed of topographical features, such as grooves, ridges, whorls and pits, ranging from nano- to micro-scale [70].

It has been postulated [71, 72] that cells sense topographical features and regulate their behaviour accordingly through focal adhesion interactions that elicit a cascade of cellular and molecular events (Figure 1.1a). Controlling cell directional growth is showing potential to overcome the issue of phenotype drift (change in phenotype over time) in culture and to direct stem cell differentiation (Figure 1.1b). Indeed, culturing tenocytes on microgrooved membranes maintains physiological cell morphology; prevents phenotype and functional losses; and can even restore lost phenotype of cells cultured on smooth substrates [73, 74]. Anisotropic substrates have also been used for bone tissue engineering application to increase activity and induce aligned matrix deposition in osteoblast cultures [67, 75]. Neurons have also been shown to be very sensitive to the substrate topography. Indeed, aligned electrospun nano-fibres [76, 77] and isoelectric focused collagen films [78] have been employed to successfully guide the outgrowth of dorsal root ganglia neurites. More recently, lithography techniques have been employed to demonstrate that nanotopographies influence neuron adhesion and functionality, with a surprising sensitivity of cells to nano-scale changes [79].
Like in somatic cell culture, topographical features have been shown to significantly influence stem cell behaviour. Specifically, it has been demonstrated that substrate topography, in synergy with biochemical cues, such as laminin coating, enhances and regulates differentiation of hippocampal neural stem/progenitors cells [80, 81]. Similarly, culturing human MSCs on nano-scale pits, made by colloidal lithography, induced osteogenesis in the absence of osteogenic media, whilst reducing the level of offset in pit placement, induced a switch from osteogenic stimulation to maintenance of MSC phenotype and multipotency [82, 83]. Numerous attempt has been tried to modify the surface of implants with unidentified surface topographies by either using coatings [e.g., using calcium phosphate coatings [84] on hip implants] or through physically modifying the surface of the implant by varying the surface roughness (e.g., by sand blasting and electropolishing [85]. Of significant importance has been a high-throughput study that revealed that surface topographies able to induce MSC proliferation or osteogenic differentiation [86]. MSCs cultured within micro-channels, formed using soft-lithography, produced oriented cartilage structures with improved mechanical properties [87]. In an attempt to reproduce the complexity of native ECM and to commit MSCs to tenogenic lineage, bio-imprints were produced, using tendon sections as template. This replica, when coated with collagen type I, supported tenogenesis of MSCs without requiring exogenous growth factors [88].

The manipulation of surface topography is a powerful tool not only for tissue engineering applications, but also for the development of cell-based patterned arrays for drug screening [89]. Indeed controlling cell-substrate interaction through topography results in the modulation of other events, such as ion channel function, differentiation and gene expression [90]. For instance, a recent study suggested the use of topographical substrates to develop neural cell-based assays for drug discovery targeting ion channel function; compared to flat polystyrene surfaces, micro-bead arrayed substrates were capable of cell spreading and enhanced voltage-gated calcium channel responsiveness of neural progenitor cells [90].

Collectively, these studies suggest that there are critical size and geometry features for each cell type-specific response. In the near future, it was anticipated that a deeper understanding of cell-substrate interactions and improvement of nano- and micro-fabrication technologies will guide the design and production of ‘intelligent’
nano-topographic surfaces that will direct cell behaviour. However, it is imperative for imprinting technologies to become financial viable, as the current associated expenditure prohibits scaling up and subsequent commercialisation of the newly developed knowledge.
Figure 1.1: a) Cells sense substrate topography through focal adhesions. Changes in geometry and size of the underlying features of the ECM influence the clustering of integrins and other cell adhesion molecules, thus altering the number and distribution of focal adhesions. Such alterations at focal adhesions may then induce changes in cytoskeletal organisation and structure, which in turn elicit a cascade of cellular and molecular events leading to modifications of cell growth, adhesion, motility and stem cell fate. b) Examples of how topographical cues influence cell behaviour in vitro.
1.5. Substrate Stiffness

Normal cells are anchorage dependent, generally not viable when suspended in a fluid [91, 92]. Such cells must adhere to a solid, but a solid can be as rigid as glass or softer than a baby's skin. An understanding of how cells sense matrix stiffness has emerged with quantitative studies of cells adhering to gels or to other cells with which elasticity can be tuned to approximate that of tissues [93]. Key roles in molecular pathways are played by adhesion complexes and the actinmyosin cytoskeleton, whose contractile forces are transmitted through transcellular structures. The feedback of local matrix stiffness on cell state likely has important implications for development, differentiation, disease, and regeneration [94-96]. Culturing cells on rigid polystyrene surfaces are one of the many orders of magnitude stiffer than most tissues, leading to abnormal cell behaviour. This abnormal change in matrix stiffness influences terminally differentiated cell signalling, motility, morphology and proliferation, as well as stem cell [72, 91, 92]. Cell response to rigidity has been correlated to the stiffness or elasticity of the tissue from which they derived from [97, 98]. At focal adhesions (Figure 1.2a), cells exert forces to the ECM through actin-myosin contractions and respond to the resistance of the matrix [91, 99] through, still poorly understood, chemical signalling mechanisms [98, 100].

Fibroblasts and epithelial cells were the first cells those reported to sense and respond to substrate stiffness [101]. It was observed that cells increased motility or lamellipodial activity on flexible substrates, whereas on stiff matrices cells were more rigid and well spread. More recently, it was demonstrated that fibroblasts preferentially move toward stiffer regions of matrix compliance, a process termed “durotaxis” [102]. Studies with stem cells demonstrate that matrix elasticity is effective, although insufficient to induce terminal differentiation of MSCs, which showed early osteogenic, myogenic and neuronal phenotypes after culture on stiff, intermediate and soft gels respectively [99] (Figure 1.2b). The ability of MSCs to sense matrix elasticity likely involves the non-muscle myosin II, as suggested by the lineage specification blockage of MSCs on any substrate after treatment with blebbistatin [99]. Further studies utilising microarrays with different rigidity impacted on the differentiation of human MSCs: osteogenic lineage was favoured on
rigid arrays, whereas adipogenic differentiation was enhanced on soft substrates [103, 104]. In neural field, soft hydrogels conjugated with full-length laminin directed differentiation of neural stem cells (NSC) toward neurons, whilst stiff hydrogels resulted in equal proportions of neurons and astrocytes. Rho GTPases activity increased with stiffness and modulated lineage specification toward astrocyte differentiation [105]. Of note, it has also been shown that the use of substrates mimicking the elasticity of in vivo stem cell niches enhances in vitro self-renewal of a variety of stem cells including muscle stem cells [106], embryonic stem cells (ESC) [107, 108] and hematopoietic stem cells [109].

Although through 2D cultures these fundamental findings have been obtained, an increasing body of evidence suggests the importance of investigating the cell-matrix interactions in a 3D microenvironment, as the different spatiotemporal distribution of cell-adhesion sites significantly influences cell behaviour [110]. Indeed, it has been shown that the different viscoelastic properties of biomaterials influence both proliferation and matrix remodelling of fibroblasts in 3D hydrogels [110]. It has been also demonstrated that the dedifferentiation process observed in chondrocytes in monolayer cultures can be reversed upon transfer into a 3D environment [111]. Understanding of how 3D matrices influence cell behaviour, including the subsequent proteolytic degradation of the biomaterial matrix, has a potential application in bio-printing technology, where living cells together with hydrogel-based scaffolds are precisely deposited in a certain 3D pattern to fabricate de novo organs [112, 113]. To move the organ printing one step closer to reality, new hydrogels should be tailored to enhance in each cell type a specific spatiotemporal response, such as cellular migration, differentiation or gradual matrix degradation.

In the field of drug discovery, there is increasing evidence that changes in matrix stiffness influence cancer cell behaviour and sensitivity to therapeutic drugs. Cancer progression in soft tissues is typically associated with an increase in ECM rigidity [114, 115]. Accordingly, hepatocellular carcinoma cell lines cultured on gels of variable stiffness showed increased proliferation and chemotherapeutic resistance on rigid matrices [114]. On the other hand, the addition of an antibody directed to an integrin associated protein had little effect on the viability of lung cancer cells on soft gels, due to the less adhesion, whereas it was effective against cells spread on
rigid substrates [116]. With respect to gene delivery applications, ECM stiffness has been found to mediate cell ability to uptake exogenous molecules. In particular, it has been shown that high substrate rigidity leads to a higher efficiency of non-viral gene delivery and expression in cells, probably through the regulation of cell proliferation [117, 118].

Overall, these observations clearly indicate the importance of controlling matrix stiffness _in vitro_ to optimise cell culture conditions and modulate the fate of terminally differentiated cells and stem cells. Despite the significant strides achieved, further multifactorial studies employing new biomaterials, advancements in nano- and micro- fabrication technologies, microfluidics systems and biochemical or biological signalling will be essential to systematically address the mechanisms of cell stiffness sensing. Appropriate design of substrate stiffness will also allow development of more realistic and efficient drug delivery systems.
Figure 1.2: a) By pulling on the matrix at focal adhesions, cells transmit forces to the substrate and respond to its resistance, which depends on the stiffness, or elasticity, of the matrix or environment. Substrate stiffness influences cell motility, morphology, proliferation and stem cell fate. The force generated by cells to deform the matrix is transmitted through the cytoskeleton to an interior target, such as the nucleus. The resulting tension induces conformational changes or unfolding of focal adhesions or other proteins, leading to activation of signalling pathways (inset).

b) MSCs become adipocyte-like, neuron-like, myocyte-like and osteoblast-like, when cultured on substrates having elasticity typical of fat, brain, muscle and cross-linked collagen of osteoid respectively.
1.6. Oxygen Tension

Joseph Priestley convincingly demonstrated the importance of molecular oxygen (O\textsubscript{2}) for animal life in 1774 when he placed a burning candle in a bell jar alongside a mouse. O\textsubscript{2} consumption by the candle had obvious deleterious effects on the unfortunate rodent, underscoring the potentially lethal outcome of exposure to low levels of O\textsubscript{2} (known as hypoxia). Even moderate hypoxia elicits immediate, transient responses, which range from rapid changes in the carbohydrate metabolism of tissues to more permanent changes in local blood vessel networks. Most organisms, including bacteria, yeasts, invertebrates and vertebrates, require O\textsubscript{2} for survival. O\textsubscript{2} is the primary electron acceptor in many intracellular biochemical reactions and is harnessed by mitochondria to generate ATP through aerobic metabolism [119]. Availability of an aqueous medium as a source of nutrients and oxygen to support metabolism is a key factors for survival of mammalian cells. Changes in aqueous environment are relatively slow while metabolic requirements are variable and may occur at a much faster rate; however, since fluid medium management and metabolic supply depend on the same system of delivery, it follows that oxygen supply and fluid exchange are, in principle, linked [120].

Physiological oxygen levels range from 5% to 13% in blood and 2 to 9% in most tissues. Current cell culture studies are generally performed at 21% O\textsubscript{2}, which should be considered as a hyperoxic environment, and similar to hypoxic environments (<2% O\textsubscript{2}) (Table 1.1) it have a strong impact on cellular biology [119]. Indeed, \textit{in vitro} and \textit{in vivo} data convincingly demonstrate that molecular oxygen levels regulate cell behaviour and play a significant role in developmental processes, such as angiogenesis, haematopoiesis, and morphogenesis [119]. Therefore, considerable research effort has been directed towards optimisation of oxygen supplementation for \textit{in vitro} engineering (\textbf{Figure 1.3}) of various tissues [121], including cartilage [122, 123], tendon [124], bone [125, 126], intervertebral disc [127], nucleus pulposus [128] and heart [129, 130].
Figure 1.3: Routinely, cells are cultured under hyperoxic conditions (21% $O_2$), although physiological conditions in several adult and developing tissues are hypoxic. Considerable effort is ongoing to adjust oxygen levels of cultured cells to physiological tissue levels.

<table>
<thead>
<tr>
<th>Terminology</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Hypoxia</td>
<td>A state in which the level of $O_2$ is decreased relative to the normal level (which is 2–9% in most mammalian cell types). Under basal conditions, cells are adjusted to an $O_2$ environment biologically read as normoxia [119, 131]</td>
</tr>
<tr>
<td>Normoxia</td>
<td>Although frequently defined in the literature as 21% $O_2$, physiological normoxia is actually in the range of 2–9% $O_2$ for most adult cells in vivo [119]</td>
</tr>
<tr>
<td>Hyperoxia</td>
<td>Level of $O_2$ is increased relative to the normal level (which is 2–9% in most mammalian cell types) [119]</td>
</tr>
</tbody>
</table>

Table 1.1: Terminologies based on oxygen supply percentages.
Oxygen homeostasis is regulated by hypoxia-inducible transcription factor 1 (HIF-1) which facilitates both O$_2$ delivery and adaptation to O$_2$ deprivation [132]. Hypoxia up-regulates the activity of specific prolyl hydroxylases involved in the stabilisation of HIF, which in turn transcriptionally activates a variety of genes linked to biological processes, such as angiogenesis and glucose metabolism [133, 134]. In fibroblast cultures, hypoxia has been shown to increase mRNA levels of procollagen $\alpha_1$(I) [135] and transforming growth factor-beta 1 (TGF-$\beta$1) [136], a fundamental regulator of ECM formation [137, 138], enhancing that way ECM production. In addition, low oxygen concentrations have been employed in cardiovascular tissue engineering to enhance ECM formation and maturation by human myofibroblasts [130].

Recent studies also demonstrate that oxygen tension is of paramount importance in maintaining stem cell niche and stem cell commitment towards a specific lineage [139]. In MSC cultures, low oxygen tension (5% O$_2$) has been shown to retain their undifferentiated and multipotent status [140, 141]. Moreover different oxygen concentrations have been employed to stabilise a chondrogenic phenotype or to promote hypertrophy of cartilaginous grafts, suggesting a possible application for cartilage repair therapies or endochondral bone repair strategies respectively [142]. Human ESCs maintain pluripotency at oxygen tensions between 3% and 5%, whilst spontaneously differentiate, when cultured at 21% [143]. This is not surprisingly considering the relatively oxygen-poor environment in which the mammalian embryo develops. Also NSCs, which physiologically reside in a relatively hypoxic niche, are able to respond to changes in redox status. Indeed, several studies have demonstrated that mild hypoxic conditions (5% O$_2$) enhance their proliferation and multipotency [144], whilst cultures at 20% oxygen lead to mitotic arrest and glial differentiation [145]. Of note, in this latter study, when human neural precursors were expanded at 5% O$_2$ and then differentiated at 20% O$_2$, oligodendrocyte maturation was greatly enhanced in comparison to cells expanded in 20% oxygen [145], indicating that dynamic control of oxygen tension may be crucial for committing NSCs to an oligodendrocyte lineage in neuro-regenerative therapies where remyelination of damaged axon is required. Recently, low oxygen tension (2%) has been successfully applied for expansion of human tendon-derived stem cells
in vitro, suggesting that hypoxia could help achieve a sufficient number of these stem cells for clinical application [124].

As oxygen tension facilitates stem cell expansion, whilst maintaining phenotype fidelity, it may also be a useful tool to generate stem cells of sufficient quantity and quality for drug screening applications [146]. Moreover several studies have shown that HIF-1 can be an important target for treating diseases such as cancer, heart failure, stroke and fibrosis [147-149]. As HIF-1 is overexpressed in hypoxic tissues, including solid tumours, several anticancer agents have been developed to inhibit HIF-1 activity and prevent cancer cells survival [148]. On the other hand, enhanced expression of HIF-1 has been used to protect cultured cardiomyocytes against simulated ischemia-reperfusion injury [150, 151]. These observations thus suggest that low oxygen levels can be used for drug screening purposes to produce physiologically relevant in vitro models of hypoxia-related pathologies.

1.6.1. Oxygen Tension in Biological Pathways

In 1977, Packer reported in “Nature” that human diploid fibroblasts grown at 10% O₂ have a longer life than cells grown at the routine 20% O₂ [152]. In 2003, it was reported that growth arrest of fibroblasts caused by 20% O₂ was reversible [153], consistent with the reports of the current study [154], and it was therefore concluded that exposure of cells to hyperoxic insult causes differentiation and not senescence. Although it is standard practice to culture cells at an ambient O₂ concentration of 20% (i.e. room air and balance of 5% CO₂) which corresponds to a pO₂ of approximately 140 mmHg at sea level, cells in the human body are exposed to much lower O₂ concentrations ranging from ~14% (100 mm Hg) in the pulmonary alveoli to 3–5% (35 mm Hg) in the heart and skin [153, 155]. Thus, it is important to recognise that standard cell culture under-conditions of 20% O₂ represents exposure of cells to hyperoxic insult, particularly for primary cells that have been freshly isolated from organs and were therefore adjusted to lower pO₂ as their physiological normoxic status [153, 155]. For cell lines cultured at 20% O₂ over a long period, it is reasonable to assume that the overall cell population represents a hyperoxia-tolerant variety which has survived hyperoxic insult over time by accepting 20% O₂ as their normoxic state. Indeed, while 20% O₂ represents normoxia for these cells, it is
important to appreciate that such state may have little connection with respect to resembling cells or cellular responses *in vivo* [156].

The responses of the cells to the hypoxic microenvironment are primarily mediated through hypoxia inducible factor (HIF) [157]. HIF transcription factors are composed of an oxygen-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit, which dimerise to produce a basic helix-loop-helix (bHLH) transcription factor that binds to hypoxia-responsive elements (HREs), thereby activating specific genes [158-160]. The N-terminal transactivation domain of HIF-1α contains an oxygen-dependent degradation domain that allows the degradation of the α-subunit under normoxic conditions [161]. As such, under hypoxic conditions, HIF-1α accumulates and can, after dimerising with HIF-1β, affect the expression levels of a large range of genes [162-164]. For example, HIF-1 has been shown to activate glycolytic genes and suppress metabolism through the Krebs cycle, hence stimulating the conversion of glucose to pyruvate and subsequently to lactate [165]. HIF-1 also plays a crucial role in directing chondrogenic differentiation [166, 167] and is, in addition, involved in the regulation of various processes that enhance delivery of oxygen to tissues, including the production of red blood cells mediated through erythropoietin and the formation of new blood vessels via the secretion of vascular endothelial growth factor (VEGF) during embryonic development and wound healing [168].

### 1.6.2. Collagen Biosynthesis and Oxygen Tension

Vertebrates have at least 27 collagen types with 42 distinct polypeptide chains (α chains), and more than 20 additional proteins that have collagen-like domains. Prolyl 4-hydroxylases (P4Hs) catalyse the formation of 4-hydroxyproline by the hydroxylation of proline residues in peptide linkages. Two families of P4H are known today: collagen P4Hs (C-P4Hs) that have a central role in the synthesis of all collagens, and HIF-P4Hs that play a key role in the regulation of oxygen homeostasis by hydroxylating critical residues in the α subunit of the hypoxia-inducible transcription factor HIF. Collagen prolyl 4-hydroxylases (C-P4Hs), lysyl hydroxylases (LHs) and lysyl oxidases (LOs) have essential roles in collagen synthesis [169-172].
Collagen prolyl 4-hydroxylases (P4Hs, EC 1.14.11.2) are located within the lumen of the endoplasmic reticulum and catalyse the formation of 4-hydroxyproline by the hydroxylation of prolines in -X-Pro-Gly- sequences in collagens and more than 15 other proteins that have collagen-like domains [170-172]. P4Hs have a central role in the biosynthesis of collagens, as 4-hydroxyproline residues are essential for the formation of the collagen triple helix (Figure 1.4). In addition, a novel and distinct family of cytoplasmic prolyl 4-hydroxylases playing a critical role in the regulation of the hypoxia-inducible transcription factor HIFα has recently been identified [134, 173-175]. This transcription factor is synthesised continuously, and a critical proline residue in a -Leu-X-X-Leu-Ala-Pro- sequence is hydroxylated under normoxic conditions [134, 175] (Figure 1.4). This proline is not hydroxylated by collagen P4Hs [175], but instead by a novel cytoplasmic HIF P4H family [173, 174]. The 4-hydroxyproline residue formed is essential for the binding of HIFα to the von Hippel–Lindau (VHL) E3 ubiquitin ligase complex and for subsequent rapid proteasomal degradation in normoxia [134, 175]. Under hypoxic conditions hydroxylation ceases, so that HIFα escapes degradation and forms a stable dimer with HIFβ [134, 175]. This dimer is translocated into the nucleus and becomes bound to the HIF-responsive elements in a number of hypoxia-inducible genes, such as those for erythropoietin, vascular endothelial growth factor, glycolytic enzymes and many others [134, 175]. Interestingly, the gene for α (I) subunit of human type I collagen P4H has been shown to be one of the hypoxia-inducible target genes of HIFα [176] (Figure 1.4).

All in all, the ability to control cell behaviour and stem cell fate by modulating oxygen levels in vitro has a tremendous potential for the development of clinically relevant tissue-engineered grafts and functional cell systems for drug discovery. Further studies to determine the in vivo physiological and pathological tissue oxygen levels should be carried out, and subsequently controlled bioreactor systems should be developed to translate these findings to a robust and reproducible culture process.
Figure 1.4: Regulation of the hypoxia-inducible transcription factor HIFα by oxygen-dependent prolyl 4-hydroxylation. Under a normoxic condition, HIFα is subject to prolyl 4-hydroxylation by HIF P4Hs. Hydroxylation is required for binding of the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex and for subsequent proteasomal degradation. Hypoxia blocks the prolyl hydroxylation, so that HIFα escapes degradation and forms a stable dimer with HIFβ. This dimer is translocated into the nucleus and binds to the HIF-responsive elements in a number of hypoxia-inducible genes.
1.7. Mechanical Stimulation

Various cellular functions including signalling pathways, gene expression, cell proliferation and differentiation and secretion of ECM proteins have been shown to influence by Mechanical forces, in the form of strain, compression or shear [177, 178]. Cells react to mechanical stimuli through a multitude of cell membrane receptors, including integrins at focal adhesions [179, 180], micro-cilia [181] and mechano-sensitive ion channels [182]. Stimuli [183, 184] can then be transferred mechanically through the cell via the actin microfilament network or by stimulating activity in chemo-cascades, such as MAPK and ERK1/2, which in turn result in a nuclear response (Figure 1.5a).

Physiological function of various cell types in vitro have been found to be benefited by mechanical loads (Figure 1.5b). Shear stress and compression have been shown to greatly increase the activity of osteoblasts on engineered scaffolds [185, 186]. Cyclic loading preconditioning of smooth muscle cells has been shown to be critical for blood vessel tissue engineering [187, 188]. Although excessive load induces chondrocyte death, morphological changes and cellular damage, a physiological cyclic load triggers morphological and ultra-structural recovery aspects [189] and significantly increases the amounts of GAGs and collagen II after two weeks in culture [190]. In addition mechanically stimulated tenocytes increase proliferation rate and ECM synthesis, maintain cell phenotype and improve tendon repair biomechanics [191-194], whilst the absence of mechanical load induces perpendicular to the substrate topography orientation of bovine tenocytes [195]. Further work has also showed that cyclic tension regulates matrix remodelling in tendon cells and fibroblast cultures [196, 197].

Mechanical loads have also been shown to induce cell differentiation. Human fibroblasts [198] and MSCs [191, 199-202] differentiate towards tenogenic lineage after mechanical stimulation. Cyclic compression is the principle mechanism required for MSCs to undergo chondrogenesis [203-205]. It has even been observed that mechanical stimulation is more potent than chemical stimuli in differentiation of human MSCs towards chondrogenic lineage [206]. Moreover, application of mechanical stimuli, such as tension, compression, fluid flow and high-frequency vibration, has been shown to up-regulate osteogenesis in MSCs [207-209].
Furthermore, the addition of mechanical stimulation has been shown to improve cell culture models for gene delivery and toxicology assessment. Indeed, endothelial cells that were exposed to a fluid shear stress, increased both uptake and expression of liposome-DNA plasmid complexes, compared to cells under static conditions, due to an enhanced supply of plasmids reaching the cellular surface and increased cellular activity [210]. Recently, a ‘lung-on-a-chip system’, utilising integrated microfluidics and cyclic mechanical strain, imitated breathing movements [211]. In this study, mechanical stress enhanced cellular uptake of nanoparticles in the alveolar epithelium and stimulated their transport into the underlying endothelium. As the effect of cyclic mechanical strain on lung nanoparticle absorption has never been detected in conventional static cell culture models, this study suggests that a mechanically active micro-device may implement current cell culture systems for toxicology applications [211]. It was anticipated that in the near future, such miniaturised lab-on-a-chip systems will replace or improve the outcome of animal studies, by enhancing the predictive power of in vitro models.

Overwhelming literature clearly demonstrates the positive effects of mechanical stimulation / conditioning on cell phenotype maintenance, stem cell differentiation and cellular drug/gene delivery uptake. However, unless the underlying mechanisms are unveiled, as well as bioreactor systems become affordable, mechanical stimulation will remain an understudied opportunity to optimise in vitro systems.
Figure 1.5: a) Mechanical forces act through integrin receptor proteins, mechanosensitive ion channels or a specialised cell surface projection called primary cilium, which has recently been shown to mediate fluid flow mechanotransduction. Forces are then applied to the nucleus via intracellular mechanisms, (e.g. actin filaments or chemo-cascades) resulting in protein transcription being activated. b) Examples of how mechanical forces have been applied in cell cultures studies and tissue engineering strategies to affect stem cell fate; ECM synthesis; and cell phenotype and proliferation.
1.8. Animal / Human Sera and Serum-Free Media

In the early stages of animal cell culture, it was discovered that a small amount of serum in the basal medium would support the growth and proliferation of cells. In the late 1940’s the first cell line (HeLa) was cultivated *in vitro* in a fluid mixture of chicken plasma, bovine embryo extract and serum from umbilical cord blood. This crude mixture was the forerunner of today’s modern cell culture media [212]. Amniotic fluid [213, 214]; milk and chicken egg yolk [215]; and bovine colostrum [216] were also tested, but without success.

Serum is a rich source for vitamins (A, D, E and K), growth factors, co-factors, hormones, attachment factors (fibronectin, laminin), transport factors (albumin, globulin, transferrin), nutrients (carbohydrates, amino acids, proteins, fatty acids, lipids, nucleosides), trace elements, minerals and other factors that limit free radicals, toxins and heavy metals (Figure 1.6) [217-219]. Additionally, serum buffers the culture medium, inactivates proteolytic enzymes, increases medium viscosity, which reduces shear stress during pipetting or stirring and conditions the growth surface of the culture vessel [220]. Thus, it remains the most effective growth product for cell culture available today. The choice of cell culture serum is extremely important, significantly affecting cell growth. To-date, animal, human and serum-free culture media are under intense investigation.
Figure 1.6: Some of the major functions of serum are to provide growth factors, hormones, attachment and spreading factors, binding proteins, lipids, and minerals. Serum supports in vitro microenvironment by controlling growth, proliferation and expression of differentiated functions of cultured cells [220-222].
Foetal bovine serum (FBS) and foetal calf serum (FCS) are the most widely used sera, due to their high content of physiologically balanced growth promoting factors (e.g. growth factors, hormones [217-219, 222, 223] and low levels of growth inhibiting factors (e.g. low gamma globulin content) [213, 214, 220, 224, 225]. Although horse serum has been investigated as an alternative to bovine sera, comparative studies have favoured bovine sera for optimal cell growth [223, 226]. To avoid inter-species transmission of disease, several studies have advocated the use of human serum, as opposed to animal sera, for adipose-derived stem cells, bone marrow stem cells, dental pulp stem cells, dental pulp stem cells, chondrocytes and myoblasts [227-238]. Unfortunately, issues associated with ill-defined composition; lot-to-lot variability; potential source of biological contaminants; and presence of immunogenic factors have triggered investigation into serum-free media [213, 214, 222, 223, 226, 228, 234, 239-249].

With the identification, cloning and recombinant production of growth factors and nutrients, a broad range of chemically defined, serum-free media have become available for numerous permanently differentiated cells and stem cells [213, 214, 217-219, 222, 250-260]. The clear advantage of serum-free culture is the well-defined composition of the media [222, 252, 261-263], which produces more consistent results [222, 261]; eliminates immunogenic / inter-species transmission of disease issues [253, 257]; and offer precise control over cellular functions [261]. Unfortunately, serum-free media are substantially more expensive and require an enormous amount of development to ensure reproducibility and regulatory clearance [222].
<table>
<thead>
<tr>
<th>Serum</th>
<th>Potential use in vitro</th>
</tr>
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<tbody>
<tr>
<td>Bovine serum</td>
<td>Propagation of viruses, <em>Helicobacter pylori</em> and <em>Plasmodium falciparum</em> [264-266].</td>
</tr>
<tr>
<td>Newborn calf serum</td>
<td>It is used in cornea preservation and cryopreservation of bovine embryos. NCS is used in cell culture to support the maturation of oocytes; to study the function of tubular epithelial cells; and to develop primary cultures of cells such as visceral adipocytes. Culture of fish cells [267-269].</td>
</tr>
<tr>
<td>Horse serum</td>
<td>Support an in vitro antibody response. Used in the production of C2 heterokaryons. Used in primary neuronal culture [270-272].</td>
</tr>
<tr>
<td>Porcine serum</td>
<td>Used with hormones in the growth of rat mammary tumor epithelial cells! Required for the growth of rat cervical epithelial cells. The use of porcine serum in chromosome studies has been found to be satisfactory and consistent and could be used in lymphocyte culture work [273-275].</td>
</tr>
<tr>
<td>Sheep serum</td>
<td>Sheep serum is useful for the propagation of long-term cultures of erythrocytic stages of the Theileria uilenbergi intracellular protozoan parasite [276].</td>
</tr>
<tr>
<td>Chicken serum</td>
<td>Preservation of corneal endothelium and in culture of <em>Leishmania</em> [267, 277].</td>
</tr>
<tr>
<td>Human serum</td>
<td>Human serum is useful for ECM deposition from lung and skin fibroblasts and corneal keratocytes [278].</td>
</tr>
</tbody>
</table>

Table 1.2: Various serum type and *in vitro* use.
1.9. Ascorbic Acid Supplementation

Sodium ascorbate / ascorbic acid / vitamin C is a key factor utilised in culture medium as one of the microenvironment modulators. It is an essential co-factor in the hydroxylation of collagenous proline and lysine [279-282] that results in increased procollagen synthesis and collagen deposition in various cells [283-287]. This reaction is also stereo-specific and independent of intracellular degradation of collagen [288]. Ascorbic acid influences the collagen synthesis, maturation, secretion and functional properties of several cell types and is therefore widely used in tissue engineering [281].

The activity of ascorbic acid on human skin fibroblast suggests that it acts only in the synthesis of hydroxyproline; however it does not increase the synthesis of collagenous polypeptides or the amount of propyl hydroxylase [289]. In the absence of ascorbic acid, prolyl residues of α-chains are not sufficiently hydroxylated to form a thermo-stable triple-helix that can be secreted in intact form [290]. Even low concentration of ascorbic acid has been shown to increase cell proliferation in both normal and malignant cells [291-295] and stem cells [296-304]. Further, ascorbic acid protects against oxidative stress damage through inhibition of the redox cycling of superoxide radicals [305].

The stimulatory effect of ascorbic acid, on the extracellular matrix (ECM) synthesis of cells in vitro has been widely investigated. L-ascorbic acid acts as an essential cofactor for enzymatic activity of lysyl hydroxylase and prolyl hydroxylase. These enzymes are an essential part of collagen biosynthesis and induce the posttranslational modification to the procollagen α-chains resulting in an increase of the thermal stability (at body temperature) of the folded procollagen triple helix in the endoplasmic reticulum. In the absence of ascorbic acid, prolyl residues of alpha-chains are not sufficiently hydroxylated to form a thermostable triple helix that can be secreted in their intact form [290]. Ascorbic acid is also reported to favour the telomerase activity, which leads to up-regulation of various ECM molecules such as; collagen type I, fibronectin, and integrin β1. Overall, this process results in increased procollagen synthesis and collagen deposition in various cells [283-287]. This reaction is also stereospecific and independent of intracellular degradation of collagen [288]. The activity of ascorbic acid on human skin fibroblast suggests that it
acts only in the synthesis of hydroxyproline; however it does not increase the synthesis of collagenous polypeptides or the amount of propyl hydroxylase [289].

There are a number of studies showing that the ascorbic acid induces various cellular responses in different cells that may be depends on the dose- and environmental parameters to modulate the various cellular responses including ECM synthesis, proliferation, and differentiation. Usually, the low concentration of ascorbic acid increases the cell proliferation and this effect is more pronounced in malignant cells than in normal cells [291-295]. It is reported that ascorbic acid can regulate cell differentiation of many cells, particularly of multilineage mesenchymal cells (adipogenesis, osteogenesis, chondrogenesis, and myogenesis). Furthermore, ascorbic acid can also stimulate the proliferation of various mesenchymal-derived cell types; such as osteoblasts, adipocytes, chondrocytes, and odontoblasts [296-304], and can also modulate cell proliferation in vitro [296, 306-311]. Tsai-Ming Lin et al. have grown the human adipose derived MSCs using a novel, low calcium cell culture medium supplemented with the antioxidant compounds N-acetyl-L-cysteine (NAC) and a stable compound of L-ascorbate 2-phosphate (Asc-2-P). This medium favors the cell proliferation and its differentiation into various lineage such as adipocytes, osteoblasts, and chondrocytes [300]. Ascorbic acid acts as a growth promoter to increase the cell proliferation as well for DNA synthesis of cells at specific concentration. However, it is also reported that ascorbic acid at markedly higher concentrations used in medium are lethal, which can inhibit cell proliferation as well as cell apoptosis. This relationship between the cellular toxicity and ascorbic acid concentration is dependent on several culture-related factors such as the type of medium used or incubator condition (CO₂ concentration) [312, 313].

1.9.1. Ascorbic Acid in Biological Pathways

Ascorbic acid plays an active role in the metabolism of various amino acids which leads to the formation of hydroxyproline, hydroxylysine, norepinephrine, serotonin, homogenistic acid, and carnitine [279, 282]. Among these, hydroxyproline and hydroxylysine are the basic components of collagens. The role of ascorbic acid is to preserve the iron cofactor in a reduced state at the active sites of the hydroxylases. The soluble form of collagen, i.e. procollagens molecules, synthesise in the rough endoplasmic reticulum similar to other secreted proteins and require posttranslational
modifications before being extruded from the cell. First of all, the procollagen molecule is formed by three polypeptide chains that assume a specific helical conformation due to the high content of glycine which occupies every third position along most of the length of the three polypeptide chains. When collagen is synthesised, proline and lysine are hydroxylated posttranslationally on the growing polypeptide chain.

Moreover, procollagen is characterised by the presence of hydroxyproline and hydroxylysine formed by specific hydrolases during the molecule assembly; furthermore, some of the hydroxylysine residues undergo O-galactosyl and O-galactosyl-b-glycosyl substitution. All these post-translational modifications are necessary for collagen to be secreted from the cells as procollagen. The presence of hydroxyproline and hydroxylysine are required for the formation of a stable extracellular matrix and cross-links in the fibers. The subsequent triple helix quaternary state of physiologically effective collagen can only be achieved if the requisite proline and lysine residues have been hydroxylated. In the extracellular space, procollagen is further modified by proteinases which cut the C- and N-terminal pro-peptides of the molecule and make tropocollagen suitable for self-assembly into fibrils. The last enzymatic modification of the collagen molecule is by lysyl oxidase, which initiates a series of reactions leading to the formation of stable intermolecular cross-links (Figure 1.7). A deficiency of ascorbic acid reduces the activity of two mixed function oxidases, prolyl-hydroxylase and lysyl hydroxylase, which hydroxylate proline and lysine. Some collagen formation take place in the absence of ascorbic acid; but the collagen fibers are abnormal, which leads to the skin lesions and blood vessel fragility, characteristics features of scurvy [173, 282, 314-316].
Figure 1.7: Biosynthesis of collagen begins from synthesis of pro-α chain that undergoes hydroxylation processed by a number of enzymatic steps within the lumen of rough endoplasmic reticulum. The proline and lysine residues are hydroxylated by prolyl and lysyl hydroxylases to form hydroxy proline and hydroxy lysine respectively. The prolyl and lysyl hydroxylases require molecular oxygen, ascorbic acid and α-keto glutarate for their action.
1.10. Macromolecular Crowding

One key distinguishing feature between living cells and the prevailing *in vitro* cellular system is the density of molecules around cellular components, which is an under-appreciated, yet important factor in the regulation of cellular dynamics [317, 318]. Macromolecular crowding, a key feature in natural cells, as it can dramatically influence biochemical kinetics via excluding volume effect, which reduce diffusion rates and enhance binding rates of macromolecules [319]. The nonspecific steric repulsion is always present, regardless of any other attractive or repulsive interactions that might occur between the solute molecules. The amount of unavailable intracellular volume to other macromolecules depends on the number, size and shape of all the molecules present in each compartment [30, 320, 321] (Figure 1.8).

1.10.1 Current Cell Culture Practice: Dilute and not Crowded

The human body is thought to be comprised of approximately 411 different cell types [322], which are reside in a dense extracellular space [323]. Cell-based therapies are based on mechanical or enzymatic isolation of cells from their native highly dense *in vivo* tissue context and expansion *in vitro* in a highly dilute culture environment [324]. This situation is far from physiological, given that the total concentration of macromolecules in prokaryotic or eukaryotic cell environment is considerably higher than the *in vitro* milieu [325-327]. Even blood has a solute concentration of 80 g/l [317]. Even the addition of serum supplements fails to create a physiologically relevant crowded environment [328]. As the cells are confronted with an environment devoid of ECM they start to rebuild their environment by producing their own ECM. While fibronectin is deposited rapidly *in vitro* [329], the deposition of a collagen matrix, the primary structural biological material in all tissues and organs, is enzymatically rate-limited. As the current culturing practices are characterised by a lack of macromolecular crowding and hence excluded volume effect, the procollagen conversion, and as a consequence, collagen matrix deposition is notoriously slow *in vitro* [328] (Figure 1.8). Thus, many weeks or even months are needed for sufficient ECM deposition to create a cohesive tissue construct [330]. This represents a major bottleneck in cell-based, scaffold-free tissue engineering.
Figure 1.8: (a) The *in vivo* illustration of heavily crowded extracellular space. (b) The deposition of ECM in the current, notably dilute and far from physiological, culturing systems is very slow. In the case of collagen type I, the most abundant ECM protein, cells will produce procollagen and the specific N- and C- proteinases. However, in the very dilute in vitro microenvironment, the conversion of the water-soluble procollagen to insoluble collagen will be very slow since either the proteinases will be deactivated before they cleave the specific N- and C- propeptides or the procollagen will be dissolved before its conversion to collagen. Only after prolonged culture time, the cells will self-crowd the media and collagen deposition will be achieved. (c) The addition of inert polydispersed macromolecules, presented as spheres with different diameters, in the culture media can create most effective excluding volume effect, and consequently increases the relative density of procollagen and proteinases in the culture media. This facilitates the cleavage of the propeptides by their respectful proteinases and lead to the fast procollagen conversion to collagen and deposition of the former.
The high total concentration of macromolecules in cellular media are termed ‘crowded’ rather than ‘concentrated’ because, in general, no single macromolecular species occurs at high concentration, but the macromolecules occupy a significant fraction (typically 20–30%) of the total volume. Thus, this fraction is physically unavailable to other molecules. Biological macromolecules evolve and function within intracellular environments and in the extracellular space that is crowded with other macromolecules. In *Escherichia coli*, for example, the concentration of total protein inside the cell is in the range of 200-300 mg/ml, whereas that of RNA is in the range of 75-150 mg/ml, making up the total concentration of 300-400 mg/ml [325, 331]. In cell culture, the total concentration of macromolecules is estimated to be only 1-10 mg/ml [317, 332].

Enormous efforts have been made in the cell culture industry to ensure quality and reproducibility of the culture-ware, as well as serum and media compositions. These efforts are reflected in textbooks that specifically deal with establishing cell cultures [220]. So far, this technology has served us well, but it has become forgotten over time that what we have in cell culture flasks and in many bioreactors is pathological: the disproportion of aqueous medium to cell mass, the substitution of an organic support by plastic, and the lack of macromolecular crowdedness as such, a hallmark of the microenvironment of cells in metazoans. In fact, if such a disproportion of fluid to cell occurs in the human body this would require immediate medical attention; yet biologists all over the world are complacently growing cells under conditions that in the clinical world would be considered as oedema or effusion. Scaffold-free approaches involving cell printing and cell sheet technology are both dependent on the presence of sufficient ECM to stabilise the structures [333, 334]. Unfortunately, however, the deposition of ECM *in vitro* with uncrowded conditions is an inefficient process. Stem cell based therapies are currently obstructed because *ex vivo* propagation of stem and progenitor cells on tissue culture polystyrene results in decaying proliferation and differentiation capacities [335, 336].

### 1.10.2. Macromolecular Crowding: A Biophysical Approach

Macromolecular crowding is more accurately termed the excluded volume effect (EVE). The unavailable volume to other macromolecules depends on the number, size and shape of all the molecules present in each compartment. The nonspecific
steric repulsion is always present, regardless of any other attractive or repulsive interactions that might occur between the solute molecules. This conclusion applies both to the bulk soluble phases in each compartment and to the smaller volumes confined by cytoskeletal elements in eukaryotic cells. The cytoskeleton constitutes a dense lattice in which the fluid part of the cytoplasm is dispersed among small elements or pores, with dimensions comparable to the size of large macromolecular complexes. Volume exclusion by the pore boundaries to the macromolecules within is a type of crowding called macromolecular confinement [320, 321, 337]. Macromolecular crowding functions by way of the excluded-volume effect (EVE) and is often referred to as the “volume of a solution that is excluded to the center of mass of a probe particle by the presence of one or more background particles in the medium” [327]. *Fractional volume occupancy* $\Psi$ (FVO) denotes the fraction of the total volume occupied by macromolecules (Figure 1.9). Thermodynamically, volume exclusion lowers the configurational and conformational freedom (entropy) leading to elevated basal free energy of the reactant macromolecules and a number of downstream effects [338]. These may be identified as: (1) folding of biopolymers (e.g. proteins and nucleic acids) into native states optimal for function [339]; (2) stronger macromolecular transition complexes with longer half-lives (for example: enzyme–substrate) leading to more product, and (3) buffering effect of crowded environments on biological function under conditions of adverse pH, temperature or ionic strength [340]. Biological reactions performed in dilute in vitro conditions proceed more slowly than in their in vivo counterparts [341]. However, MMC or EVE have not been appreciated in the biological domain and applications are yet to be fully developed and implemented [324, 325].
**Figure 1.9:** Schematic illustration of macromolecular crowding / excluding volume effect. The diagram reflects the situation that a test molecule (purple) will encounters in a given volume of box. The crowders (red) occupy the volume \((4/3\pi r^3)\) inside box; this possession known as fractional volume occupancy (FVO, \(\psi\)). Apart from FVO, electrostatic repulsion, hydration, intermolecular charge also excludes the volume from box, which makes additional unavailable volumes for the test molecule.
1.10.3. The Ideal Crowding Agent

For being an ideal crowder, the macromolecule should have a molecular weight in the range of 50 – 600 kDa, be highly water-soluble and not be prone to self-aggregation. The molecular shape has to be globular, rather than extended, to prevent solutions becoming too viscous to handle [342]. The crowder should be easily available in highly purified form so that the use of high concentrations does not introduce problems associated with contaminants. Most importantly, the agent should not interact with the system under test, except via steric repulsion. This requirement is the most difficult to meet. It is essential to establish that any effects observed when using crowding agents are not the result of unintentional changes in other factors, such as pH, ionic strength or redox potential [317]. This requirement eliminates the possibility of using highly concentrated cell extracts as crowding agent, since any interpretation will be complicated by specific interactions, hydrolase activity and the presence of denatured proteins. Commonly used crowding agents include Ficolls®, dextrans, polyethylene glycol and polyvinyl alcohol [317].

1.10.4. Size, Charge and Density of Macromolecular Crowder

Structurally and chemically, the macromolecule could be derived from any representative of the carbohydrate, protein, nucleic acid, lipid family. However, the crowding behavior is entirely dependent on the physical property of the macromolecule and basic cellular physiology in vitro. The density or crowdedness of macromolecules is determined by the approximation of a solute content. For the interior of cells, concentrations of macromolecules have been determined to be in a range from 50 to 400 mg/mL [321]. This concentration refers to the cumulative concentration of all species of macromolecules present, rather than the concentration of any single macromolecule. The presence of these macromolecules means that they significantly occupy space in the given medium, and biophysicists refer to such medium as “crowded” or “confining” [321]. MMC can be mimicked experimentally by adding inert synthetic or natural macromolecules to a system in vitro [325]. As a result of the mutual impenetrability of solute molecules (Pauli Exclusion Principle), steric repulsions are generated and this affects the activity of a solute — which is highly dependent on its steric freedom or the volume that is available to the centre of the mass of each molecular species. This is the most fundamental of all interactions.
between macromolecules in solution and always occurs in finite concentrations, independent of the magnitude of additional electrostatic or hydrophobic interactions. It is conceivable that the addition of the inert crowders to occupy a significant volume fraction in the medium will place constraints on the active factors present in the microenvironment, thus driving associations and reactions [321, 325]. One important concept in crowding theory is that macromolecular crowding is most effective if the crowder (better, the volume it occupies) and the test molecule (better, the volume it occupies) are of similar size [343, 344]. Therefore to select any crowder for a biological reaction, it becomes essential to choose crowders of small size to the reactant molecules (enzymes/substrates). Hence, prior knowledge of the hydrodynamic radii of the crowders and the substrate molecules become critical and herein lies the importance of our biophysical approach to select suitable crowders [324].

1.10.5. Dispersity of Macromolecular Crowder

With the exception of a few recent studies [341, 345, 346], almost all of the theoretical studies have modeled crowding agents as monodisperse spherical particles. Such a description may be adequate in rationalizing experimental results that are obtained using Ficoll, polyethylene glycol, and dextran as crowding agents. It should be emphasised that to obtain quantitative agreement with experiments, it may be necessary to consider not only the shapes of crowding particles but also nonspecific attractive interactions between crowding agents and proteins [347, 348]. Indeed, as pointed out by McGuflce et al. [345], the nonspherical shapes of macromolecules as well as polydispersity due to variations in composition and sizes of macromolecules in cells could have a dramatic effect on protein stability. Similarly, the shape of crowding particles can not only influence protein stability but also have a profound effect on protein association [349] and oligomer formation in an amyloidogenic peptide [346, 350].
**Figure 1.10:** Illustration of polydispersed model which represent intracellular and extracellular molecules of various sizes. The macromolecules are represented as a single sphere and they are highlighted in different colour to represent diverse volume.
A cellular milieu is crowded with macromolecules of various sizes and shapes, or polydispersed [351, 352], whereas synthetic crowders provide a rather homogenous environment. It has been shown that the sizes and shapes of crowders can greatly affect the folding process of a protein [353, 354]. In addition, recent experiments have shown a sizeable range of folding kinetics at different cytoplasmic regions of a cell [355]. Thus, a polydispersed model (Figure 1.10) that can adequately represent the composition inside a cell is needed for understanding protein folding in vivo [356].

1.10.6. Potential Macromolecular Crowder and Rationale

i) **Dextran Sulfate Sodium Salt**
   - Mimics natural mucopolysaccharide (chondroitin sulphate, dermatan sulphate).
   - A poly-anion, which is freely soluble in water.

   ![Dextran Sulfate Sodium Salt](image)

ii) **Ficoll®**
   - Uncharged (neutral), highly branched.
   - Chemically inert and not found to interact with proteins.

   ![Ficoll®](image)
iii) **Polysodium-4-Styrene Sulfonate**
- Anionic polyelectrolyte.
- Freely soluble in water.

![Polysodium-4-Styrene Sulfonate](image)

iv) **Carrageenan**
- Linear polymers of 1,3α-1,4β-galactans.
- Poly-anionic and high degree of polydispersity.

![Carrageenan](image)

- **Kappa (K) 1 Sulfate for 2 Sugar Units**
- **Iota (I) 2 Sulfates for 2 Sugar Units**
- **Lambda (Λ) 3 Sulfates for 2 Sugar Units**
v) **Agarose**
- Polymer of agarbios (D-galactose and 3,6-anhydro-L-galactopyranose).
- Uncharged, high degree of polydispersity and temperature stable.

vi) **Sepharose® CL**
- Sepharose is a beaded agarose.
- Low content of ionisable groups’ pH (3-14) and temperature stable.
1.10.7. Evaluation of Macromolecular Crowding on DNA and Nucleus

Molecular crowding influences DNA replication and several aspects of DNA metabolism among them: oriC replication [357], blunt–end DNA ligation by DNA ligase [358], activities of phage T4 DNA kinase [359], the association of accessory proteins [360], and the assembly of a phage T4 DNA polymerase holoenzyme [361]. Macromolecular crowding has also been used as a tool to detect interactions within the T4 replisome [362]. To assess the interactions between DNA molecules, Parsegian and co-workers applied osmotic pressure in ordered arrays of DNA molecules and used X–ray diffraction to measure the structural changes [363, 364]. Crowding determines genome structure and function by its effects on both the organization of DNA into nucleosomes by folding-packaging and the interactions between DNA and histones [365, 366]. Crowding has been shown to contribute to enhance the free energy of binding between two individual DNA strands or between a protein and a DNA interaction [340, 367]. Crowding thus influences the structural and functional integrity of nuclear compartments in vivo [368]. Moreover, it has been reported that the initiation of DNA replication depends on nucleus formation [369, 370], which emphasises the role of crowding for the operation of the replication machinery, as shown by in vitro experiments [360, 369].

1.10.8. Evaluation of Macromolecular Crowding on Protein Folding, Aggregation and Stability

Crowding achieves protein stability by compressing it from expanded states and enhancing associations of protein macromolecules [317, 325]. Crowding tends to compel binding of enzyme to the substrate so increased strength of binding could, in principle, stabilise both components to denaturation [371]. Macromolecular crowding stabilises the native state of a protein by destabilizing the unfolded state, thereby compensating for the energetically unfavorable “folded” conformation [372, 373]. Crowding also influences the rate of protein folding [374-378] and acts as a regulator of the thermal stability [379]. The thermal stability of the proteins found in the interior of an eye lens increases with concentration [380], and the exceptional heat stability of an intact lens has been attributed in part to the stabilizing effects of macromolecular crowding inside the lens cell [381]. The increased thermal stability of α-lactalbumin was indicated by a rise in melting temperature by 25°C – 30°C by
encapsulation in a silica matrix [382, 383], an example also of the ‘caging effect’ that crowding exerts on proteins [384]. Studies of the crowding effect on the refolding rates of proteins have been done on reduced lysozyme [385, 386], glucose-6-phosphate dehydrogenase [387], glyceraldehyde-3-phosphate dehydrogenase [388], protein disulfide isomerase (PDI) [387] and GroEL [389]. Apart from improvement of protein folding, macromolecular crowding also induces protein aggregation [317, 378]. Several experiments show that the oligomerisation of actin [390], spectrin [391], tubulin [392] and FtsZ-GDP [393] were augmented under crowded conditions, as well as the self-association of fibrinogen [392]. In the intracellular context, it was shown that the association of ribosomal particles was increased under crowded conditions [394]. The aggregation under macromolecular crowding is due to increase in the thermodynamic activity of partially folded polypeptide chains, and this effect is particularly pronounced for small as well as for slow-folding chains [325].

1.10.9. Evaluation of Macromolecular Crowding on Intra-cellular Trafficking and Cellular Homeostasis

The viscoelastic properties of a cell determine how it reacts to the environment [395]. Macromolecular Crowding contributes to the viscoelasticity of the cytoplasm and nucleoplasm which results in anomalous sub-diffusion of macrosolutes [395, 396] leading to slow apparent translational diffusion of molecules in the cytosol [397]. Crowding has also been associated with cell signal transduction pathways [398]. Crowding in cells greatly favors macromolecular associations involved in signal transmission, frequently increasing the binding strength by at least an order of magnitude [399]. The living cell depends entirely on the enzyme-driven metabolic reactions as suggested by Arthur Kornberg as one of the ten ‘commandments’ in enzymology [400]. The seventh ‘commandment’, according to Kornberg describes the crowded nature of the cell and is a reminder that all enzymatic reactions in vitro with cell free systems need to include this crowding element [400]. All the enzymes function at optimal conditions of salt concentration, pH and temperature. If any of these conditions are altered, the enzyme function is disrupted. Nevertheless, under crowded conditions the enzymatic activity is still maintained. For nick-translation and polymerizing functions of E. Coli DNA polymerases the enzyme activity was inhibited under high salt (KCl) concentration but under crowded conditions (Ficoll
the enzyme activity was still easily detectable [371]. This was explained to be due to the sustained binding of the polymerases to their template-primer complexes under Macromolecular Crowding conditions even in highly unfavorable conditions (in this case, the ionic strength) that are introduced, the enzymes remaining active and hence widening the range of conditions/environments in which the cell can remain viable. It has also been found that crowding effects resulting from changes in the amount of water seem to compensate for the effects of changes in cytoplasmic $\text{K}^+$ ions and contribute to maintenance of protein–nucleic-acid equilibria and kinetics in the range required for function in vivo [367].

1.10.10. Macromolecular Crowding Accelerate Procollagen Conversion

Macromolecular crowding has a potential biophysical role in collagen deposition in vitro. The principal of the approach is that when adequately provided with ascorbic acid, fibrogenic cells secrete substantial amounts of procollagens [290]. The deposition of a collagen matrix depends on the conversion of de novo synthesised procollagen to collagen in the crowded extracellular space or immediately before its release into the same [401]. However, before procollagen can participate in supramolecular assembly, it has to be proteolytically cleaved to collagen by procollagen C-proteinase (PCP), which in turn is regulated by an allosteric binding molecule, procollagen C-endopeptidase enhancer (PCOLCE). In vivo, the extracellular space is highly crowded and this step takes place very fast. However, it is still not widely known that the activity of PCP is low under current dilute, non-crowded conventional cell culture system. As a result, only small amounts of insoluble collagen matrix are formed, while most of the (water soluble) procollagen is lost during culture medium changes and in bioreactor outflows [328, 402].

This thesis research work proposes that the addition of inert polydispersed macromolecules (presented as spherical objects of variable diameter in (Figure 1.8c and 1.10) in the culture media will facilitate amplified deposition and subsequent production of insoluble ECM-rich living substitutes. This method was applied to human fibroblasts (lung and skin), tenocytes and osteoblasts and characterised the resulting matrices biochemically, biophysically, structurally and functionally. This approach can be readily implemented to different cell types to create tissue-specific ECM-rich substitutes for regenerative medicine applications. This technology can
also be used to develop *in vitro* pathophysiological models for drug discovery purposes. It therefore opens new avenues for studying *in vitro* cell-produced ECM assemblies and offers an opportunity for a more rational design in engineering cohesive tissue modules.
Table 1.1: In last 30 year of research using macromolecular crowding, most of the studies have been utilised this technology for various biophysical phenomena of proteins (stability, aggregation, folding and rate kinetics) and DNA (binding, conformational behaviour, Influence on PCR performance). Macromolecular crowding as a great tool for tissue engineering application, only few reports have been suggested its potential. Examples of macromolecular crowders used and their in vitro consequence [increased (↑) or decreased (↓)] in culture of various cells.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Macromolecular Crowder</th>
<th>Purpose</th>
<th>In vitro system</th>
<th>Consequence</th>
<th>Year/References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ribonuclease A, beta-lactoglobulin, BSA &amp; PEG 20kDa</td>
<td>Effect of MMC upon the structure &amp; function of GAPD</td>
<td>………………</td>
<td>Formation of tetramer ↑</td>
<td>1981 [373]</td>
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<tr>
<td>2</td>
<td>PEG 8kDa, dextran T-70, Ficoll® 70kDa, BSA</td>
<td>To evaluate the role of DNA polymerase binding to DNA</td>
<td>Escherichia coli</td>
<td>Non specific enzyme interaction↑ The enzymatic activity ↑</td>
<td>1987 [371]</td>
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<td>3</td>
<td>Dxs 500kDa</td>
<td>Effect on pro-collagen processing</td>
<td>Human skin fibroblasts</td>
<td>Complete proteolytic processing of procollagen to collagen</td>
<td>1990 [403]</td>
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<tr>
<td>4</td>
<td>Dextran 40 kDa</td>
<td>Assessment on ECM deposition</td>
<td>Human skin fibroblasts</td>
<td>Deposition of collagen type III ↑</td>
<td>1991 [404]</td>
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<td>5</td>
<td></td>
<td>Dextran sulphate</td>
<td>To characterize the biosynthesis &amp; matrix deposition in</td>
<td>Fibroblasts culture</td>
<td>Insoluble matrix deposition ↑</td>
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<td></td>
<td></td>
<td></td>
<td>Osteogenesis Imperfecta</td>
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<td>Relative amount of fibronectin ↑</td>
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<td></td>
<td>Ficoll® 70kDA, dextran 70kDa</td>
<td>The effect of MMC on chaperonin mediated protein folding</td>
<td>Escherichia coli</td>
<td>Complete chaperonin folding in the presence of excluded volume</td>
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<td>7</td>
<td></td>
<td>PEG 6kDa &amp; 35kDa</td>
<td>Effect of total enzyme concentrations on flux through phosphotransferase system (PTS)</td>
<td>Escherichia coli strain (PJ4004)</td>
<td>Enzyme complex dissociation rate constant ↓</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>Thus the enzyme complex stability ↑</td>
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<td>8</td>
<td></td>
<td>PEG 10kDa</td>
<td>To understand the conformational behaviour of a giant duplex-DNA</td>
<td>...............</td>
<td>Enhancement of unimolecular condensation of large linear DNA by PEG</td>
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<td></td>
<td></td>
<td></td>
<td>chain</td>
<td></td>
<td>The discrete folding transition of the DNA↑</td>
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<td></td>
<td>BSA, chicken albumin, Ficoll® 70kDa, dextran T70</td>
<td>To study the effect on protein folding &amp; aggregation</td>
<td>Micrococcus lysodeikticus cell</td>
<td>Protein refolding rates ↑</td>
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<td>Material</td>
<td>Effect on α-synuclein fibrillation</td>
<td>Formation of stable, compact, salt-induced molten globule state, transition temperature ↑, overall, protein stability &amp; conformation↑</td>
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<td>10</td>
<td>PEG 300Da</td>
<td>Protein structure &amp; protein stability</td>
<td>↑ in the hydrophobic interaction in the presence of excluded volume influence the folding equilibrium</td>
<td>2001 [382, 383]</td>
<td></td>
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<td>11</td>
<td>Dextran (138KDa), PEG (200, 400, 600 &amp; 3350 Da), Ficolls® (70kDa &amp; 400kDa)</td>
<td>Effect on α-synuclein fibrillation</td>
<td>α-synuclein fibrillation (6-fold with lysozyme; 5-fold with BSA)</td>
<td>2002 [410]</td>
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<td>12</td>
<td>Dextran (0-300 g/l)</td>
<td>Effect on lysozyme protein stability &amp; conformation</td>
<td>Formation of stable, compact, salt-induced molten globule state, transition temperature ↑, overall, protein stability &amp; conformation↑</td>
<td>2003 [411]</td>
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<td>13</td>
<td>Dextran sulphate (500kDa) &amp; neutral dextran (670 kDa)</td>
<td>To evaluate the deposition of collagen matrix</td>
<td>WI 38 lung fibroblasts</td>
<td>Collagen deposition ↑</td>
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<td>Interaction</td>
<td>Details</td>
<td>Year [Ref]</td>
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<td>14</td>
<td>Ficoll® 70 kDa &amp; Ficoll® 70kDa/400kDa</td>
<td>Influence on PCR performance</td>
<td>WI-38 derived RNA samples</td>
<td>Sensitivity (8-10 folds) ↑, Polymerase processivity ↑, Amplicon yield ↑, Primer annealing ↑, Specificity ↑, DNA polymerase thermal stability ↑</td>
<td>2007 [412]</td>
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<td>Dextran sulphate (500kDa, 10kDa), Polysodium -4-Styrene sulfonate (200kDa), Ficoll® (400 kDa &amp; 70kDa)</td>
<td>To evaluate the deposition of collagen matrix</td>
<td>WI 38</td>
<td>23-fold &amp; 36-fold collagen deposition ↑ after DxS (500 kDa) &amp; PSS (200 kDa)</td>
<td>2007 [402]</td>
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<td>16</td>
<td>PEG-3.5kDa &amp; Ficoll® 70 kDa</td>
<td>To evaluate the effect of crowing on fibrillation of proteins specific to conformational &amp; aggregation behavior of fibrillating proteins</td>
<td>…………..</td>
<td>Fibrillation of oligomeric proteins ↓, Fibrillation of monomeric natively unfolded Proteins ↑</td>
<td>2008 [413]</td>
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<td>No.</td>
<td>Compound/Composition</td>
<td>Experiment/Effect</td>
<td>Reference</td>
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<td>17</td>
<td>PEG 8kDa</td>
<td>To evaluate the encapsulation effect in Giant Lipid Vesicles</td>
<td>Encapsulation efficiency of poorly encapsulated macromolecules in fluorescently labeled polymers †</td>
<td>2008 [414]</td>
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<td>18</td>
<td>Ficoll® 70 kDa</td>
<td>To evaluate the influence of MMC on shape &amp; proteins folding dynamics</td>
<td>Borrelia burgdorferi VlsE model</td>
<td>Distinct conformational changes in VlsE proteins are accompanied by secondary structure changes</td>
<td>2008 [415]</td>
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<td>Dextran</td>
<td>Effect on the structure &amp; condensation of DNA molecule confined in nanochannel</td>
<td>DNA molecules progressively elongate &amp; condense into a compact structure</td>
<td>2009 [416]</td>
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<td>20</td>
<td>Dextran sulphate (500 kDa), Ficoll® (400 kDa &amp; 70kDa)</td>
<td>Development of novel in vitro model to assess the effect of antifibrotic agents</td>
<td>WI-38 cells</td>
<td>The in vitro fibrosis model was developed which can be used in fast, quantitative &amp; non-destructive way.</td>
<td>2009 [417]</td>
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<td>21</td>
<td>Ficoll® 70 kDa &amp; dextran 70kDa</td>
<td>To understand the role of a crowded physiological environment in the pathogenesis of neurodegenerative diseases</td>
<td>Escherichia coli</td>
<td>Accelerate the nucleation step of fibril formation of human Tau fragment/human prion protein/human α-synuclein (a significant decrease in the lag time)</td>
<td>2009 [418]</td>
</tr>
<tr>
<td>22</td>
<td>Ficoll® (70kDa &amp; 400kDa)</td>
<td>Effect on ECM deposition</td>
<td>10 fold increase in collagen type I deposition in 2D</td>
<td>2010 [419]</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Ficoll&lt;sup&gt;®&lt;/sup&gt; (400 kDa &amp; 70 kDa)</td>
<td>To evaluate the impact of induced crowding on extra- &amp; intra-cellular protein organization of human MSCs</td>
<td>MSCs derived ECM</td>
<td>Supramolecular assembly ↑, Alignment of ECM ↑ alignment of the intracellular actin cytoskeleton ↑, MMC induced deposited matrix promote adhesion, proliferation &amp; migration of MSCs</td>
<td>2012 [420]</td>
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<tr>
<td>24</td>
<td>Dextran sulphate (500kDa) &amp; Ficoll&lt;sup&gt;®&lt;/sup&gt; (400 kDa &amp; 70 kDa)</td>
<td>Stable pluripotent feeder-free propagation of human embryonic stem cells (hESCs)</td>
<td>WI 38 derived ECM</td>
<td>ECMs deposited by fibroblast under MMC build suitable microenvironment for stable hESC propagation</td>
<td>2012 [421]</td>
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<tr>
<td>25</td>
<td>Ficoll&lt;sup&gt;®&lt;/sup&gt; (70kDa &amp; 400kDa)</td>
<td>Effect on ECM deposition (2D Vs 3D)</td>
<td>Pig chondrocytes</td>
<td>Collagen type II deposition ↑</td>
<td>2013 [422]</td>
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<tr>
<td>26</td>
<td>Ficoll&lt;sup&gt;®&lt;/sup&gt; 400 kDa</td>
<td>To evaluate the rate of collagen I nucleation, fiber growth &amp; proliferation of MSCs</td>
<td>MSCs</td>
<td>Gel pore size, protein permeability, transparency &amp; resistance to enzymatic degradation can be controlled, Mechanical stability of MMC induced gel ↑, Proliferation of MSC culture ↑</td>
<td>2014 [423]</td>
</tr>
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<td></td>
<td>Polyvinylpyrrolidone (PVP)</td>
<td>Use of PVP as a novel crowder molecules to assess the cell behavior</td>
<td>Bone marrow-derived MSCs, Dermal fibroblasts</td>
<td>Cell proliferation ↑, ECM deposition ↑</td>
<td>2014 [424]</td>
</tr>
</tbody>
</table>
1.11. Project Rationale and Hypothesis

The cellular and the extracellular milieu is a crowded with macromolecules of various sizes and shapes environment [351, 352]. These macromolecules greatly affect the folding process of proteins [353-356]. To better understand the biological consequences of crowding, numerous theoretical studies have been carried out over the years [341, 345-350] and various crowding molecules have been proposed. However, only a handful of these agents have been assessed to-date in human cell culture and no rational has been provided.

Herein, this PhD work hypothesise that modulation of the in vitro microenvironment with polydispersed macromolecules will enhance the deposition of extracellular matrix proteins, ultimately leading to the faster construction of extracellular matrix-rich tissue equivalents.
1.12. Aims and Objectives

**Aim 1:** Identification of optimal culture period, serum origin and serum concentration for maximum ECM deposition in human skin and lung fibroblast culture.

**Objectives**
1. Evaluation of macromolecular crowding on ECM deposition from human skin fibroblasts (WS-1) and lung fibroblasts (WI-38) as a function of culture time (2, 4 and 6 days);
2. Evaluation of macromolecular crowding on ECM deposition from human skin fibroblasts (WS-1) and lung fibroblasts (WI-38) as a function of FBS and HS concentration (0.0, 0.5, 1.0, 2.0, 5.0 and 10.0%).

**Aim 2:** Identification of optimal macromolecular crowder for maximum ECM deposition in human skin fibroblast culture.

**Objectives**
1. Evaluation of size and dispersity of various sulphated (e.g. Dxs, CR) and non-sulphated (e.g. FC® 70 and 400) macromolecular crowders;
2. Evaluation of ECM deposition from human skin fibroblasts (WS-1) using various sulphated (e.g. Dxs, CR) and non-sulphated (e.g. FC® 70 and 400) macromolecular crowders.

**Aim 3:** Production and characterisation of ECM-rich human skin fibroblast-sheets.

**Objectives**
1. Evaluation of attachment and detachment of ECM-rich cell-sheets;
2. Evaluation of changes in the proteome under macromolecular crowding condition.

**Aim 4:** Assessment of the influence macromolecular crowding in human osteoblast and tenocyte culture.

**Objectives**
1. Evaluation of macromolecular crowding on ECM deposition from human osteoblast and tenocytes as a function of culture time (2, 4 and 6 days);
2. Evaluation of macromolecular crowding on ECM deposition from human osteoblast and tenocyte as a function of FBS concentration (0.0, 0.5, 5.0 and 10.0%).
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Chapter 2 – Modulation of In Vitro Microenvironment
Using Macromolecular Crowding

Parts of this chapter have been published at:

2.1. Introduction

Advancements in molecular and cell biology have led to the development of cell-based therapies to treat injured or degenerated tissues [1-4]. The rationale of this concept is that functional regeneration can be achieved best by using the innate capacity of cells to create their own tissue-specific extracellular matrix (ECM) avoiding the shortfalls of man-made devices. Although direct cell injections have demonstrated very promising preclinical and clinical outcomes [5-7], the mode of administration offers little control over local retention and distribution of the injected cell suspensions [8, 9] leading to scattered therapeutic efficiency. This deficiency has led to the development of living substitutes for skin [10-14] and blood vessel [15] composed of cells seeded on a collagen scaffold. Notwithstanding the efficacious results in preclinical models and clinical trials, it soon became apparent that the presence of the scaffold hinders tissue remodelling and function [16-20]. These drawbacks led to the development of the scaffold-free cell-sheet tissue engineering (CSTE) [21, 22] or tissue engineering by self-assembly (TESA) [23], a therapy that offers the fabrication of a contiguous cell sheet that is stabilised by cell-cell contacts and endogenously produced ECM. Despite the documented, in preclinical and clinical setting, positive outcomes for skin [24-26], blood vessel [27-31], cornea [32, 33], heart [34-36], lung [37], liver [38], and bone [39] replacement, only Epicel® (Genzyme, USA) for skin and LifeLine™ for blood vessel (Cytograft, USA) have been commercialised so far. This limited technology transfer from bench-top to clinic has been attributed to the substantial long period of time required for ex vivo culture (e.g. 14-35 days for corneal epithelium [33]; 84 days for corneal stromal [40, 41]; 28 days for corneal endothelium [42]; 70 days for lung cell-sheet [37]; and 196 days for blood vessel [28] that often leads to cell senescence and loss of native phenotype [43, 44].

A biophysical approach is proposed here, termed macromolecular crowding (MMC) that increases thermodynamic activities and biological processes by several orders of magnitude [45-57], as means to create ECM-rich tissue equivalents (e.g. binding of DNA polymerase to DNA [56, 57], RT-PCR performance [48], regulation of cellular volume [50, 57], protein folding [53], aggregation [51, 52], nucleation [55] and collagen deposition [46, 47, 49]). The principle of MMC is derived from the notion
that in vivo cells reside in a highly crowded/dense extracellular space and therefore the conversion of the de novo synthesised procollagen to collagen I is rapid [58]. However, in the even substantially more dilute than body fluids (e.g. urine: 36-50g/l; blood: 80g/l) culture conditions (e.g. HAM F10 nutrient medium: 16.55g/l; DMEM/F12 medium: 16.78g/l; DMEM high glucose and L-glutamine medium: 17.22g/l), the rate limiting conversion of procollagen to collagen I is very slow (Figure 2.1a). It was proposed that the addition of inert polydispersed macromolecules (presented as spherical objects of variable diameter in Figure 2.1b) in the culture media will facilitate amplified production of ECM-rich living substitutes.
Figure 2.1: Modulation of the in vitro microenvironment using MMC to imitate the in vivo dense extracellular space. (a) The deposition of ECM in the current, notably dilute and far from physiological, culturing systems is very slow. In the case of collagen type I, the most abundant ECM protein, cells will produce procollagen and the specific N- and C- proteinases. The enzymes procollagen C- and N-proteinases specifically cleave carboxyl- and amino-terminal propeptides of procollagens. However, in the very dilute in vitro microenvironment, the conversion of the water-soluble procollagen to insoluble collagen will be very slow since either the proteinases will be deactivated before they cleave the specific N- and C- propeptides or the procollagen will be dissolved before its conversion to collagen. Only after prolonged culture time, the cells will self-crowd the media and collagen deposition will be achieved. (b) Here, it was hypothesise that the addition of inert polydispersed macromolecules, presented as spheres with different diameters, in the culture media will create most effective volume occupancy, and will consequently increase the relative density of procollagen and proteinases in the culture media. This will facilitate the cleavage of the propeptides by their respectfull proteinases and will lead to the fast procollagen conversion to collagen and deposition of the former.
2.2. Materials and Methods

2.2.1. Cell Culture

Human lung fibroblasts (WI-38; ATCC, LGC Standards, UK), human skin fibroblasts (WS-1; ATCC, LGC Standards, UK) and human tenocytes (DV Biologics, USA) were cultured in Dulbecco’s modified Eagle medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO₂. Human osteoblasts (Lonza, UK) were maintained in low-glucose Dulbecco’s modified Eagle medium and same conditions as above. Cells were seeded at 25,000 cells/cm² in 24-well plates and were allowed to attach for 24 hours. After 24 hours the medium was changed with medium containing macromolecular crowders (100 µg/ml Dxs 500 kDa; 37.5 mg/ml Ficoll™ 70 and 25 mg/ml Ficoll™ 400; and 75 µg/ml carrageenan (CR) with various percentages of FBS (0%, 0.5%, 1%, 2%, 5%, 10% for skin and lung fibroblasts and 0%, 0.5%, 5%, 10% for osteoblasts and tenocytes). To induce collagen synthesis fibroblasts, osteoblasts and tenocytes were supplemented with 100 µM L-ascorbic acid phosphate. After optimisation of culture conditions, commercially available human serum was used as supplement to replace FBS. Experiments were performed in quadruplicate (n=4) (Figure 2.2).

![Figure 2.2: All the cell culture experiments were performed in quadruplicate (n=4) in 24 well tissue culture plates.](image-url)
2.2.2. Phase ContrastMicroscopy

The influence of various crowders and serum (FBS and HS) percentages on cell morphology was evaluated using phase-contrast microscopy at day 2, 4 and 6. Images of the cells were taken using an inverted microscope (Leica microsystem, Germany) and images were analysed with the LAS EZ 2.0.0 software.

2.2.3. Cell Metabolic Activity (alamarBlue®)

Principle: AlamarBlue® assay was performed to quantify the influence of various crowders and serum present on metabolic activity of the fibroblasts, osteoblasts and tenocytes. When added to cell cultures, the oxidized form of the AlamarBlue® enters the cytosol and is converted to the reduced form by mitochondrial enzyme activity by accepting electrons from NADPH, FADH, FMNH, NADH as well as from the cytochromes. When cells are alive they maintain a reducing environment within the cytosol of the cell. Resazurin, the active ingredient of alamarBlue® reagent, is a nontoxic, cell permeable compound that is blue in color and virtually non-fluorescent. Upon entering cells, resazurin is reduced to resorufin, a compound that is red in color and highly fluorescent. Viable cells continuously convert resazurin to resorufin, increasing the overall fluorescence and color of the media surrounding cells. This redox reaction can be easily measured by colourimetric or fluorometric reading. Briefly, at the end of culture time points, cells were washed with Hanks’ Balanced Salt solution (HBSS) and then diluted alamarBlue® was added directly to each well and the plates were incubated at 37° C to allow cells to convert resazurin to resorufin. After 4 hours of incubation at 37° C, absorbance signal was measured at 570 nm, using 600 nm as a reference wavelength (normalised to the 600 nm values) with help of Varioskan Flash spectral scanning multimode reader (Thermo Scientific). Cell metabolic activity was measured from 3 different wells (n=3) for all the samples according to the supplier’s protocol and expressed in terms of percentage reduction of alamarBlue® dye.

2.2.4. Cell Viability (Live/Dead® assay)

Principle: Live/Dead® assay was used to measure the influence of various crowders, serum present and thermal responsive polymers on cell viability, as per manufacturer
protocol. Ubiquitous intracellular esterase activity and an intact plasma membrane are distinguishing characteristics of live cells. The LIVE/DEAD® Viability/Cytotoxicity Kit quickly discriminates live from dead cells by simultaneously staining with green-fluorescent calcein-AM to indicate intracellular esterase activity and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity. It is adaptable to most eukaryotic cells where cytotoxic conditions produce these cellular effects. The assay is useful with a variety of fluorescence-detection methodologies.

- The Live cell dye labels intact, viable cells green. It is membrane permeant and non-fluorescent until ubiquitous intracellular esterases remove ester groups and render the molecule fluorescent. The Excitation (max) and Emission (max) are 494 nm and 515 nm, respectively (similar to FITC).
- The Dead cell dye labels cells with compromised plasma membranes red. It is membrane-impermeant and binds to DNA with high affinity. Once bound to DNA, the fluorescence increases >30-fold. The Excitation (max) and Emission (max) are 528nm and 617nm, respectively.

Briefly, at the end of culture time points, cells were washed with HBSS and then Live/Dead® staining solution was added (2μmol/L calcein-AM-green and 0.5μmol/L ethidium homodimer-1) in a pH-adjusted buffer. The cells were allowed to incubate for 30 minutes at 37°C and 5% CO₂. Fluorescence images were captured from 3 different wells (n=3) for all the samples with an Olympus IX-81 inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan).

2.2.5. Collagen Extraction and Pepsin Digestion

The telopeptides of collagens are susceptible to proteolytic attack, whereas the intact triple-helical domain is generally resistant to most proteolytic enzymes. The majority of covalent cross-links stabilizing the fibrillar collagens involve the telopeptides and, consequently, pepsin treatment of insoluble cross-linked fibers tends to release the triplehelical domain, which can be recovered in its native conformation. The most widely used approach is pepsin digestion at pH 1–2 of tissues at 4°C to 15°C, which relies on the resistance of the collagen triple helix to enzymic degradation below its denaturation temperature, but the susceptibility of virtually all other proteins to
digestion. The collagen triple helix is unique in being both resistant to pepsin and soluble at acid pH, so that the end product of digestion is a mixture of solubilized collagen types. The identification of the collagen types is initially achieved through their characteristic mobilities on SDS-polyacrylamide gel electrophoresis.

At the end of culture time points, culture media were collected into separate vials, whereas cell layers were washed twice with HBSS. Washed cell layer were digested with porcine gastric mucosa pepsin in a final concentration of 1 mg/ml in 0.5M acetic acid. Samples were incubated at 37°C for 2 hours with gentle shaking followed by neutralization with 0.1N sodium hydroxide.

2.2.6. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

**Principle:** SDS-PAGE systems are used to resolve and characterise the number and size of protein chains or protein subunit chains in a protein preparation. The detergent SDS binds to all regions of proteins and unravels all intra-molecular protein associations. This results in total disruption of proteins and total disruption of associated subunit organisation and then yields SDS-carrying, highly-anionic polypeptide chains. The anionic denatured protein chains are then resolved electrophoretically in a buffered environment that contains SDS and a high concentration of polyacrylamide gel. The SDS maintains the denatured state of the disrupted proteins or protein subunits. The SDS bound to the proteins generates a constant anionic charge-to-mass (friction) ratio for all the unravelled protein chains, while the high concentration gel generates molecular sieving, in which the viscosity and pore size of the gel define mobility. As a result, the relative mobility of each of the anionic, denatured polypeptide chains is a log function of the molecular weight of the polypeptide chain. SDS-gel systems are a simple and useful qualitative and quantitative gel-electrophoresis tool.

Cell layer samples [from 3 different wells (n=3) for each samples] were analysed by SDS-PAGE under non-reducing conditions with Mini-Protean 3 (Bio-Rad Laboratories, UK). For loading control, the total number cell (25,000 cell/ cm²) at the beginning of cell culture was constant in all the experiment. 100-500 µg/ml of bovine collagen type I was used as standard control with every gel. Protein bands were stained with the SilverQuest® kit according to the manufacturer’s protocol. The sensitivity of silver staining kit was evaluated using various concentration of collagen
type I standard (Figure 2.3). Densitometric analysis of gels was performed using GeneTools® or Image J analysis software. Collagen bands were quantified by defining each band with the rectangular tool with background subtraction (please see the detail steps in section 4.2.11). Scanned gel images for densitometric analysis were normalised with collagen type I standard in each gel.

![Collagen bands](image.png)

**Figure 2.3**: Evaluation of sensitivity of silver staining kit using various concentration of collagen type I standard. Collagen type I consists of alpha subunits [two α1(I) chains and one α2(I)] and cross-linked beta subunits (β1 1 and β1 2).

### 2.2.7. Immunocytochemistry

**Principle**: Immunocytochemistry (ICC) or immunofluorescence (IF) is a method used to assess the presence of a specific protein or antigen in cells. This technique uses a specific antibody, which binds to the protein of interest, thereby allowing visualization and examination under a microscope. The primary antibody allows visualization of the protein under a fluorescence microscope when it is bound by a secondary antibody that has a conjugated fluorochrome. It is a valuable tool for the determination of intra-cellular or extra-cellular contents from individual cells. Samples that can be analyzed include blood smears, aspirates, swabs, cultured cells and cell suspensions.
Chapter 2

Cells were seeded on 4 or 8-well Lab-Tek™ II chamber slides at 25,000 cells/cm² and after 24 hours of seeding, cells were treated with crowders. At the end of cell culture time points, medium was removed and cell layers were washed with HBSS and fixed with 2% paraformaldehyde (PFA) at room temperature for 15 minutes. After several washes in phosphate-buffered saline (PBS), nonspecific sites were blocked with 3% bovine serum albumin (BSA) in PBS for 30 minutes. The cells were incubated for 90 minutes at room temperature or overnight at 4° C simultaneously with the primary antibody of collagen type I, III, IV, V, VI, VII, laminin, elastin, fibronectin, α-smooth muscle actin, tenomodulin, epithelial keratin, tubulin, hyaluronic acid, chondroitin sulphate, keratin sulphate, heparin sulphate, aggrecan, biglycan, decorin, Endosialin / Tumor endothelial marker 1 (TEM-1), lysyl oxidase, transglutaminase-2, fibrillin, and interleukin-10. Bound antibodies were visualised using AlexaFluor®488 chicken anti-rabbit and AlexaFluor®555 goat anti-mouse 1:400 in PBS for 30 minutes. Post-fixation was done with 2% PFA for 15 minutes. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and slides were mounted with Vectashield® mounting media. For dilution factors, please see table 4.4 and 4.5. Images were captured and fluorescence intensity measurements from 3 different wells (n=3) were performed with an Olympus IX-81 inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan).

2.2.8. Gelatin Zymography

Principle: Zymography is a technique to assess the enzymatic activity of proteins either in situ or by separating them with electrophoresis. The enzyme converts the substrate into a product which is detected by different staining methods. One of the most popular techniques is by separating the protein mixture by Polyacrylamide gel electrophoresis in which a substrate is incorporated within the polyacrylamide gels. These protein substrates present in the gel are degraded by the proteases present in the sample which are activated during the incubation period. Staining with Coomassie blue shows the proteolytically cleaved sites as white clear bands on a dark blue background. Necessary precautions are taken to prevent the enzyme from denaturation during the process. Hence SDS is generally avoided. But in gelatinase zymography, SDS is used to activate the gelatinases. Gelatin Zymography is used to
detect gelatinase activity, specially MMP-2 and -9. MMP-2 and -9 remain inactive while they are with their pro-domains. They need proteolytic processing or denaturation to get activated. SDS in SDS-PAGE can do that activation by denaturing the MMP_2 and -9. MMP-2 (gelatinase A, 72 kD) and MMP-9 (gelatinase B, 92 kD) can be detected on gelatin zymograms as two-three white bands (pro and active forms) after staining with Coomassie Blue staining. Zymography was performed to analyse expression of matrix metalloproteinase-2 (MMP-2). At the end of cell culture time points, culture media [from 3 different wells (n=3) for all the samples] were collected and separated on 10% SDS-PAGE gels containing 1 mg/ml gelatin. After electrophoresis, SDS was removed from the gels by two incubations in 2.5% Triton X-100 for 30 minutes. Gels were incubated overnight at 37° C in 2.5% Triton X-100, 50mM Tris (pH 7.4), 5mM CaCl₂, 1 μM ZnCl₂. Gels were then stained for 20 minutes with Coomassie Brilliant Blue and destained for 2 hours. Uncultured medium with various percentages of FBS and HS were used as controls.

2.2.9. Dynamic Light Scattering (DLS) Measurements

**Principle:** Dynamic Light Scattering measures the size of particles typically in the sub micron region. Particles suspended within a liquid undergo Brownian motion. The larger the particle, the slower the Brownian motion will be. DLS monitors the Brownian motion with light scattering. It is used to characterize size of various particles including proteins, polymers, micelles, carbohydrates, and nanoparticles. If the system is monodisperse, the mean effective diameter of the particles can be determined. This measurement depends on the size of the particle core, the size of surface structures, particle concentration, and the type of ions in the medium.
DLS measurements of macromolecules were carried out at 25° C using Zetasizer Nano ZS90 (Malvern Instruments, UK). Molecules were dissolved [in triplicate (n=3) for all the samples] in double distilled water for size measurements of macromolecular crowders [Z-Ave; d.nm (average hydrodynamic diameter in nanometer)]. The results were analyzed using the Zetasizer software 6.12 (Malvern Instruments, UK).

2.2.10. Nanoparticle Tracking Analysis (NTA)

**Principle:** Nanoparticle Tracking Analysis (NTA) utilizes the properties of both light scattering and Brownian motion in order to obtain the particle size distribution of samples in liquid suspension. NTA utilizes the properties of both light scattering and Brownian motion in order to obtain the particle size distribution of samples in liquid suspension. A laser beam is passed through the sample chamber, and the particles in suspension in the path of this beam scatter light in such a manner that they can easily be visualized via a 20x magnification microscope onto which is mounted a camera. The camera which operates at approximately 30 frames per second (fps) captures a video file of the particles moving under Brownian motion within the field of view of approximately 100 µm x 80 µm x 10 µm.

NTA measurements were performed using NanoSight LM10 (NanoSight, Amesbury, UK) equipped with a sample chamber with a 405nm blue laser. The samples were injected in sample chamber with sterile syringes until the liquid reached the tip of the
nozzle. All measurements were performed at room temperature (26.3°C – 26.5°C). The software used for capturing and analysing the data was the NTA 2.0 Build 127. The samples were measured for 196 - 215s with manual shutter and gain adjustments. The 'single shutter and gain mode' was used to capture the dispersity of the samples based on their viscosity in mm²/sec (Centistokes). Three measurements of the same sample were performed for all samples. The mean size and SD values obtained by the NTA software correspond to the arithmetic values calculated with the hydrodynamic sizes of all the particles analysed by the software.

**Note:** NTA measurement was done by Dr. Héctor Peinado and Prof. David Lyden at Children's Cancer and Blood Foundation Laboratories, Departments of Pediatrics, Cell and Developmental Biology, Weill Cornell Medical College, New York, USA.

### 2.2.11. Total Protein Extraction

Human skin fibroblasts were cultured in 24-well plates (25,000 cells/cm²). After 24 hours the medium was changed with medium containing macromolecular crowder (75 µg/ml CR), 0.5% HS and 100 µM L-ascorbic acid phosphate. After 2 days the media were collected and cell layer were washed three times with ice cold PBS. Total protein extraction from cell layer [pooled 4 different wells] was carried out using Qproteome™ mammalian protein preparation kit (Qiagen, UK). Briefly, cell layers were gently scraped using ice cold PBS and transferred in to pre-chilled 1.5ml tubes (Protein LoBind Tubes, Eppendorf, UK). The cell suspension was centrifuged for 5 minutes at 450g and 4°C. The supernatant was discarded and pellet was re-suspended in mammalian cell lysis buffer containing Benzonase® nuclease and protease inhibitor, followed by incubation in a rotary shaker for 5 minutes at 4°C. The suspension was centrifuged for 10 minutes at 14,000g and 4°C. The supernatant was transferred into new pre-chilled 1.5 ml tubes (Protein LoBind Tubes).

### 2.2.12. SDS-PAGE and Gel Band Excision for Proteomic Analysis

Total protein from cell layer and medium samples were separated on 4-12% Bis-Tris Gels (NuPAGE®Novex®, 1.0-mm thick, 10-well) under reducing conditions (NuPAGE® reducing agent) at 70°C for 10 minutes. After reducing the samples, iodoacetamide was added to final concentration of 2mM and incubated for 30
minutes at room temperature in the dark. Gels were run in 1X NuPAGE® MOPS 3-(N-morpholino) propanesulfonic acid SDS running buffer containing NuPAGE® antioxidant in the upper buffer chambers of XCell SureLock™ Mini-Cell electrophoresis system (Invitrogen, UK). 5 µl of precision plus™ protein standards was used as a molecular weight marker with the range of 250kDa to 10kDa. Protein bands were stained with the Coomassie brilliant blue and destained with destaining solution (40% ethanol, 10% acetic acid and 50% deionised water). Band excision [from 3 different SDS-PAGE gels lanes (n=3)] was done in a laminar flow cabinet with the help of light box. Gel bands were excised with scalpel and cut each slice into 1mm cubes and transfer into protein LoBind tubes.

2.2.13. Trypsin Digestion

For peptide extraction from SDS-PAGE gel lanes (for mass spectrometric analysis), all the gel lanes were excised and cut into twenty equal portions and digested with trypsin. Briefly the gel bands were destained, reduced with DTT and then alkylated with iodoacetamide. Gel bands were digested with trypsin for 16 hours at 37° C. Peptides were extracted with sequential washes of acidified aqueous acetonitrile and concentrated in a vacuum centrifuge and then purified. Samples were pooled for each group (with CR and without CR) mass spectrometric analysis.

2.2.14. Mass Spectrometry

**Principle:** Mass spectrometry is a powerful analytical technique used to quantify known materials, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of different molecules. The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances. This technique basically studies the effect of ionizing energy on molecules. It depends upon chemical reactions in the gas phase in which sample molecules are consumed during the formation of ionic and neutral species. A mass spectrometer generates multiple ions from the sample under investigation; it then separates them according to their specific mass-to-charge ratio (m/z), and then records the relative abundance of each ion type. The first step in the mass
spectrometric analysis of compounds is the production of gas phase ions of the compound, basically by electron ionization. This molecular ion undergoes fragmentation. Each primary product ion derived from the molecular ion, in turn, undergoes fragmentation, and so on. The ions are separated in the mass spectrometer according to their mass-to-charge ratio, and are detected in proportion to their abundance. A mass spectrum of the molecule is thus produced. It displays the result in the form of a plot of ion abundance versus mass-to-charge ratio. Ions provide information concerning the nature and the structure of their precursor molecule. In the spectrum of a pure compound, the molecular ion, if present, appears at the highest value of m/z (followed by ions containing heavier isotopes) and gives the molecular mass of the compound.

Mass Spectrometric Proteomics analysis was performed to evaluate proteins under crowding and non crowding conditions. Samples were run on a LTQ XL Orbitrap mass spectrometer (Thermo Scientific, UK) coupled to an Ultimate 3000 RSLC nano HPLC system (Dionex, UK). The system was run in direct injection using a home-packed 15 cm long by 75 μm internal diameter picotip emitter (New Objective, UK) containing ReproSil-Pur C18-Aq, 3 μm bead (Dr. Maisch GmbH HPLC, Germany). Samples were analysed on a 120 minutes gradient with the mass spectrometer operated in a data dependent acquisition mode in which the top 5 2+, 3+ and 4+ ions was selected for fragmentation.

Note: Mass Spectrometry was done by Dr. Benjamin Thomas at Sir William Dunn Pathology School, Oxford University, Oxford, UK.

2.2.15. Proteomics Data Analysis

All data were analysed in the Central Proteomics Facilities Pipeline (CPFP) [59]. Briefly, data were merged from all gel bands in a sample and were searched using Mascot, X!tandem and OMSSA. Results from the search engines were merged and the False Discovery Rate (FDR) calculated. Protein abundance fold changes were estimated using Spectral Index Normalised Quantitation (SINQ) label-free quantitation.

Note: Mass Spectrometric Proteomics data was generated by Dr. Benjamin Thomas (Sir William Dunn Pathology School, Oxford University, Oxford, UK) and analysed by Abhigyan Satyam.
2.2.16. Proteomic Validation by Immunocytochemistry

To validate proteomics results, proteins of interest were analysed and validated by fluorescence intensity measurements of immunocytochemistry images using protocol described in section 4.2.14.2.

2.2.17. Preparation of Cell-Sheets using Thermo-Responsive Culture Surfaces

pNIPAAm and pNTBA were added in anhydrous ethanol in the ratio of 40 $\mu$g/ml and 20 $\mu$g/ml and left for continuous shaking overnight. Petri dishes were subsequently coated with the appropriate amount of polymer in a desiccator. The dishes were further dried in vacuum oven at 60°C overnight. Fibroblasts were seeded at 25,000 cells/cm$^2$ after drying and mild UV sterilization of the petri dishes for 2 hours. After 24 hours of seeding, cells were treated with media containing macromolecular crowder. After 2 days of culture, the fibroblast sheets were harvested by decreasing the temperature of culture to 20°C for 30 minutes. The efficiency of poly(NIPAAM-co-NTBA) copolymer to produce intact ECM-rich cell sheets is attributed to the decreased number of N – H residues, due to the additional steric hindrance induced by the addition of the NTBA group, which decreased hydrogen bonding and consequently decreased protein adsorption, making cell detachment easier.

2.2.18. Scanning Electron Microscopy (SEM)

A normal scanning electron microscope operates at a high vacuum. The basic principle is that a beam of electrons is generated by a suitable source, typically a tungsten filament or a field emission gun. The electron beam is accelerated through a high voltage (e.g.: 20 kV) and pass through a system of apertures and electromagnetic lenses to produce a thin beam of electrons., then the beam scans the surface of the specimen by means of scan coils (like the spot in a cathode-ray tube "old-style" television). Electrons are emitted from the specimen by the action of the scanning beam and collected by a suitably-positioned detector.

After 2 days of culture, cell-sheets were washed with HBSS and fixed with 2% paraformaldehyde at room temperature for 15 minutes. Subsequently, the cell-sheets
were washed three times with PBS and serially dehydrated with 30%, 50%, 70%, 90% and 100% ethanol. The dehydrated cell-sheets were placed on adhesive carbon tabs mounted on SEM specimen stubs and then were dried. The specimens were subsequently coated with gold using an Emitech K550 coating system. SEM images [from 3 different slides (n=3)] were obtained using a Hitachi S-4700 field emission microscope operating with a beam voltage of 15kV.

2.2.19. Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) is a technique to obtain images and other information from a wide variety of samples, at extremely high (nanometer) resolution. AFM works by scanning a very sharp (end radius ca. 10 nm) probe along the sample surface, carefully maintaining the force between the probe and surface at a set, low level. Usually, the probe is formed by a silicon or silicon nitride cantilever with a sharp integrated tip, and the vertical bending (deflection) of the cantilever due to forces acting on the tip is detected by a laser focussed on the back of the cantilever. The laser is reflected by the cantilever onto a distant photodetector. The movement of the laser spot on the photodetector gives a greatly exaggerated measurement of the movement of the probe. This set-up is known as an optical lever. The probe is moved over the sample by a scanner, typically a piezoelectric element, which can make extremely precise movements. The combination of the sharp tip, the very sensitive optical lever, and the highly precise movements by the scanner, combined with the careful control of probe-sample forces allow the extremely high resolution of AFM.

WS-1 fibroblasts were seeded on 4-well Lab-Tek™ II chamber slides at 25,000 cells/cm² and after 24 hours of seeding, cells were treated with crowders. After 2 days of culture, medium was removed and cell layers were washed with HBSS and fixed with 2% paraformaldehyde at room temperature for 15 minutes. The cell layers were washed three times with phosphate-buffered saline (PBS) and serially dehydrated with 30%, 50%, 70%, 90% and 100% ethanol. AFM was performed on a commercial microscope (MFP-3D, Asylum Research) using triangular cantilevers (DNP C, Bruker) each having a nominal resonance frequency of 56 KHz and a spring constant of 0.24 N/m. Images were recorded using contact mode in an ambient environment after drying the sample with nitrogen gas. Images recorded
using amplitude modulation mode yielded similar results, as did images recorded in PBS on samples, which remained fully hydrated. All images were first-order XY plane-fit flattened (Igor Pro, Wavemetrics) except for Figure 25f, 26c, which were also zero-order line-by-line flattened along the fast scan direction (X).

### 2.2.20. Hematoxylin and Eosin (H & E) Staining

Cell-sheet was stained with Hematoxylin for 10 minutes and then rinse 3 times in tap water. It was differentiated with 0.3% acid alcohol to removes non-specific Hematoxylin staining. After rinsing in scott’s tap water the cell-sheet were stained with eosin and that was followed dehydration through a series of ethanol baths (70%-80%-90%-100%). The final dehydration was done in xylene for least 5 minutes and the slide was mounted using DPX. Images [from 3 different slides (n=3) for all the samples] were captured with an Olympus IX-81 inverted fluorescence microscope.

### 2.2.21. Masson’s Trichrome Staining

Masson's trichrome is a three-colour staining protocol for the detection of collagen fibers in tissues. The collagen fibers will be stained blue and the nuclei will be stained black and the background is stained red.

Cell-sheets were fixed in Bouin's solution for 1 hour at 56° C. After rinsing running tap water it was stained in Weigert's iron Hematoxylin working solution for 10 minutes and then stained in biebrich scarlet-acid fuchsin solution. Cell-sheets were differentiated in phosphomolybdic-phosphotungstic acid solution and then directly (without rinse) transferred to aniline blue solution. After brief rinse in distilled water, it was differentiated in 1% acetic acid solution. Washing in distilled water followed by dehydration through a series of ethanol baths (70%, 80%, 90%, and 100%). The final dehydration step was carried out in xylene for least 5 minutes and the slides were mounted using DPX. Images were captured [from 3 different slides (n=3) for all the samples] with an Olympus IX-81 inverted microscope.
2.2.22. Picro-Sirius Red Staining

The Picro-Sirius Red staining is for visualization of collagen I and III fibers in addition to muscle in tissue sections. This staining may be viewed using standard light microscopy or polarized light resulting in birefringence of the collagen fibers to distinguish between type I and type III.

Staining Interpretation

**Light Microscopy:** Collagen: Red; Muscle Fibers: Yellow; Cytoplasm: Yellow

**Polarized Light Microscopy:** Type I (Thick fibers): Yellow-Orange Birefringence; Type III (Thin fibers): Green Birefringence

Cell-sheets were stained with Weigert’s Hematoxylin for 8 minutes and then rinsing in tap water it was stained with 0.2% phosphomolybdic acid hydrate. After rinsing in distilled water, the cell-sheets were stained with Picro-Sirius Red followed by wash in acidified water. Dehydration was done through a series of ethanol baths (70%, 80%, 90%, and 100%). The final dehydration step was carried out in xylene for least 5 minutes and the slides were mounted using DPX. Images were captured [from 3 different slides (n=3) for all the samples] with an Olympus IX-81 inverted microscope.

2.2.23. Statistical Analyses

Numerical data is expressed as mean ± SD. Analysis was performed using statistical software (MINITAB™ version 16, Minitab Inc., USA). One way analysis of variance (ANOVA) for multiple comparisons (with Tukey or Bonferroni post-hoc tests) and 2-sample t-test for pair wise comparisons were employed after confirming the following assumptions: (a) the distribution from which each of the samples was derived was normal (Anderson-Darling normality test); and (b) the variances of the population of the samples were equal to one another (Bartlett’s and Levene’s tests for homogeneity of variance). Non-parametric statistics were used when either or both of the above assumptions were violated and consequently Kruskal-Wallis test for multiple comparisons or Mann-Whitney test for 2-samples were carried out. Statistical significance was accepted at $p<0.05$. Experiments were performed in triplicate or quadruplicate.
2.3. Results

2.3.1. Identification of Optimal Culture Period, Serum Origin and Serum Concentration for Maximum ECM Deposition

To acquire maximum ECM deposition under MMC conditions from human fibroblasts; optimal culture period, serum origin and serum concentration were assessed. SDS-PAGE (Figure 2.5 a) and complementary densitometric analysis (Figure 2.5 b) revealed that the maximum (p<0.0001) collagen type I deposition was achieved after culturing WI38 human lung fibroblasts for 2 days at 0.5% foetal bovine serum (FBS) in the presence of 100\(\mu\)g/ml 500kDa dextran sulphate (DxS). In fact, an almost 30-fold increase in collagen deposition was observed in comparison to the non-MMC control counterparts. The reduction in collagen content as a function of increased FBS concentration and time in culture was attributed to the inherently high matrix metalloproteinase-2 (MMP-2) content of FBS, as revealed by gelatin zymography (Figure 2.6a and Figure 2.12c). Immunocytochemistry (ICC) analysis corroborated the high collagen type I deposition at low FBS concentrations and also confirmed the fibrillar pattern of collagen and fibronectin (Figure 2.6b).

Similar results were obtained in WS1 human skin fibroblast culture under MMC conditions (100\(\mu\)g/ml 500kDa DxS at 0.5% FBS after 2, 4 and 6 days in culture), as revealed by SDS-PAGE and complementary densitometric analysis (Figure 2.7), gelatin zymography (Figure 2.8a) and ICC analysis (Figure 2.8b). In this case, an almost 70-fold increase in collagen deposition was observed, in comparison to the non-MMC control counterparts. Phase contrast microscopy (Figure 2.9) revealed that WI38 and WS1 fibroblasts maintained their spindle-shaped morphology independent of the presence of DxS, FBS concentration (0-10%) for all time points (2, 4 and 6 days).

To avoid xenogeneic contaminants, the influence of human serum (HS), as an alternative to FBS, was assessed. MMC (100\(\mu\)g/ml 500kDa DxS) resulted in a 30- and 80-fold increase in collagen deposition for the WI38 and the WS1 fibroblasts respectively after 2 days in culture using 0.5% HS (Figure 2.10 and 2.11 respectively); a reduction in collagen content as a function of increased HS
concentration and time in culture due to the inherently high MMP-2 content of HS (Figure 2.12); and maintenance of cell morphology (Figure 2.13). There was no change in morphology of WI38 and WS1 observed in the presence of FBS or HS.
Figure 2.5: WI38 lung fibroblasts in the presence of DxS and low FBS concentration induced the highest collagen type I deposition after only 2 days in culture. (a) Representative SDS-PAGE and (b) Densitometric analysis demonstrated that WI38 fibroblasts in the presence of 100 μg/mL 500 kDa DxS deposited the highest (p < 0.0001; n=3) amount of collagen I after 2 days in culture in the presence of 0.5% FBS. Standard (STD): 50 μg/mL collagen type I. (See section 4.2.11. for Densitometry of SDS-PAGE)
Figure 2.6 (a): As the FBS concentration was increased, the collagen type I content was found to decrease. Gelatin zymography detected multiple forms of gelatinolytic enzymes; the 68–72kDa identified as proMMP-2, whilst the 62 kDa identified as the active form. Both forms of enzyme were found to increase as the FBS concentration was increased.
Figure 2.6 (b): ICC experimentation after 2 days in culture confirmed the high deposition of collagen type I, in the presence of DxS, and the reduction in collagen content as a function of increased FBS concentration. It also confirmed high deposition of collagen type I at low FBS concentrations, however fibronectin deposition was not altered, as a function of FBS concentration.
Figure 2.7: (a) Representative SDS-PAGE and (b) complementary densitometric analysis showed that WS1 human skin fibroblasts also deposit the highest amount (p<0.0001; n=3) of collagen type I in the presence of 100µg/ml 500kDa DxS at 0.5% FBS after 2 days in culture.
Figure 2.8 (a): Gelatin zymography for WS1 human skin fibroblasts confirmed that the enhanced MMP-2 content as a function of increased FBS concentration.
Figure 2.8 (b): ICC analysis after 2 days of culture of WS1 human skin fibroblasts confirmed the higher collagen type I deposition in the presence of 100μg/ml 500kDa DxS. It also confirmed high deposition of collagen type I at low FBS concentrations. Fibronectin deposition was unchanged as a function of FBS concentration.
Figure 2.9 (a): Phase contrast microscopy revealed that (a) WI38 fibroblasts maintained their spindle-shaped morphology independent of the presence of DxS, FBS concentration (0-10%) for all time points (2, 4 and 6 days).
Figure 2.9 (b): Phase contrast microscopy revealed that (a) WI38 and (b) WS1 fibroblasts maintained their spindle-shaped morphology independent of the presence of Dxs, FBS concentration (0-10%) for all time points (2, 4 and 6 days).
Figure 2.10: (a) Representative SDS-PAGE and (b) complementary densitometric analysis showed that in the presence of 100μg/ml 500kDa DxS, WI38 fibroblasts deposit the highest (p<0.0001; n=3) amount of collagen type I in the presence of 0.5% human serum after 2 days in culture. Specifically, an over 30-fold increase in collagen deposition was observed after 2 days in culture using 0.5% HS.
Figure 2.11: (a) Representative SDS-PAGE and (b) complementary densitometric analysis showed that in the presence of 100μg/ml 500kDa Dxs, WS1 fibroblasts deposit the highest amount of collagen type I in the presence of 0.5% HS after 2 days in culture (p<0.0001; n=3). Specifically, an over 80-fold increase in collagen deposition was observed after 2 days in culture using 0.5% HS.
Figure 2.12: Gelatin zymography detected multiple forms of gelatinolytic enzymes; the 68–72kDa identified as proMMP-2, whilst the 62 kDa identified as the active form. Gelatin zymography for (a) WI38 and (b) WS1 fibroblasts confirmed the enhanced MMP-2 content as a function of increased HS concentration. (c) Zymography analysis of FBS and HS without cell culture treatments demonstrates the inherently high MMP content of sera.
Figure 2.13 (a): Phase contrast microscopy revealed that WI38 fibroblasts maintained their spindle-shaped morphology independent of the presence of DxS, human serum concentration (0-10%) for all time points (2, 4 and 6 days).
Figure 2.13 (b): Phase contrast microscopy revealed that WS1 fibroblasts maintained their spindle-shaped morphology independent of the presence of Dxs, human serum concentration (0-10%) for all time points (2, 4 and 6 days).
2.3.2. Identification of Optimal MMC for Maximum ECM Deposition

Having identified the 0.5% HS as the optimal serum concentration and 2 days as the optimal culture time for maximum ECM deposition in the presence of 100μg/ml 500kDa DxS, it was ventured to identify the optimal macromolecular crowder for maximum ECM deposition. It was hypothesised that molecular polydispersity is the key modulator of excluding volume, and its complement available volume, and therefore polydispersed macromolecules will induce maximum ECM deposition. Dynamic light scattering (DLS) analysis of neutral (Ficoll™70, Ficoll™400 and combination of thereof) and negatively (DxS 500kDa and carrageenan, CR) charged macromolecules revealed that CR has the highest hydrodynamic diameter (Figure 2.14), whist high-resolution particle size distribution, using Nanoparticle Tracking Analysis (NTA), demonstrated that CR has the highest polydispersity (Figure 2.15).

SDS-PAGE (Figure 2.16a) densitometry (Figure 2.16b) and phase contrast microscopy (Figure 2.17) identified the 75μg/ml as the optimal carrageenan (CR) concentration. Subsequent culture of WI38 and WS1 fibroblasts with 0.5% FBS and HS with variable dispersity macromolecules and subsequent SDS-PAGE (Figure 2.18a), densitometry (Figure 2.18b) and ICC (Figure 2.19) analysis validated our hypothesis that maximum ECM deposition can be achieved by the most polydispersed macromolecule, distinguishing CR as the most suitable molecule. Phase contrast microscopy revealed that WI-38 (Figure 2.20) and WS-1 fibroblasts (Figure 2.21) maintained their spindle-shaped morphology independent of the crowder species used. alamarBlue® (Figure 2.22 for HS and Figure 2.23 for FBS) and Live/Dead® (Figure 2.24 for HS and Figure 2.25 for FBS), analysis demonstrated that MMC did not affect cell metabolic activity and viability, respectively.
Figure 2.14: Dynamic light scattering (DLS) analysis for size (Z-ave, d.nm) of various negatively charged (DxS and CR) and neutral (Ficoll™70, Ficoll™400) macromolecules revealed that negatively charged macromolecules were with higher hydrodynamic diameter than neutral counter part (p<0.0001; n=3). CR exhibited the highest average hydrodynamic diameter than any other macromolecule.
Figure 2.15: Macromolecular polydispersity modulates ECM deposition. (a) High-resolution particle size distribution and Nanoparticle Tracking Analysis (NTA) demonstrated that CR (75 μg/mL) had higher size dispersion (ranges from 10 nm to 2 μm) than any other macromolecule tested [100 μg/ml DxS 500 kDa; 37.5 mg/ml Ficoll™ 70 and 25 mg/ml Ficoll™ 400; and mixture of Ficoll™ 70 (37.5 mg/ml) and Ficoll™ 400 (25 mg/ml)].
Figure 2.16: (a) Representative SDS-PAGE and (b) complementary densitometric analysis of WS1 fibroblasts after 2 days in culture and at 10% FBS demonstrated that 75μg/ml CR induced maximum collagen type I deposition (p<0.001; n=3). Lower than 75μg/ml CR concentrations do not create an effective excluding volume effect, whilst higher concentrations over-crowd the media prohibiting cleavage of procollagen and therefore reduce collagen type I deposition. STD: Symatese collagen type I (100μg/ml).
Figure 2.17: Phase contrast microscopy revealed that presence of CR does not change the morphology of WS1 fibroblasts. It maintained their spindle-shaped morphology independent of the CR (µg/ml), 10% FBS for 2 days.
Figure 2.18: (a) Representative SDS-PAGE and (b) complementary densitometric analysis demonstrated that WI-38 and WS-1 fibroblasts in the presence of 75 μg/mL carrageenan, the most polydispersed macromolecule, induced the highest collagen type I deposition ($p < 0.0001; n=3$) after 2 days in culture supplemented with 0.5% FBS or HS.
Figure 2.19 (a): ICC experimentation after 2 days in culture of WI-38 and WS-1 fibroblasts confirmed the high deposition of collagen type I and fibronectin in the presence of MMC with 0.5% FBS.
Figure 2.19 (b): ICC experimentation after 2 days in culture of WI-38 and WS-1 fibroblasts confirmed the high deposition of collagen type I and fibronectin in the presence of MMC with 0.5% human serum (HS). [Collagen type I (green), fibronectin (red) and DAPI (blue)]
Figure 2.20 (a): Phase contrast microscopy revealed that WI-38 fibroblasts maintained their spindle-shaped morphology independent of macromolecular crowder used, FBS concentration (0-10%) for all time points (2, 4 and 6 days).
Figure 2.20 (b): Phase contrast microscopy revealed that WI-38 fibroblasts maintained their spindle-shaped morphology independent of macromolecular crowder used, HS concentration (0-10%) for all time points (2, 4 and 6 days).
Figure 2.21 (a): Phase contrast microscopy revealed that WS-1 fibroblasts maintained their spindle-shaped morphology independent of macromolecular crowder used, FBS concentration (0-10%) for all time points (2, 4 and 6 days).
Figure 2.21 (b): Phase contrast microscopy revealed that WS-1 fibroblasts maintained their spindle-shaped morphology independent of macromolecular crowder used, HS concentration (0-10%) for all time points (2, 4 and 6 days).
Figure 2.22: No significant differences in cellular metabolic activity were detected under macromolecular crowding conditions (p<0.05; n=3). AlamarBlue® analysis revealed that WI-38 and WS-1 fibroblasts maintained their cellular metabolic activity after macromolecular crowding, 0.5% HS for all time points (2, 4 and 6 days).
Figure 2.23: No significant differences in cellular metabolic activity were detected under macromolecular crowding conditions (p<0.05; n=3). AlamarBlue® analysis revealed that WI-38 and WS-1 fibroblasts maintained their cellular metabolic activity after macromolecular crowding, 0.5% FBS for all time points (2, 4 and 6 days).
Figure 2.24: No significant differences in cellular viability were detected under macromolecular crowding conditions (p<0.05; n=3). Live/Dead® assay for cell viability demonstrated that the various macromolecules did not affect cellular viability after macromolecular crowding, 0.5% HS for all time points (2, 4 and 6 days).
Figure 2.25: No significant differences in cellular viability were detected under macromolecular crowding conditions (p<0.05; n=3). Live/Dead® assay for cell viability demonstrated that the various macromolecules did not affect cellular viability after macromolecular crowding, 0.5% FBS for all time points (2, 4 and 6 days).
2.3.3. Production and Characterisation of ECM-Rich Cell-Assembled Constructs

After optimisation of MMC culture condition, ECM-rich cell-assembled constructs were produced and characterised. Due to the abundance in ECM deposition in the presence of 0.5% HS and 75μg/ml CR, traditional thermo-responsive N-isopropyl acrylamide (pNIPAAm) coating alone was not suitable for the detachment of intact ECM-rich WS-1 fibroblasts sheets, even only after 2 days in culture (Figure 2.26a-d). A 65% N-isopropyl acrylamide / 35% N-tertbutyl acrylamide (pNIPAAm + pNTBA) copolymer allowed for very first time attachment (Figure 2.26e) and detachment (Figure 2.26f) of intact ECM-rich WS-1 fibroblast sheets, produced after 2 days in the presence of 0.5% HS and 75μg/ml CR. Further time-lapse microscopy indicates that the detachment rate of the WS-1 fibroblasts sheets under MMC conditions (0.5% HS and 75μg/ml CR, 2 days in culture; Figure 2.27a-c, g) was slower than their non-MMC counterparts (Figure 2.27d-f, g), due to the abundance in deposited ECM. ICC (Figure 2.26i-n), Masson’s Trichrome (Figure 2.27h, i), Picro Sirius red (Figure 2.26o, r) and scanning electron microscopy (Figure 2.26p, s) analysis further corroborate the enhanced ECM deposition, the fibrillar pattern and tissue like organisation of cell-sheets produced under MMC crowding conditions (0.5% HS and 75μg/ml CR) within 2 days of culture. Atomic force microscopy (Figure 2.26q, w; Figure 2.28) revealed the presence of significantly more fibrous structure in the intercellular regions on the cell sheet surface under MMC crowding conditions, consistent with the presence of collagen. The fibrous ECM (Figure 2.29) was visible also under no MMC crowding conditions.
Figure 2.26: ECM-rich fibroblast-sheet detachment and characterisation after MMC treatment with 75 μg/mL CR, 2 days in culture and 0.5% HS. pNIPAAm alone (a, d) was not able to detach fibroblast cell sheet. A pNIPAAm + pNTBA copolymer allowed attachment (b, e) and detachment of intact rich in ECM cell sheets (c, f). ICC analysis clearly demonstrates a less cohesive ECM patent in the absence of CR (i–k), whilst the abundant and fibrillar in nature deposited ECM is evidenced in the presence of CR (l–n). Picro Sirius red staining (o, r) further corroborates the high amount of deposited ECM in the presence of CR. SEM analysis (p, s) revealed a dense ECM patent of the cell sheets produced under MMC conditions. Representative AFM images (q, w) of intercellular regions on the surface of the cell sheet illustrated the presence of a fibrous network under MMC conditions [Z scale, 100 nm for (q) and (w)].
Figure 2.27: A 65% N-Isopropyl acrylamide + 35% N-tert-butyl acrylamide copolymer was found appropriate for cell attachment and detachment of intact fibroblast sheets. Due to high ECM deposition under MMC conditions, a slower detachment rate was observed using time-lapse analysis (a-f) and subsequent plotting of % detachment area against time (g). Masson’s Trichrome staining (h, i) further corroborated the high/dense collagen matrix deposition under MMC condition (i).
Figure 2.28: Representative (a-c) large (80 × 80μm²), medium (25 × 25μm²) and small (7 × 7μm²) area AFM images of the human skin fibroblast (WS-1) cell sheet prepared without CR. Corresponding (d-f) large (90 × 90μm²), medium (25 × 25μm²) and small (8 × 8μm²) area AFM images of the cell sheet prepared with CR. (g-i) large (90 × 90μm²), medium (50 × 50μm²) and small (5 × 5μm²) area AFM images of the cell sheet prepared with CR from another location. The most striking difference between the no CR and CR images is the presence of a fibrous mesh in the intercellular region for cell sheets prepared under MMC conditions. Z scales: (a, g) 1μm; (b) 380nm; (c, f, i) 100nm; (d) 1.4μm; (e) 580nm; and (h) 220nm.
Figure 2.29: Representative (a-c) large (80 × 80μm²), medium (20 × 20μm²) and small (5 × 5μm²) area AFM images of the human skin fibroblast (WS-1) cell sheet prepared without CR, which was cultured for 2 days. Corresponding (d-f) large (90 × 90μm²), medium (15 × 15μm²) and small (5 × 5μm²) AFM area images of the cell sheet prepared with CR, which was cultured for 2 days. Thus, the presence of a fibrous mesh in the intercellular region for cell sheets prepared under MMC conditions is also apparent at this earlier time step. Z scales: (a, b, d) 600nm; (c) 20nm; (e) 300nm; and (f) 80nm.
2.3.4. Proteomics Analysis of ECM-Rich Cell-Assembled Constructs

To assess the influence of MMC (0.5% HS and 75μg/ml CR, 2 days in culture) on molecular functions, cellular components and biological processes; mass spectrometric proteomics analysis (Table 2.1-2.8) was carried out, which was subsequently validated with ICC (Figure. 2.30a; Figure 2.31a) and complementary fluorescence intensity (Figure 2.30b; Figure 2.31b) measurements. Data obtained confirmed significant upregulation (p<0.0001) of collagenous proteins (e.g. collagen type I, III, IV, V, and VI); enzymes associated with biogenesis of connective tissue proteins and cross-linking of collagen and elastin (e.g. lysyl oxidase); basement membrane proteins (e.g. laminin); glycoproteins (e.g. fibronectin); glycosaminoglycans (e.g. hyaluronic acid); and proteoglycans (e.g. decorin). Other ECM proteins (e.g. collagen type VII, elastin, fibrillin-1); enzymes associated with collagen maturation and cross-linking (e.g. transglutaminase-2); cytoskeletal proteins (e.g. α-smooth muscle actin, epithelial keratin, tubulin); glycosaminoglycans (e.g. chondroitin sulphate, keratin sulphate, heparin sulphate); proteoglycans (e.g. aggrecan, biglycan); and cytokines (e.g. TEM-1/CD248, IL-10) remained unaffected (Figure 2.31), indicating that MMC does not impair physiological function.
Figure 2.30: MMC enhances deposition of numerous ECM molecules. (a) Immunocytochemistry analysis and (b) complementary fluorescence intensity measurements confirmed significantly higher level (p < 0.0001; n=3) of collagenous proteins (type I, III, IV, V, and VI), basement membrane proteins (e.g., laminin), glycoproteins (e.g., fibronectin), glycosaminoglycans (hyaluronic acid), proteoglycans (decorin), and enzymes (lysyl oxidase) under MMC conditions.
Figure 2.31: Proteomics validation using ICC (a) and complementary fluorescence intensity measurements (b) confirmed no significant change (p<0.05; n=3) in ECM proteins (e.g. collagen type VII, elastin, fibrillin-1); cytoskeletal proteins (e.g. α-smooth muscle actin, epithelial keratin, tubulin); proteoglycans (e.g. aggrecan, biglycan); glycosaminoglycans (e.g. chondroitin sulphate, keratin sulphate, heparin sulphate); enzymes (e.g. transglutaminase-2); and cytokines (e.g. TEM-1/CD248, IL-10).
2.3.5. Production and Characterisation of Bone and Tendon ECM-Rich Cell-Assembled Constructs

The high ECM deposition procedure can be readily applied to other cell types, irrespective of the amounts of ECM secreted. Here, it was generated cohesive tissue modules from human tenocytes (Figure 2.32) and osteoblasts (Figure 2.33). SDS-PAGE (Figure 2.32a) and complementary densitometric analysis (Figure 2.32b) revealed that the maximum (p<0.0001) collagen type I deposition was achieved after culturing human tenocytes for 6 days at 5% FBS in the presence of 75μg/ml CR. In fact, an almost 15-fold increase in collagen type I deposition was observed in comparison to the non-MMC control counterparts. ICC analysis corroborated the high deposition of collagenous proteins (collagen type I, III, IV, V and VI), glycoproteins (e.g. fibronectin) and basement membrane proteins (e.g. laminin), whilst tenomodulin expression remained unaffected (Figure 2.32c). Analogous was the situation with human osteoblasts. Specifically, SDS-PAGE (Figure 2.33a) and complementary densitometric analysis (Figure 2.33b) revealed that the maximum (p<0.0001) collagen type I deposition was achieved after culturing human osteoblasts for 4 days at 5% FBS in the presence of 75μg/ml CR. In fact, an almost 20-fold increase in collagen type I deposition was observed in comparison to the non-MMC control counterparts. ICC analysis corroborated the high deposition of collagenous proteins (e.g. collagen type I, III, IV, V and VI), glycoproteins (e.g. fibronectin) and basement membrane proteins (e.g. laminin), whilst tenomodulin expression remained unaffected (Figure 2.33c). alamarBlue® analysis demonstrated that CR did not affect tenocyte (Figure 2.34a) and osteoblast (Figure 2.34b) metabolic activity.
Figure 2.32: (a) Representative SDS-PAGE and (b) densitometric analysis of demonstrated that human tenocytes in the presence of 75 μg/mL CR deposited the highest (p < 0.0001, n=3) amount of collagen I after 6 days at 5% FBS. Standard (STD): 100 μg/mL collagen type I. (c) ICC experimentation confirmed the high deposition of collagenous proteins (collagen type I, III, IV, V and VI), glycoproteins (e.g., fibronectin) and basement membrane proteins (e.g., laminin), whilst tenomodulin expression remained unaffected.
Figure 2.32 (c): ICC experimentation of human tenocytes in the presence of 75 μg/mL CR confirmed the high deposition collagenous proteins (collagen type I, III, IV, V and VI), glycoproteins (e.g., fibronectin) and basement membrane proteins (e.g., laminin), whilst tenomodulin expression remained unaffected.
Figure 2.33: MMC can be readily applied to other cell types to induce high ECM deposition. (a) Representative SDS-PAGE and (b) densitometric analysis demonstrated that human osteoblasts in the presence of 75 μg/mL CR deposited the highest (p < 0.0001; n=3) amount of collagen I after 4 days at 5% FBS. Standard (STD): 100 μg/mL collagen type I.
Figure 2.33(c): ICC experimentation of human osteoblasts in the presence of 75 μg/mL CR confirmed the high deposition of collagenous proteins (collagen type I, III, IV, V and VI), glycoproteins (e.g., fibronectin) and basement membrane proteins (e.g., laminin), whilst tenomodulin expression remained unaffected.
Figure 2.34: AlamarBlue® analysis revealed that (a) human tenocytes and (b) osteoblasts maintained their cellular metabolic activity (n=3) after macromolecular crowding, 5-10% FBS for all time points (2, 4 and 6 days).
Mass spectrometry results of up-regulated (↑) and down-regulated (↓) proteins (Table 2.1-2.8) from human skin fibroblast (WS-1) in the presence of 0.5% HS under crowding condition with carrageenan (+CR) and non-crowding (-CR) conditions. Role and functions of proteins are obtained from The Universal Protein Resource [UniProt, European Bioinformatics Institute (EMBL-EBI), Swiss Institute of Bioinformatics (SIB) and the Protein Information Resource (PIR)] and National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine. “ND” indicates - not detected; “Cell -CR Only” indicates – WS-1 culture without crowder; “Cell +CR Only” indicates - WS-1 culture with crowder (carrageenan).

Table 2.1: Collagen and collagen related proteins in cell layer

<table>
<thead>
<tr>
<th>Cell layer Proteins</th>
<th>Gene Symbol</th>
<th>Role/ Function</th>
<th>PSMs</th>
<th>% Coverage</th>
<th>Ratio Cell +CR/-CR</th>
<th>log2 Fold change</th>
<th>↓↑</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen alpha-1(IV)</td>
<td>COL4A1</td>
<td>Type IV collagen is the major structural component of glomerular basement membranes (GBM), forming a 'chicken-wire' meshwork together with laminins, proteoglycans and entactin/nidogen.</td>
<td>12</td>
<td>7.5</td>
<td>Cell-CR Only</td>
<td>ND</td>
<td>Cell -CR Only</td>
</tr>
<tr>
<td>Collagen alpha-1(XVIII)</td>
<td>COL18A1</td>
<td>Collagen alpha-1(XVIII) is one of the multiplexins, extracellular matrix proteins that contain multiple triple-helix domains (collagenous domains) interrupted by non-collagenous domains. COLA18A probably plays a major role in</td>
<td>6</td>
<td>5.8</td>
<td>Cell-CR Only</td>
<td>ND</td>
<td>Cell -CR Only</td>
</tr>
<tr>
<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Cell +CR/-CR</td>
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</tr>
<tr>
<td>Collagen alpha-1(VI)</td>
<td>COL6A1</td>
<td>determining the retinal structure as well as in the closure of the neural tube. It may inhibit angiogenesis by binding to the heparan sulfate proteoglycans involved in growth factor signaling.</td>
<td>112</td>
<td>42.5</td>
<td>0.087</td>
<td>-3.515</td>
<td>↓</td>
</tr>
<tr>
<td>Collagen type VI, alpha 2</td>
<td>COL6A2</td>
<td>Collagen VI is a major structural component of microfibrils and acts as a cell-binding protein. The protein encoded by this gene is the alpha 1 subunit of type VI collagen (alpha 1 (VI) chain)</td>
<td>7</td>
<td>40.9</td>
<td>0.260</td>
<td>-1.939</td>
<td>↓</td>
</tr>
<tr>
<td>Collagen alpha-1(III)</td>
<td>COL3A1</td>
<td>involved in extensible connective tissues such as skin, lung, uterus, intestine and the vascular system, frequently in association with type I collagen. It Involved in regulation of</td>
<td>11</td>
<td>6.6</td>
<td>0.490</td>
<td>-1.026</td>
<td>↓</td>
</tr>
<tr>
<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Cell +CR/-CR</td>
<td>log2 Fold change</td>
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</tr>
<tr>
<td>Collagen alpha-2(IV)</td>
<td>COL4A2</td>
<td>This is one of the six subunits of type IV collagen, the major structural component of basement membranes. It inhibits proliferation and migration of endothelial cells, reduces mitochondrial membrane potential, and induces apoptosis.</td>
<td>7</td>
<td>5.3</td>
<td>0.523</td>
<td>-0.932</td>
<td>↓</td>
</tr>
<tr>
<td>Procollagen galactosyltransferase 1</td>
<td>GLT25D1</td>
<td>Glycosyl transferase 25 domain 1 (GLT25D1) has galactosyltransferase activity towards collagens and mannose binding lectin (MBL). It transfers beta-galactose to hydroxylysine residues of collagen.</td>
<td>39</td>
<td>34.6</td>
<td>0.536</td>
<td>-0.899</td>
<td>↓</td>
</tr>
<tr>
<td>Collagen alpha-1(I)</td>
<td>COL1A1</td>
<td>This is a pro-alpha1 chains of type I collagen whose triple helix comprises two alpha1 chains and one alpha2 chain. Type I is a fibril-forming collagen found in most connective tissues and is abundant in bone, cornea, dermis and tendon.</td>
<td>99</td>
<td>20.4</td>
<td>1.409</td>
<td>0.495</td>
<td>↑</td>
</tr>
<tr>
<td>Collagen alpha-3(VI)</td>
<td>COL6A3</td>
<td>The alpha-3 chain is one of the three alpha chains of type VI collagen, a beaded filament collagen found in most connective tissues. Collagen VI acts as a cell-binding protein</td>
<td>781</td>
<td>56.4</td>
<td>2.798</td>
<td>1.484</td>
<td>↑</td>
</tr>
<tr>
<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Cell +CR/-CR</td>
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</tr>
<tr>
<td>Collagen alpha-1(VIII)</td>
<td>COL8A1</td>
<td>This is one of the two alpha chains of type VIII collagen, a major component of the basement membrane of the corneal endothelium. Also component of the endothelia of blood vessels. Necessary for migration and proliferation of vascular smooth muscle cells and thus, has a potential role in the maintenance of vessel wall integrity and structure, in particular in atherogenesis.</td>
<td>2</td>
<td>3.9</td>
<td>2.938</td>
<td>1.555</td>
<td>↑</td>
</tr>
<tr>
<td>Collagen alpha-1(XII)</td>
<td>COL12A1</td>
<td>This is an alpha chain of type XII collagen, a member of the FACIT (fibril-associated collagens with interrupted triple helices) collagen family. Type XII collagen is a homotrimer found in association with type I collagen, an association that is thought to modify the interactions between collagen I fibrils and the surrounding matrix.</td>
<td>577</td>
<td>55.2</td>
<td>3.241</td>
<td>1.696</td>
<td>↑</td>
</tr>
<tr>
<td>Collagen alpha-1(XI)</td>
<td>COL11A1</td>
<td>This is one of the two alpha chains of type XI collagen, a minor fibrillar collagen. May play an important role in fibrillogenesis by controlling lateral growth of collagen II fibrils.</td>
<td>4</td>
<td>3.1</td>
<td>3.488</td>
<td>1.802</td>
<td>↑</td>
</tr>
<tr>
<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Cell +CR/-CR</td>
<td>log2 Fold change</td>
<td>↓↑</td>
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</tr>
<tr>
<td>Collagen alpha-2(I)</td>
<td>COL1A2</td>
<td>This is a pro-alpha2 chain of type I collagen whose triple helix comprises two alpha1 chains and one alpha2 chain. Type I is a fibril-forming collagen found in most connective tissues and is abundant in bone, cornea, dermis and tendon.</td>
<td>82</td>
<td>19.7</td>
<td>5.539</td>
<td>2.469</td>
<td>↑</td>
</tr>
<tr>
<td>Collagen alpha-1(V)</td>
<td>COL5A1</td>
<td>Type V collagen is a member of group I collagen (fibrillar forming collagen). It is a minor connective tissue component of nearly ubiquitous distribution. Type V collagen binds to DNA, heparan sulfate, thrombospondin, heparin, and insulin.</td>
<td>56</td>
<td>13.8</td>
<td>6.617</td>
<td>2.726</td>
<td>↑</td>
</tr>
<tr>
<td>Collagen triple helix repeat-containing protein</td>
<td>CTHRC1</td>
<td>This protein plays a role in the cellular response to arterial injury through involvement in vascular remodeling. It may act as a negative regulator of collagen matrix deposition.</td>
<td>17</td>
<td>33.3</td>
<td>6.697</td>
<td>2.743</td>
<td>↑</td>
</tr>
<tr>
<td>Collagen alpha-2(V)</td>
<td>COL5A2</td>
<td>This is an alpha chain for one of the low abundance fibrillar collagens. Fibrillar collagen molecules are trimers that can be composed of one or more types of alpha chains. Type V collagen is found in tissues containing type I collagen and</td>
<td>14</td>
<td>8.5</td>
<td>11.167</td>
<td>3.481</td>
<td>↑</td>
</tr>
<tr>
<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Cell +CR/-CR</td>
<td>log2 Fold change</td>
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</tr>
<tr>
<td>Collagen alpha-1(XVI)</td>
<td>COL16A1 1</td>
<td>The alpha chain of type XVI collagen is a member of the FACIT collagen family (fibril-associated collagens with interrupted helices). Members of this collagen family are found in association with fibril-forming collagens such as type I and II, and serve to maintain the integrity of the extracellular matrix. It involved in mediating cell attachment and inducing integrin-mediated cellular reactions, such as cell spreading and alterations in cell morphology.</td>
<td>1</td>
<td>2.3</td>
<td>Cell+CR Only</td>
<td>ND</td>
<td>Cell+CR Only</td>
</tr>
<tr>
<td>Collagen alpha-1(XIV)</td>
<td>COL14A1 1</td>
<td>This is an alpha chain of type XIV collagen, a member of the FACIT (fibril-associated collagens with interrupted triple helix) family.</td>
<td>9</td>
<td>6.8</td>
<td>Cell+CR Only</td>
<td>ND</td>
<td>Cell+CR Only</td>
</tr>
<tr>
<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Cell +CR/-CR</td>
<td>log₂ Fold change</td>
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<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Cell +CR/-CR</td>
<td>log₂ Fold change</td>
<td>↓↑</td>
</tr>
<tr>
<td>Cell+CR Only</td>
<td>ND</td>
<td>Cell+CR Only</td>
<td>23</td>
<td>46.5</td>
<td>Cell+CR Only</td>
<td>ND</td>
<td>Cell+CR Only</td>
</tr>
</tbody>
</table>

helices) collagen family. Type XIV collagen interacts with the fibril surface and is involved in the regulation of fibrillogenesis. Plays an adhesive role by integrating collagen bundles. It is probably associated with the surface of interstitial collagen fibrils via Collagen I.

Fibrillar collagen types I-III are synthesized as precursor molecules known as procollagens. These precursors contain amino- and carboxyl-terminal peptide extensions known as N- and C-propeptides, respectively, which are cleaved, upon secretion of procollagen from the cell, to yield the mature triple helical, highly structured fibrils. This is a glycoprotein which binds and drives the enzymatic cleavage of type I procollagen and enhances procollagen C-proteinase activity of type I.
# Table 2.2: Fibronectin in cell layer

<table>
<thead>
<tr>
<th>Cell layer Proteins</th>
<th>Gene Symbol</th>
<th>Role/ Function</th>
<th>PSMs</th>
<th>% Coverage</th>
<th>Ratio Cell +CR/-CR</th>
<th>log2 Fold change</th>
<th>↓↑</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin type-III domain-containing protein 3B</td>
<td>FNDC3B</td>
<td>Fibronectin Type III Domain Containing 3B predominantly expresses in white adipose tissue (WAT), especially in the stromal vascular cells. Expression increased in the early stage of adipogenesis.</td>
<td>7</td>
<td>6.6</td>
<td>Cell-CR Only</td>
<td>ND</td>
<td>Cell-CR Only</td>
</tr>
<tr>
<td>Fibronectin type-III domain-containing protein 3A</td>
<td>FNDC3A</td>
<td>Fibronectin type-III domain-containing protein 3A expresses in the odontoblast and nerves in the dental pulp. Also expressed in trachea and to a lesser extent in the brain, liver, lung and kidney. It mediates spermatid-Sertoli adhesion during spermatogenesis.</td>
<td>1</td>
<td>4.2</td>
<td>Cell-CR Only</td>
<td>ND</td>
<td>Cell-CR Only</td>
</tr>
<tr>
<td>Fibronectin 1 (FN1)</td>
<td>FN1</td>
<td>Fibronectin, a glycoprotein present in a soluble dimeric form in plasma, and in a dimeric or multimeric form at the cell surface and in extracellular matrix. Fibronectins bind cell surfaces and various compounds including collagen, fibrin, heparin, DNA, and actin. Fibronectins are involved in cell adhesion, cell</td>
<td>1305</td>
<td>70.9</td>
<td>1.605</td>
<td>0.682</td>
<td>↑</td>
</tr>
<tr>
<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Cell +CR/-CR</td>
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<tr>
<td>motility, opsonization, wound healing, and maintenance of cell shape. Involved in osteoblast compaction through the fibronectin fibrillogenesis cell-mediated matrix assembly process, essential for osteoblast mineralization. Participates in the regulation of type I collagen deposition by osteoblasts.coagulation, host defense, and metastasis.</td>
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</tbody>
</table>
Table 2.3: Glycosaminoglycans (GAGs) and proteoglycans (PGs) in cell layer

<table>
<thead>
<tr>
<th>Cell layer Proteins</th>
<th>Gene Symbol</th>
<th>Role/ Function</th>
<th>PSMs</th>
<th>% Coverage</th>
<th>Ratio Cell +CR/-CR</th>
<th>log2 Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basement membrane-specific heparan sulfate proteoglycan core protein</td>
<td>HSPG2</td>
<td>This is an integral component of basement membranes also known as perlecan protein, which consists of a core protein to which three long chains of glycosaminoglycans (heparan sulfate or chondroitin sulfate) are attached. The perlecan protein is a large multidomain proteoglycan that binds to and cross-links many extracellular matrix components and cell-surface molecules. It has been shown that this protein interacts with laminin, prolarigin, collagen type IV, FGFBP1, FBLN2, FGF7 and transthyretin, etc., and it plays essential roles in multiple biological activities. Perlecan is a key component of the vascular extracellular matrix, where it helps to maintain the endothelial barrier function.</td>
<td>283</td>
<td>42.8</td>
<td>0.002</td>
<td>-8.959</td>
</tr>
<tr>
<td>Chondroitin sulfate proteoglycan 4</td>
<td>CSPG4</td>
<td>Chondroitin sulfate proteoglycan 4 plays role in cell proliferation and migration which stimulates endothelial cells motility during microvascular morphogenesis. It may also</td>
<td>73</td>
<td>28.1</td>
<td>0.480</td>
<td>-1.057</td>
</tr>
<tr>
<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Cell +CR/-CR</td>
<td>log2 Fold change</td>
</tr>
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</tr>
<tr>
<td></td>
<td></td>
<td>inhibit neurite outgrowth and growth cone collapse during axon regeneration.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyaluronan &amp; proteoglycan link protein 1</td>
<td>HAPLN1</td>
<td>Hyaluronan and proteoglycan link protein 1 stabilizes the aggregates of proteoglycan monomers with hyaluronic acid in the extracellular cartilage matrix</td>
<td>12</td>
<td>32.5</td>
<td>7.505</td>
<td>2.908</td>
</tr>
<tr>
<td>Keratan sulphate</td>
<td>CHST1</td>
<td>Keratan sulfates are large, highly hydrated proteoglycan (PG) molecules which in joints can act as a cushion to absorb mechanical shock. Keratan sulfate chains are attached to cell-surface or extracellular matrix proteins, termed core proteins (Lumican, Keratocan, Mimecan, Fibromodulin, PRELP, Osteoadherin and Aggrecan).</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>ACAN</td>
<td>This proteoglycan is a major component of extracellular matrix of cartilagenous tissues. A major function of this protein is to resist compression in cartilage. It binds avidly to hyaluronic acid via an N-terminal globular region.</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Biglycan</td>
<td>BGN</td>
<td>Biglycan is a small cellular or pericellular matrix proteoglycan</td>
<td>7</td>
<td>28.3</td>
<td>Cell-CR</td>
<td>ND</td>
</tr>
</tbody>
</table>
that is closely related in structure to two other small proteoglycans, decorin and fibromodulin. It plays a role in assembly of collagen fibrils and muscle regeneration. Biglycan interacts with several proteins involved in muscular dystrophy, including alpha-dystroglycan, alpha- and gamma-sarcoglycan and collagen VI, and it is critical for the assembly of the dystrophin-associated protein complex.

<table>
<thead>
<tr>
<th>Cell layer Proteins</th>
<th>Gene Symbol</th>
<th>Role/ Function</th>
<th>PSMs</th>
<th>% Coverage</th>
<th>Ratio Cell +CR/-CR</th>
<th>log2 Fold change</th>
<th>↓↑</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decorin</td>
<td>DCN</td>
<td>Decorin is a component of the extracellular matrix, which is the intricate lattice of proteins and other molecules that forms in the spaces between cells. Decorin is found in the extracellular matrix of a variety of connective tissues, including skin, tendon, bone, and cartilage. It is involved in the fibrils formation and organization of collagens.</td>
<td>2</td>
<td>9.2</td>
<td>Cell-CR Only</td>
<td>ND</td>
<td>Cell-CR Only</td>
</tr>
</tbody>
</table>
Table 2.4: Laminin in cell layer

<table>
<thead>
<tr>
<th>Cell layer Proteins</th>
<th>Gene Symbol</th>
<th>Role/ Function</th>
<th>PSMs</th>
<th>% Coverage</th>
<th>Ratio Media +CR/-CR</th>
<th>log2 Fold change</th>
<th>↓↑</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin subunit gamma-1</td>
<td>LAMC1</td>
<td>Laminins, a family of extracellular matrix glycoproteins, are the major noncollagenous constituent of basement membranes. They have been implicated in a wide variety of biological processes including cell adhesion, differentiation, migration, signaling, neurite outgrowth and metastasis. Binding to cells via a high affinity receptor, laminin mediates the attachment, migration and organization of cells into tissues during embryonic development by interacting with other extracellular matrix components. The biological functions of the different chains and trimer molecules are largely unknown, but some of the chains have been shown to differ with respect to their tissue distribution, presumably reflecting diverse functions in vivo.</td>
<td>38</td>
<td>22.4</td>
<td>1.889</td>
<td>0.918</td>
<td>↑</td>
</tr>
<tr>
<td>Laminin subunit beta-1</td>
<td>LAMB1</td>
<td>62</td>
<td>29.6</td>
<td>2.452</td>
<td>1.294</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Laminin subunit beta-2</td>
<td>LAMB2</td>
<td>5</td>
<td>3.3</td>
<td>3.770</td>
<td>1.914</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Isoform 1 of Laminin subunit alpha-4</td>
<td>LAMA4</td>
<td>18</td>
<td>11.8</td>
<td>3.804</td>
<td>1.927</td>
<td>↑</td>
<td></td>
</tr>
</tbody>
</table>
### Table 2.5: Tubulins in cell layer

<table>
<thead>
<tr>
<th>Cell layer Proteins</th>
<th>Gene Symbol</th>
<th>Role/ Function</th>
<th>PSMs</th>
<th>% Coverage</th>
<th>Ratio Media</th>
<th>log2 Fold change</th>
<th>↓↑</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubulin beta-3 chain</td>
<td>TUBB3</td>
<td>Microtubules of the eukaryotic cytoskeleton perform essential and diverse functions and are composed of a heterodimer of alpha and beta tubulins. The genes encoding these microtubule constituents belong to the tubulin superfamily, which is composed of six distinct families. Genes from the alpha, beta and gamma tubulin families are found in all eukaryotes. The alpha and beta tubulins represent the major components of microtubules, while gamma tubulin plays a critical role in the nucleation of microtubule assembly. There are multiple alpha and beta tubulin genes, which are highly conserved among species. Beta tubulins are one of two core protein families (alpha and beta tubulins) that heterodimerize and assemble to form microtubules. This protein is primarily expressed in neurons and may be involved in neurogenesis and axon guidance and maintenance. Tubulin chains binds two moles of...</td>
<td>23</td>
<td>72.4</td>
<td>0.0962</td>
<td>-3.377</td>
<td>↓</td>
</tr>
<tr>
<td>Tubulin beta-4 chain</td>
<td>TUBB4</td>
<td></td>
<td>13</td>
<td>76.8</td>
<td>0.378</td>
<td>-1.402</td>
<td>↓</td>
</tr>
<tr>
<td>Tubulin alpha-1C chain</td>
<td>TUBA1C</td>
<td></td>
<td>32</td>
<td>78.2</td>
<td>0.381</td>
<td>-1.390</td>
<td>↓</td>
</tr>
<tr>
<td>Tubulin beta-8 chain</td>
<td>TUBB8</td>
<td></td>
<td>4</td>
<td>28.6</td>
<td>0.422</td>
<td>-1.243</td>
<td>↓</td>
</tr>
<tr>
<td>Tubulin alpha-1A</td>
<td>TUBA1A</td>
<td></td>
<td>39</td>
<td>82.5</td>
<td>0.529</td>
<td>-0.916</td>
<td>↓</td>
</tr>
<tr>
<td>Tubulin beta-2A chain</td>
<td>TUBB2A</td>
<td></td>
<td>18</td>
<td>82.9</td>
<td>1.150</td>
<td>0.202</td>
<td>↑</td>
</tr>
<tr>
<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Media +CR/-CR</td>
<td>log2 Fold change</td>
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<tr>
<td>Tubulin beta-2C chain</td>
<td>TUBB2C</td>
<td>GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain.</td>
<td>6</td>
<td>82.9</td>
<td>1.442</td>
<td>0.528</td>
<td>↑</td>
</tr>
<tr>
<td>Tubulin gamma-1 chain</td>
<td>TUBG1</td>
<td></td>
<td>2</td>
<td>6.7</td>
<td>Cell+CR Only</td>
<td>ND</td>
<td>Cell+CR Only</td>
</tr>
</tbody>
</table>

Chapter 2
Table 2.6: Other ECM and cytoskeleton proteins

<table>
<thead>
<tr>
<th>Cell layer Proteins</th>
<th>Gene Symbol</th>
<th>Role/ Function</th>
<th>PSMs</th>
<th>% Coverage</th>
<th>Ratio Media +CR/-CR</th>
<th>log2 Fold change</th>
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</thead>
<tbody>
<tr>
<td>Elastin</td>
<td>ELN</td>
<td>Elastin is a major structural protein of tissues such as aorta and nuchal ligament, which must expand rapidly and recover completely. It is a Molecular determinant of the late arterial morphogenesis, stabilizing arterial structure by regulating proliferation and organization of vascular smooth muscle</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Actin, aortic smooth muscle</td>
<td>ACTA2</td>
<td>The actin family of proteins, which are highly conserved proteins, play a role in cell motility, structure and integrity. Alpha, beta and gamma actin isoforms have been identified, with alpha actins being a major constituent of the contractile apparatus, while beta and gamma actins are involved in the regulation of cell motility. This actin is an alpha actin that is found in skeletal muscle.</td>
<td>7</td>
<td>74.5</td>
<td>0.229</td>
<td>-2.122</td>
<td>↓</td>
</tr>
<tr>
<td>Isoform 1 of Keratin</td>
<td>KRT13</td>
<td>The protein encoded by this gene is a member of the keratin gene family. The keratins are intermediate filament proteins responsible for the structural integrity of epithelial cells and are</td>
<td>14</td>
<td>32.6</td>
<td>Cell-CR Only</td>
<td>ND</td>
<td>Cell-CR Only</td>
</tr>
<tr>
<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Media</td>
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<tr>
<td>Subdivided into cytokeratins and hair keratins. Most of the type I cytokeratins consist of acidic proteins which are arranged in pairs of heterotypic keratin chains. This type I cytokeratin is paired with keratin 4 and expressed in the suprabasal layers of non-cornified stratified epithelia.</td>
<td>10.7</td>
<td>Cell-CR Only</td>
<td>ND</td>
<td>Cell-CR Only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keratin, type I cytoskeletal 18</td>
<td>KRT18</td>
<td>Keratin 18, together with its filament partner keratin 8, are perhaps the most commonly found members of the intermediate filament gene family. They are expressed in single layer epithelial tissues of the body. Involved in the uptake of thrombin-antithrombin complexes by hepatic cells (By similarity). When phosphorylated, plays a role in filament reorganization. Involved in the delivery of mutated CFTR to the plasma membrane. Together with KRT8, is involved in interleukin-6 (IL-6)-mediated barrier protection.</td>
<td>7</td>
<td>10.7</td>
<td>Cell-CR Only</td>
<td>ND</td>
<td>Cell-CR Only</td>
</tr>
<tr>
<td>Keratin, type II</td>
<td>KRT3</td>
<td>The type II cytokeratins consist of basic or neutral proteins which are arranged in pairs of heterotypic keratin chains</td>
<td>5</td>
<td>17</td>
<td>0.192</td>
<td>-2.376</td>
<td>↓</td>
</tr>
<tr>
<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Media +CR/-CR</td>
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<tr>
<td>cytoskeletal 3</td>
<td>FBN1</td>
<td>Fibrillins are structural components of 10-12 nm extracellular calcium-binding microfibrils, which occur either in association with elastin or in elastin-free bundles. Fibrillin-1-containing microfibrils provide long-term force bearing structural support. Regulates osteoblast maturation by controlling TGF-beta bioavailability and calibrating TGF-beta and BMP levels, respectively.</td>
<td>170</td>
<td>29.3</td>
<td>5.459</td>
<td>2.448</td>
<td>↑</td>
</tr>
<tr>
<td>Keratin 12</td>
<td></td>
<td>coexpressed during differentiation of simple and stratified epithelial tissues. This type II cytokeratin is specifically expressed in the corneal epithelium with family member Keratin 12.</td>
<td>77</td>
<td>100</td>
<td>2.448</td>
<td>1.892</td>
<td>↓</td>
</tr>
<tr>
<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Media +CR/-CR</td>
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<tr>
<td>Interleukin enhancer-binding factor 3</td>
<td>ILF3</td>
<td>Interleukin enhancer-binding factor 3 forms a heterodimer with a 45 kDa transcription factor (NF45, ILF2) required for T-cell expression of interleukin 2. It can act as a translation inhibitory protein which binds to coding sequences of acid beta-glucosidase (GCase) and other mRNAs and functions at the initiation phase of GCase mRNA translation, probably by inhibiting its binding to polysomes. It can also regulate protein arginine N-methyltransferase 1 activity.</td>
<td>49</td>
<td>28.5</td>
<td>0.182</td>
<td>-2.454</td>
<td>↓</td>
</tr>
<tr>
<td>Interleukin enhancer-binding factor 2</td>
<td>ILF2</td>
<td>Interleukin enhancer-binding factor 2 is the 45 kDa component of nuclear factor of activated T-cells (NFAT), a heterodimer of 45 kDa and 90 kDa proteins. NFAT is a transcription factor required for T-cell expression of the interleukin 2 gene. Appears to function predominantly as a heterodimeric complex with ILF3. This complex may regulate transcription of the IL2 gene during T-cell activation. It can also promote the formation</td>
<td>40</td>
<td>54.1</td>
<td>0.861</td>
<td>-0.215</td>
<td>↓</td>
</tr>
<tr>
<td>Cell layer</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Media</td>
<td>log2 Fold change</td>
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<tr>
<td>Proteins</td>
<td></td>
<td>of stable DNA-dependent protein kinase holoenzyme complexes on DNA. Essential for the efficient resshuttling of ILF3 (isoform 1 and isoform 2) into the nucleus.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interleukin-36 gamma</td>
<td>IL36G</td>
<td>Interleukin-36 gamma is a member of the interleukin 1 cytokine family. The activity of this cytokine is mediated by interleukin 1 receptor-like 2 (IL1RL2/IL1R-rp2), and is specifically inhibited by interleukin 1 family, member 5 (IL1F5/IL-1 delta). Interferon-gamma, tumor necrosis factor-alpha and interleukin 1, beta (IL1B) are reported to stimulate the expression of this cytokine in keratinocytes.</td>
<td>4</td>
<td>44.8</td>
<td>Cell+CR Only</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Interleukin-10</td>
<td>IL10</td>
<td>Interleukin-10 is a cytokine produced primarily by monocytes and to a lesser extent by lymphocytes. This cytokine has pleiotropic effects in immunoregulation and inflammation. It down-regulates the expression of Th1 cytokines, MHC class II Ags, and costimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody</td>
<td>ND</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Media</td>
<td>log2 Fold change</td>
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<tr>
<td>Endosialin (TEM1)</td>
<td>CD248</td>
<td>production. This cytokine can block NF-kappa B activity, and is involved in the regulation of the JAK-STAT signaling pathway.</td>
<td>20</td>
<td>22.7</td>
<td>0.016</td>
<td>-5.904</td>
<td>↓</td>
</tr>
<tr>
<td>Lysyl oxidase homolog 2</td>
<td>LOXL2</td>
<td>Lysyl oxidases are essential for the biogenesis of connective tissue, encoding an extracellular copper-dependent amine oxidase that catalyses the first step in the formation of crosslinks in collagens and elastin. It also involved in E-cadherin repression following hypoxia, a hallmark of epithelial to mesenchymal transition believed to amplify tumor aggressiveness, suggesting that it may play a role in tumor</td>
<td>19</td>
<td>14.6</td>
<td>0.719</td>
<td>-0.474</td>
<td>↓</td>
</tr>
<tr>
<td>Cell layer</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Media</td>
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<tr>
<td>Progression. Acts as a regulator of chondrocyte differentiation, probably by regulating expression of factors that control chondrocyte differentiation.</td>
<td>Transglutaminase 2</td>
<td>TGM2</td>
<td>Transglutaminases are enzymes that catalyze the crosslinking of proteins by epsilon-gamma glutamyl lysine isopeptide bonds. Trans-glutaminase 2 acts as a monomer, is induced by retinoic acid, and appears to be involved in apoptosis.</td>
<td>ND</td>
<td>-</td>
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</tr>
</tbody>
</table>
Table 2.8: Matrix metalloproteinases (MMPs) and tissue inhibitor metalloproteinases (TIMPs) in cell layer

<table>
<thead>
<tr>
<th>Cell layer Proteins</th>
<th>Gene Symbol</th>
<th>Role/Function</th>
<th>PSMs</th>
<th>% Coverage</th>
<th>Ratio Cell +CR/-CR</th>
<th>log2 Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrix metalloproteinase</td>
<td>MMP14</td>
<td>Matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. Seems to specifically activate progelatinase A. May be involved in actin cytoskeleton reorganization by cleaving PTK7. Acts as a positive regulator of cell growth and migration via activation of MMP15. Involved in the formation of the fibrovascular tissues in association with pro-MMP2.</td>
<td>26</td>
<td>21.1</td>
<td>2.001</td>
<td>1.001</td>
</tr>
<tr>
<td>Cell layer</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Cell +CR/-CR</td>
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<tr>
<td>72 KDa type IV collagenase</td>
<td>MMP2</td>
<td>MMP2 degrades type IV collagen, the major structural component of basement membranes. The enzyme plays a role in endometrial menstrual breakdown, regulation of vascularization and the inflammatory response. It involved in diverse functions such as remodeling of the vasculature, angiogenesis, tissue repair, tumor invasion, inflammation, and atherosclerotic plaque rupture.</td>
<td>44</td>
<td>46.5</td>
<td>2.390</td>
<td>1.257</td>
</tr>
<tr>
<td>Interstitial collagenase</td>
<td>MMP1</td>
<td>MMP1 breaks down the interstitial collagens, types I, II, and III. Also cleaves collagens of types VII and X. In case of HIV infection, interacts and cleaves the secreted viral Tat protein, leading to a decrease in neuronal Tat's mediated neurotoxicity</td>
<td>50</td>
<td>43.5</td>
<td>Cell+CR Only</td>
<td>ND</td>
</tr>
<tr>
<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Cell +CR/-CR</td>
<td>log2 Fold change</td>
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<tr>
<td>Metallo-proteinase inhibitor 1</td>
<td>TIMP1</td>
<td>Metallo-proteinase inhibitors (TIMPs) are natural inhibitors of the matrix metalloproteinases (MMPs). TIMPs promote cell proliferation in a wide range of cell types, and may also have an anti-apoptotic function. Complexes with metalloproteinases (such as collagenases) and irreversibly inactivates them by binding to their catalytic zinc cofactor. Also mediates erythropoiesis in vitro; but, unlike IL-3, it is species-specific, stimulating the growth and differentiation of only human and murine erythroid progenitors. Known to act on MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13 and MMP-16. Does not act on MMP-14.</td>
<td>15</td>
<td>76.9</td>
<td>5.409 ND</td>
<td>↑</td>
</tr>
<tr>
<td>Cell layer Proteins</td>
<td>Gene Symbol</td>
<td>Role/ Function</td>
<td>PSMs</td>
<td>% Coverage</td>
<td>Ratio Cell+CR/CR</td>
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<tr>
<td>Metallo-proteinase inhibitor 3</td>
<td>TIMP3</td>
<td>Expression of TIMP3 and TIMP2 is induced in response to mitogenic stimulation and this netrin domain-containing protein is localized to the ECM. It may form part of a tissue-specific acute response to remodeling stimuli. Known to act on MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-13, MMP-14 and MMP-15.</td>
<td>2</td>
<td>11.4</td>
<td>Cell+CR Only</td>
<td>ND</td>
</tr>
<tr>
<td>Metallo-proteinase inhibitor 2</td>
<td>TIMP2</td>
<td></td>
<td>4</td>
<td>41.3</td>
<td>Cell+CR Only</td>
<td>ND</td>
</tr>
</tbody>
</table>
2.4. Discussions

Current tissue-engineering approaches exploit various strategies which range from scaffold based biodegradable materials to scaffold free cell based therapy. Several scaffold based schemes exist for transplantation (e.g. urinary bladder [60], bone [61] cardiac tissue [62], urethras [63] and lung [64]) but the clinical effectiveness of construct has been limited by graft rejection, inflammation, the possibility of interspecies disease transmission and poor mechanical properties. Recent advancement in the field has also been mirrored in the scaffold free constructs for replacement of cornea, blood vessels, hepatic tissue among others [28, 33, 38]. More advanced therapies like TESA or CSTE utilise the inherent capacity of cells to create three-dimensional tissue-specific equivalents, bypassing the drawbacks associated with biodegradable scaffolds [8, 65, 66]. Moreover, the presence of insoluble cell-produced ECM, through secretion of paracrine and proliferative signals, minimises cell apoptosis, thus improving vascularisation and facilitating tight adherence onto the host tissues [67-70]. Despite the significant strides that have been made to-date, scaffold-less regeneration approaches require extended *ex vivo* culture that is far from physiological culture systems failing to emulate the native tissue microenvironment, resulting in phenotypic drift, growth arrest, cellular senescence, and loss of stem cell multipotency; impeding that way to clinical translation and commercialization [71]. Irrespective of considerable clinical significance, the fundamental challenge in the field has been to reduce time to prepare an engineered implantable construct.

In physiological conditions, the cells are surrounded by the dense array of macromolecules with limited supply of oxygen [57, 72, 73]. Maintenance of cells in extremely dilute culture conditions that is lack of macromolecular crowding, elevated oxygen tension, high serum supplementation, lack of vitamin C altogether consequences in to slower matrix deposition *in vitro* [47, 49]; causes exceedingly protracted production of implantable construct [28, 33, 38, 60-64]. Due to lower solute concentration of serum, its addition in regular culture media fails to create a crowded environment [72]. The modulation of *in vitro* microenvironment for faster construction of rich-in-ECM cell-sheets would be a significant development for future clinical applications. In current study, a technology was developed based on macromolecular crowding (a biophysical phenomenon with energetic consequences
in biological processes [73] and tissue-engineering by self assembly approaches that created within 48 hours and low serum content; a cohesive and rich in tissue-specific ECM cell-sheet, using human fibroblasts (lung and skin), tenocytes and osteoblasts.

When used at appropriate concentrations serum supplies growth factors, hormones, attachment and spreading factors, binding proteins, lipids, and minerals [74]. Fetal bovine serum (FBS) is the most widely used growth supplement for cell culture media because of its high content of physiologically balanced growth promoting factors and low levels of growth inhibitory factors for variety of cell types. The main role of serum in cell proliferation is as a provider of complex hormones [75-77] and other growth factors influencing attachment (e.g., fibronectin), propagational factors (e.g., insulin), transport factors (e.g., transferrin), and trace metals [78]. Together with its rich content of growth factors and low gamma-globulin content in comparison with other animal sera [79, 80], this has led to its adoption as the standard medium supplement. Fetal bovine serum has been shown to be superior to calf serum, newborn calf serum, and horse serum for use in cell cultivation [78, 81]. FBS typically constitutes 0.5%-10% vol/vol of the growth medium for cells [82]. In consistent with previous observations [83], high serum content for longer culture periods resulted in ECM degradation, due to the high proteolytic activity of MMPs present, a family of enzymes responsible for many developmental processes, including morphogenesis, angiogenesis, collagen metabolism and tissue remodeling [80, 83, 84]. The use of non-human origin serums for human tissue regeneration has been a major disadvantage of current in vitro culture practice that lays open a path for inter-species disease transmission, severe immune reactions and might be one of the causes of graft rejections. The uses of human serum not only enables more abundant ECM deposition, due to the lower MMP content, but also obviate issues associated with the use of FBS. This work corroborates previous studies advocating the use of human serum for cell-based therapies [85, 86].

Ascorbic acid, an important cofactor of collagen biosynthesis pathway, has a potential role in various tissue-engineering strategies. Ascorbic acid is a crucial prerequisite to achieve full post-translational modifications of prolyl and lysyl residues resulting in thermostability, secretion and extracellular crosslinking of collagens [87-89]. The omission of ascorbic acid in cell culture [90-92] results in
minimal production and deposition of collagen on the cell layer. In other words, scurvy can exist in cell culture. One of the novel applications is the cell sheet development in tissue engineering due to high ECM production. Lee et al. developed a skin dermal equivalent using skin fibroblasts treated with ascorbic acid without other foreign materials [93]. Liu et al., 2013 demonstrated that scaffold-free skin equivalents having capillary-like networks can be developed using co-culture system supplemented with ascorbic acid using various cells; keratinocytes, dermal fibroblasts, and dermal microvascular endothelial cells. The co-culture supplemented with ascorbic acid has advantages of rich in various vascularisation-associated growth factors, including VEGF, bFGF, and PDGF that of mono layer [94]. Several other studies demonstrated that the principal failure in wound healing during vitamin C deficiency is due to the limited synthesis and secretion of collagen because of impaired hydroxylation of proline residues in collagen types I and III [95, 96]. In the present work, adequate amount of ascorbic acid (100µM) was provided in all the culture media. L-ascorbic acid has a short half-life in culture and completely oxidises after 3 days, so use of the stable form of ascorbate, such as a magnesium salt of L-ascorbic acid 2-phosphate hexahydrate, is highly recommended [88].

Collagens are the most abundant proteins in the human body and are extremely important components of tissue engineering such as adhesion, signaling matrix and creating cohesion between single cells and cell-matrix layers [97, 98]. The confounding factor of cell culture in standard aqueous media is its characteristic difference from the natural, ‘crowded’ state of tissue. Thus, the enzymatic conversion of procollagen to collagen by C-proteinase is too slow to allow the deposition of a substantial collagen matrix in a useful time window [47, 49]. A low procollagen conversion rate is the default state of cell culture systems, which means an excess of procollagen in the culture medium and poor collagen matrix formation resulting in low productivity for tissue building. This work demonstrated here by applying the biophysical principle of MMC can overcome the cell culture-intrinsic problems. The data suggests that crowding of the in vitro culture medium with neutral (FC®70 and FC®400) and negatively charged (DxS, CR) macromolecules confined the space of molecules of comparable size and thus increased the interaction of the substrate.
This work hypothesised that molecular polydispersity is the key modulator of ECM deposition. Although the \textit{in vivo} milieu is a highly crowded environment composed of numerous molecules with varying size and shape (a rather polydispersed and heterogeneous setting), protein folding and stability under MMC conditions is solely assessed under homogeneous conditions and only a few \textit{in vitro} (none has used living human or animal cells; primarily bacteria cytoplasm is used as a model) and \textit{in silico} models have studied polydispersity [99-107]. As first, using living cells, this work provided the evidence that macromolecular polydispersity is a key modulator of ECM deposition, as it can most effectively exclude the volume. It was also identified that CR as the most suitable, inherently polydispersed macromolecule for accelerated tissue-specific ECM deposition. Indeed, the inherent polydispersity of CR most effectively occupied the available volume in the culture media, thereby preventing the diffusion of procollagen and proteinases in the media.

The abundant deposited ECM prohibited detachment of intact living substitutes from commercially available thermal responsive pNIPAAm dishes, whilst a pNIPAAm + pNTBA copolymer allowed for the first time the production of dense and cohesive tissue modules with intact cell-cell and cell-ECM junctions; tissue like morphology; and positively upregulated molecular functions, ECM and cellular components and biological processes. Significantly increased deposited collagenous proteins, enzymes associated with biogenesis of connective tissue proteins and cross-linking of collagen and elastin, basement membrane proteins, glycoproteins, glycosaminoglycans and proteoglycans that provide structural guidance and influence cell differentiation, migration, adhesion, phenotype maintenance and cell survival created \textit{ex vivo} a complex, yet orchestrated network. This variety of proteins detected suggests that the developed living substitutes have gone through physiological processes (e.g. post-translational modification, fibrillogenesis and molecular remodelling) provide a suitably complex milieu that resembles native tissue supramolecular assemblies. In addition to the accelerated and ample ECM deposition, this technology not only requires a lower cell number than multilayer cell sheets, which are often not available, but also bypasses the need of multi-layer cell sheets altogether, which due to poor nutrient transport, hypoxia and waste accumulation would result in cell necrosis in the central cell-layers [108].
To further advocate the potential of MMC as a means to engineer functional in vitro microenvironments that will maintain tissue specific function through deposition of tissue-specific ECM, human tenocytes and osteoblasts were cultured under MMC conditions. Results of this study indicated that, current method is readily applicable to other cell types, as MMC (75µg/ml CR) facilitated accelerated tissue-specific ECM deposition at low serum concentration (5% FBS) in human tenocyte and osteoblast culture. However, the rate of matrix deposition depends on the innate capacity of cells used to secrete matrix. Further, possibly, MMC intensifies the efficacy of survival factors present in sera, making the use of high serum supplementation redundant. This is of major importance, given that high serum concentration is often associated with phenotypic drift and unintended trans-differentiation [64, 109-115].

This work has provided evidences for supporting the notion that MMC, by imitating native tissue localised density, can be utilised to effectively modulate in vitro microenvironments and ultimately produce ECM-rich cell substitutes at low serum concentration, within hours rather than days or months in culture, without compromising fundamental cellular functions for regenerative medicine applications. Apart from skin, tendon, bone, cornea, stem cell and neuronal tissue engineering this system can also be used to develop pathophysiological models for drug discovery purposes.
2.5. References


Chapter 3 – Conclusions and Future Directions
3.1. Conclusions

The results of this study suggest that modulation of *in vitro* microenvironment with polydisperse macromolecular crowders enhances ECM deposition even under low serum supplementation and facilitates production of intact ECM rich cell-sheets, when combined with novel thermo-responsive polymer coating.

This project has resulted in the following conclusions:

- Macromolecular crowding enhances ECM deposition from human lung, skin, bone and tendon cells;
- Macromolecular crowding enhances ECM deposition, even at low serum concentration (0.5% FBS or Human Serum for fibroblasts and 5% FBS for tenocytes and osteoblasts);
- The dispersity of carrageenan was highest among all tested crowder molecules (DxS, FC 70, and FC 400) and facilitated higher ECM deposition. The results of this study advocate that maximum ECM deposition can be achieved by the most polydispersed macromolecule, confirming macromolecular polydispersity is key modulator of ECM deposition.
- Macromolecular crowding does not affect fundamental cellular properties, such as morphology, metabolic activity, viability, proliferation and protein expression.
- Macromolecular crowding accelerates the construction of ECM-rich cell sheet using thermo-responsive co-polymers.
3.2. Future Directions

Although a number of questions were addressed during this work, numerous more were also identified that may be subject of further research.

3.2.1. Evaluation of Presence of Macromolecular Crowder in Cell Layer

Macromolecular crowing has been postulated to be a biophysical phenomenon with numerous biological consequences. To this end, the addition of macromolecules in the cell culture media assumes that at the end of the culture time, the exact same amount of crowders added is removed. However, no study has shown as yet this or that the crowders are not internalised by the cells used. Further, no study has demonstrated whether crowders are entrapped within the cell layer, which may lead to foreign body response / inflammation, upon subsequent implantation of the cell-sheet. Overall, there is a substantial amount of basic research that still needs to be conducted to enable clinical translation and commercialisation of Macromolecularly crowded media.

3.2.2. Designing of Polydispersed and Variable Shape Crowding Models

Only a few studies have modelled polydispersed molecules as crowding agents [1-3]. Further, all these studies have modelled spherical shapes as crowders. Such a description may be adequate in rationalising experimental results that are obtained using Ficoll, polyethylene glycol, and dextran as crowding agents. Non-spherical shapes of macromolecules, as well as polydispersity due to variations in composition and sizes of macromolecules could have a dramatic effect on protein stability [2-5]. Thus, more accurate models should be developed.

3.2.3. Identifying Carrageenan Alternatives

This study demonstrated that carrageenan, a poly-anionic linear polymer with high degree of polydispersity, facilitated the highest ECM deposition. However, some studies have raised concerns with respect to the biomedical potential of carrageenan
by carrageenan. Thus, FDA approved alternatives to carrageenan should be identified to bring such technology to clinical practice.

3.2.4. Oxygen Tension and Macromolecular Crowding

Oxygen levels in human body range from 0.5% to 14%, depending on how far cells are located from the closest blood vessel that distributes oxygen [11]. Further, physiological oxygen tension has been shown to maintain cellular phenotype and increase extracellular matrix synthesis [12-14]. Thus, a potential future direction could be the co-assessment of oxygen tension and macromolecular crowding.
3.3. References


Chapter 4 – Appendices
### 4.1. List of Reagents, Chemicals, Kits and Antibody Used

#### 4.1.1. List of Reagents, Chemicals and Kits Used

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4.2. List of Protocols

4.2.1. Cell Culture Media Preparation

i. Human Lung Fibroblasts (WI-38), Skin Fibroblasts (WS-1) and Tenocytes
   1. Dulbecco’s Modified Eagle’s Medium (DMEM) - high glucose (4500 mg/L)
   2. FBS or HS (0.5% - 10%; according to requirement)
   3. Penicillin streptomycin (1%)

ii. Human Osteoblasts
   1. Dulbecco’s Modified Eagle’s Medium (DMEM) - low glucose (1000 mg/L)
   2. FBS (0.5% - 10%; according to requirement)
   3. Penicillin streptomycin (1%)

iii. Macromolecular Crowding Media
   1. Normal culture media (as mentioned above)
   2. L-Ascorbic Acid (100μM)
   3. Crowder Molecules
      – Dxs: 100μg/ml
      – CR: 75μg/ml
      – Mixture of Ficoll®70 + Ficoll®400: 37.5mg/ml + 25mg/ml
4.2.2. Cell Thawing and Culturing

1. Wear protective gloves and face shield and remove the tube containing the cells from liquid nitrogen cylinder.
2. Thaw the contents of tube by gentle moving in water bath at 37° C.
3. Transfer the contents of the tube in 15ml falcon tube containing 10ml of media and centrifuge the tube at 1500 rpm for 5 minutes.
4. Remove the supernatant and discard it.
5. Add 1ml of pre-warmed media in to the same falcon tube and mix properly with gentle aspiration to distribute the cells homogeneously.
6. Count the cells using haemocytometer.
7. Transfer 1ml of the cell suspension (known cell density) to a new T75 flask or as appropriate.
8. Add 9ml of appropriate culture media.
9. Label the flasks with name date and cell type with passage no.
10. Change the fresh media every 2-3 days as per requirements.
11. Monitor cells using phase contrast microscope.
12. If cells are starting to float or colour of media is changing, remove old media and rinse the cells using HBSS. Add 10ml of fresh culture media.
13. Monitor cells for confluence (cover more than 80% of the culture flask).
14. When cells are ready to split (cover more than 80% of the culture flask), remove the media and rinse the cells using 10ml of HBSS.
15. Add 5ml of trypsin/EDTA and incubate at 37° C for 5 minutes.
16. When trypsin start to lift off the cells from the flask, tap the bottom of flask gently and bring cells to corner. Use cell scraper, if needed.
17. Add 5ml of serum containing media to neutralise the action of trypsin/EDTA.
18. Rinse the bottom of flask with this solution several times.
19. Transfer the contents in 15ml tube and centrifuge the tube at 1500rpm for 5 minutes.
20. Discard the supernatant and re-suspend the cells pellet in 3ml or appropriate amount of fresh warm media.
21. Count the cells using haemocytometer
22. Plate the cells (with known density) in three-four new flasks (depends on type and number of cells) with appropriate culture media.
4.2.3. Cell Counting

1. Trypsinise the cells as above (in section 1.1).
2. Add equal amount of serum containing media, aspirate several time and transfer the cell suspension to a new falcon tube.
3. Ensure the haemocytometer is clean using 70% ethanol.
4. Take 10 µl of cell suspension and add 10 µl of trypan blue and mix them properly.
5. Take 20 µl of this solution and add 10 µl to each side of haemocytometer under the cover slip.
6. Allow the sample to be drawn out of the pipette by capillary action, the fluid should run to the edges to the grooves only.
7. Focus on the grid lines of haemocytometer using 10X objective of the microscope.
8. Focus on one set of 16 corners square.
9. Count the cells in this area of 16 squares.
10. Count only healthy cells unstained by trypan blue.
11. Count the cells that are within the square and any positioned on the right hand or bottom boundary line.
12. Dead cells stained blue with trypan blue can be counted separately for viability count.
13. Move the hemocytometer to another set of 16 corner square and carry on counting until all 4 sets of 16 corners are counted.
14. Get the average count and then multiply by two to adjust for the 1:2 dilution in trypan blue.
15. This is equivalent to number of cells * 10^4 ml.
16. Finally total number of cells can be obtained by multiplying the above number by the volume of cells suspension.
4.2.4. Cell Freezing

1. Trypsinise cells as above (in section 1.1) and centrifuge to form a cells pellet.
2. Re-suspend the pellet in adequate amount of freezing medium (40% FBS + 10% DMSO + 50% culture media) so as to make a cell suspension of 1x10^6 cells per ml.
3. Pipette up and down several times gently to ensure homogenous suspension.
4. Transfer 1ml of cell suspension to each freezing vial to adjust 10^6 cells per vial.
5. Label each tube with date cell name, passage and initials of the name.
6. Transfer the vial to -80 freezers overnight in MR frosty and after then to liquid nitrogen.
4.2.5. Cell Seeding for *In Vitro* Study

1. Preheat culture media for 30 minutes in a water bath at 37° C.
2. One confluent T75 flask is needed for approximately three 24 well plates (depends on number and percentage confluency of cells).
3. Trypsinise and count the cells as above (in section 1.1).
4. Add the required volume of appropriate medium in to cell suspension to get 50,000 cells per ml.
5. For Live/Dead® assay put the poly-l-lysine coated glass cover slip inside the 24 well plates before cell seeding.
6. 25,000 cells/cm² are needed. Seed 1 ml of cell suspension (50,000 cells per ml) in one well of 24 well plate or one well of 4 well chamber slide. For 48 well plates or 8 well chamber slides add 500µl of the cell suspension (50,000 cells per ml) in each well.
7. Incubate the cells in humidified incubator at 37° C and 5% CO₂ for 24 hours.
8. The culture will be ready for further experiment after 24 hours.
4.2.6. Macromolecular Crowding

1. Seed the cells as above at 25,000 cells/cm$^2$ with normal culture media.
2. Incubate the cells in humidified incubator at 37$^\circ$ C and 5% CO$_2$ for 24 hours to allow the cells to attach on the culture.
3. After 24 hours of cell seeding, change the media with fresh crowded media containing L ascorbic acid (100µM) and macromolecular crowder (DxS-100µg/ml or CR-75µg/ml or Ficoll®70+ Ficoll®400(37.5mg/ml+25mg/ml)
4. To analyse effect of serum, variable percentage (0.0%, 0.5%, 1.0%, 2.0%, 5.0% and 10%) of serum (FBS or HS) can be added in to the media with/without crowder.
5. The macromolecular crowding treatment can be allowed for various time points i.e. 2, 4 and 6 days.
4.2.7. Preparation of Pepsin Solutions

1. Aspirate the medium from the cell culture, store in appropriate tubes (medium portion).
2. Wash the cell layer portion with Hank’s Balanced Salt Solution (HBSS).
3. Prepare 1mg/ml pepsin in 0.5M Acetic Acid.----Solution A.
4. Use pepsin within 30 minutes after dissolving.
5. For the cell layer portion: make 1/10 (v/v) dilution of 1mg/ml pepsin in HBSS to make up a final concentration of 100µg/ml ------Solution B
6. Add solution B accordingly to the cell layer portion (150µl/well of 24 well plates).
7. For the medium portion: Add 1mg/ml pepsin (solution A) to each tube containing culture medium (100µl of solution A in 1ml of medium sample)
8. Vortex briefly and put samples on rotating shaker at 37°C for 2 hours with continuous shaking at 200 rpm.
9. After 2 hours of incubation medium portion can go for neutralization.
10. For cell layer: Scrape off the cell layer portion using tips and transfer to prelabeled tubes. Cell layer sample from 3-4 wells can be pooled in a 1.5ml tube.
11. Neutralization of cell layer and medium portion At the end of two hours, stop the pepsin activity by over-normalizing with 1N NaOH
12. For the cell layer portion:
   i. Add 5µl of phenol red solution in 100µl of cell layer sample. The samples turned in to yellow colour.
   ii. Add 5µl of 1N NaOH in 100µl of cell layer sample. Repeat this step until the samples turned in to pink colour
   iii. Vortex briefly
13. For the medium portion:
   i. Add 20 µl of 1N NaOH in 1ml of medium sample. Repeat this step until the samples turned in to pink colour
   ii. Vortex briefly
14. Store at 4° C for short-term storage or at -20° C for long-term storage.
4.2.8. Sample Preparation for SDS-PAGE

1. Collagen standard: dissolve 1.00mg of collagen type I (Symatese) in 1ml of 0.5M acetic acid. Make further dilution in 0.5M acetic acid, if needed. Neutralise the standard with 1N NaOH as described above.

2. Take the cell layer samples, medium samples and collagen standard in a fresh 1.5ml centrifuge tube (24µl each).

3. Add 24µl of double distilled water in these samples.

4. Add 12µl of 5X sample buffers to get 5:1 dilution.

5. Vortex the samples and centrifuge them briefly. Store them at 4°C.

6. Prior to SDS-PAGE, denature the samples and standard by heating at 95°C for 5 minutes.

7. Vortex and then centrifuge the samples briefly. Load 15µl per well.
4.2.9. SDS-PAGE

Materials

1. 1.875M Tris-HCl, pH 8.8. Dissolve 22.70g Tris-base (Bio-Rad, 161-0716) in 80ml ddH₂O; add 2ml concentrated HCl (37%), leave it overnight to equilibrate, adjust pH to 8.8 with a few drops concentrated HCl, make it up to 100ml with ddH₂O. Keep it at 4-8° C.

2. 1.25M Tris-HCl, pH 6.8. Dissolve 15.14g Tris-base in 70ml ddH₂O; add 7ml concentrated HCl (37%), leave it overnight to equilibrate, adjust pH to 6.8 with a few drops concentrated HCl, make it up to 100ml with ddH₂O. Keep it at 4-8° C.

3. 5x sample buffer. Dissolve completely 0.25g SDS (Bio-Rad 161-0301) in 0.625ml 1.25M Tris-HCl, pH 6.8 and 2ml ultrapure water. Leave it overnight for the foam to settle. Top up with glycerol (Bio-Rad) to 5ml (approximately 2.3ml). Add 2.5mg bromophenol blue (Bio-Rad 161-0404) per 10ml buffer.

4. 5x running buffer. Dissolve 15.1g Tris-base (Bio-Rad 161-0716), 72g glycine (Bio-Rad 161-0718) and 5g SDS (Bio-Rad 161-0301) in 1lt ddH₂O. Store at 4°C. 1x running buffer is made to run the gel from 5x running buffer by diluting in ddH₂O. Alternatively, 10x Tris-Glycine-SDS 5lt tube can be purchased (Bio-Rad 161-0772). 1x running buffer is made to run the gel from 10x running buffer by diluting in ddH₂O.

5. 30% Acrylamide/Bis (37.5:1) (Bio-Rad, 161-0158)

6. 10% SDS (Bio-Rad, 161-0416)

7. 100mg/ml Ammonium Persulphate (Bio-Rad, 161-0700) in ddH₂O. Dissolve 500mg APS in 5ml ddH₂O, aliquot it in Eppendorf tubes and keep it at - 20°C. The solution is active for a few months.

8. TEMED (Bio-Rad, 161-0800)

9. 10% and 70% Ethanol in dH₂O

Procedure for making the gels

1. Clean glass plates with 70% ethanol and wipe dry with microscope tissue papers.
2. Set the gel making apparatus ensuring that the glass plates fit snugly to the platform (mini gel: 1mm space using appropriate spacers).

3. Check for any leaks by pouring water prior to making the gels.

4. Add the gel ingredients to make the 5% resolving gel. This can be done in a 15ml conical tube (Falcon).

5. Using a Pasteur pipette, pour the prepared mixture carefully into the space between the 2 glass plates to reach about 1 cm (mini gel) from the bottom of the wells etched out by the comb (keep the excess solution to check how quickly the gels will be polymerised).

6. Overlay the gel with 10% ethanol solution to cut off oxygen in contact to the gels.

7. Leave it aside for approximately 30 minutes (check with solution remained).

8. During the setting period, prepare the 3% stacking gel (do NOT add APS & TEMED until the gel solution is ready for pouring).

9. A line at the ethanol-gel interface that initially had disappeared will reappear after the 30 minutes period indicating that polymerization is complete.

10. Carefully aspirate the ethanol out of the glass plates using a syringe and imbibe any traces using filter paper.

11. Now add the APS and TEMED to the stacking gel solution and carefully pour it on top of the polymerised resolving gel. Immediately insert the comb taking care to avoid trapping any air bubbles (keep the excess solution to check how quickly the gels will be polymerised).

12. Allow it to gelation for 10-15 minutes.
Table 4.1: Preparation of 5% separation gel and 3% stacking gel

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<td>collagen for mini gel</td>
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</tr>
<tr>
<td>1.875M Tris-HCl pH 8.8</td>
<td>1000 µl</td>
<td>2000 µl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>50 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>ddH2O</td>
<td>3070 µl</td>
<td>6140 µl</td>
</tr>
<tr>
<td>APS (100mg/ml)</td>
<td>42 µl</td>
<td>84 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>5000 µl</strong></td>
<td><strong>10000 µl</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1 Gel</th>
<th>2 Gels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3% Stacking Gel</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1mm thickness) for</td>
<td></td>
<td></td>
</tr>
<tr>
<td>collagen for mini gel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Protein II Bio-Rad)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30% Acrylamide/Bis (37.5:1)</td>
<td>200 µl</td>
<td>400 µl</td>
</tr>
<tr>
<td>1.25M Tris-HCl pH 6.8</td>
<td>200 µl</td>
<td>400 µl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>33 µl</td>
<td>66 µl</td>
</tr>
<tr>
<td>ddH2O</td>
<td>1550 µl</td>
<td>3100 µl</td>
</tr>
<tr>
<td>APS (100mg/ml)</td>
<td>17 µl</td>
<td>33 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>3 µl</td>
<td>6 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2000 µl</strong></td>
<td><strong>4000 µl</strong></td>
</tr>
</tbody>
</table>
SDS-PAGE Run

1. After the gels have been set, remove slowly the combs from the gels.
2. Assemble the electrophoresis apparatus, for small gel apparatus, fit the gel plates on the electrode bar and fit the set into the inner chamber and clamp them.
3. Fill the upper/inner chamber with 1x running buffer.
4. Wash the wells by squirting buffer into the wells with a hypodermal needle syringe to remove all air bubbles.
5. Load the standards, samples and markers using Hamilton syringe (50 µl). Wash the syringe in between using the running buffer in the chamber (at least 5-times).
6. Put the upper chamber on the main chamber, close the lid and run the gel(s)
7. For the mini gel→ run at constant voltage: 50V until the front reaches the end of the stacking gel (± 30-40 min), then 120V until the front reaches the end of the separating gel (±1 hour).
8. Remove the glass using the wonder wedge, cut the lower right hand corner and release the gel slowly into dH₂O.
9. Proceed Silver staining (SilverQuest®, Invitrogen Protocols respectively)
4.2.10. Silver Staining

Follow the steps below for silver staining by “SilverQuest™ Silver Staining Kit” of a mini gel.

**Table 4.2: Steps for silver staining**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Reagent</th>
<th>Volume</th>
<th>Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixing</td>
<td>Ethanol 40ml, Acetic Acid 10ml and Ultra pure water to 100 ml</td>
<td>100ml</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Washing</td>
<td>Ethanol 30ml, Ultra pure water to 100ml</td>
<td>100ml</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Sensitizing</td>
<td>Ethanol 30ml, Sensitiser 10ml Ultra pure water to 100ml</td>
<td>100ml</td>
<td>10 minutes</td>
</tr>
<tr>
<td>First wash</td>
<td>Ethanol 30ml, Ultra pure water to 100ml</td>
<td>100ml</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Second wash</td>
<td>Ultra pure water 100ml</td>
<td>100ml</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Staining</td>
<td>Stainer 1ml, Ultra pure water to 100ml</td>
<td>100ml</td>
<td>15 minutes</td>
</tr>
<tr>
<td>Wash</td>
<td>Ultra pure water 100ml</td>
<td>100ml</td>
<td>1 minutes</td>
</tr>
<tr>
<td>Developing</td>
<td>Developer 10ml, Developer enhancer 1 drop, Ultra pure water to 100ml</td>
<td>100ml</td>
<td>4-8 minutes</td>
</tr>
<tr>
<td>Stopping</td>
<td>Stopper 10ml, Add directly to Developing solution</td>
<td>10ml</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Final wash</td>
<td>Ultra pure water 100ml</td>
<td>100ml</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>
4.2.11. Densitometry of SDS-PAGE

1. Scan the SDS-PAGE gels on Scanner (HP Scanjet 7400C Series) with Active Transparency Adapter (HP Scanjet XPA c7671b).
2. Measure the band density of α1(I) and α2(I) bands with ImageJ software.
3. Open ImageJ
4. Go to File→Open→(your image)
5. If the image look too dark or too light
   - Image→Adjust→Brightness/contrast
   - Save the image with an updated name
6. Go to Analyze → Set measurements → Tick the following boxes
7. Area, Mean Gray Value, Standard Deviation
8. Select the rectangle tool, and draw a box around the lane
9. Go to Analyze → Measure
10. A new box with all the results will appear.
11. Repeat the step 7 and 8 for all the bands of your interest.
12. Be consistent with the area of rectangle box.
13. Copy all results and Paste in to Microsoft Excel sheet.
14. Add the mean band intensity of α1(I) with α2(I) band.
15. Normalize the mean value of Collagen type I standard. (For example- if the mean band density is 20 for 100µg/ml of Collagen type I standard, multiply all the mean value of sample with 5).
16. Plot the bands intensity as µg/ml of sample and standard bands.
4.2.12. **almarBlue® Assay**

1. At the end of culture time points, remove the medium.
2. Wash the cell layer with HBSS.
3. Add 200µl (for 24 well plate) of HBSS containing 10% almarBlue® reagent.
4. Add 200µl of 10% almarBlue® reagent and HBSS (in triplicate) in an empty 24 well plate.
5. Incubate the samples in a incubator for 4 hours at 37° C and 5% CO₂.
6. Transfer the 100µl of incubated samples into a black 96-well plate.
7. Also transfer the 100µl 10% almarBlue® reagent and HBSS from an empty 24 well plate.
8. Read the fluorescence of the media using a microplate reader (Varioskan Flash, Thermo Scientific) at excitation and emission 570 nm 600 nm wavelength.
9. Calculate the metabolic activity of the samples using the percent reduction of dye, according to the supplier’s protocol and compared with the respective control samples.
4.2.13. Live/Dead® Assay

1. At the end of culture time points, remove the medium.
2. Rinse the cell layer using HBSS for 5 minutes.
3. Add the calcein-AM and ethidium homodimer solution (2 µM calcein-AM and 4 µM EthD-1) in PBS according to manufacturer’s staining protocol. Incubate the samples in a humidified incubator for 30 minutes.
4. Wash the cell layer after this incubation using the PBS to remove the excess dyes.
5. Take at least three images per sample for live as well as dead cells in the same samples using an Olympus IX81 inverted fluorescence microscope.
6. Use the FITC filter and DX red filter for and at least three images per sample were obtained for live as well as dead cells.
7. Calculate the percent of live cells according to the manufacturer protocol.
4.2.14. Immunocytochemistry/Immunofluorescence

1. Culture cells at 25,000 cells/cm² in Labtec® II chamber slides.
2. At the end of culture time points, aspirate the medium.
3. Wash 1x with HBSS.
4. Fix with 2% paraformaldehyde (pre-cooled at 4°C) for 15 minutes. 300µl/well for 4-well chamber slides or 150µl/well 8-well chamber.
5. To make 2% PFA (in glass bottle with magnetic stirrer): Weight 0.2g of PFA and add 10ml of PBS. Put on a magnetic stirrer with heater. Leave it for around 1 hr (put the cap on, but loosen a little bit). Cool it and keep it 4°C.
6. Drain away fixative and wash 3x with PBS, 5 minutes each.
7. Block with 3% (w/v) BSA in 1x PBS for 30 minutes at RT. 300µl/well for 4-well chamber slides or 150µl/well 8-well chamber. Put the lid on.
8. Incubate with primary antibodies in 1x PBS for 1hr 30 minutes at room temperature or overnight at 4°C. 150µl/well for 4-well chamber slides or 75µl/well 8-well chamber. Put the lid on (refer to table for antibody titration).
9. Wash 3x with 1x PBS, 5 minutes each.
10. Incubate with secondary antibodies in 1x PBS (mixed) for 30 minutes at RT. 150µl/well for 4-well chamber slides or 75µl/well 8-well chamber. Put the lid on (refer to table for antibody titration).
11. Wash 3x with 1x PBS, 5 minutes each.
12. Post-fix with 2% PFA for 15 minutes at room temperature.
13. Wash 1x with PBS, 5 minutes each.
14. Incubate with methanol containing 0.5µg/ml DAPI for 5 minutes at room temperature. 150µl/well for 4-well chamber slides or 75µl/well 8-well chamber. Put the lid on.
15. Wash 3x with PBS, 5 minutes each.
16. Keep in PBS or Mount with Vectashield mounting media (Vector Laboratories Ltd, Peterborough, UK).
17. Take the images on Olympus IX-81 inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan).
18. Evaluate the fluorescence intensity measurements by ImageJ image analysis software.
19. **Fluorescence Intensity Measurements**

- Open ImageJ
- Go to File→Open→(your image)
- Go to Analyze → Set measurements → Tick the following boxes
  - Area, Mean Gray Value, Standard Deviation
- Go to Analyze → Measure
- A new box with all the results will appear.
- Copy all results and Paste in to Microsoft Excel sheet.
- Plot the “mean” intensity of slides.
- Normalize the mean intensity value of proteins with respect to control sample to make 100. (For example- if the mean intensity value is 200 for control, multiply all the mean value of sample with 0.5).
- Plot the fluorescence intensity of control and test sample.
Table 4.3: Primary antibody, source and dilution

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Collagen III</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Collagen V</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Collagen VI</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Collagen VII</td>
<td>Mouse monoclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>Laminin</td>
<td>Rabbit polyclonal</td>
<td>1:100</td>
</tr>
<tr>
<td>Elastin</td>
<td>Rabbit polyclonal</td>
<td>1:50</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Mouse monoclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Mouse monoclonal</td>
<td>1:400</td>
</tr>
<tr>
<td>α-Smooth Muscle Actin</td>
<td>Mouse monoclonal</td>
<td>1:500</td>
</tr>
<tr>
<td>Epithelial Keratin</td>
<td>Mouse monoclonal</td>
<td>1:50</td>
</tr>
<tr>
<td>Hyaluronic Acid</td>
<td>Sheep polyclonal</td>
<td>1:300</td>
</tr>
<tr>
<td>Keratan Sulfate</td>
<td>Mouse monoclonal</td>
<td>1:500</td>
</tr>
<tr>
<td>Chondroitin Sulfate</td>
<td>Mouse monoclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Heparan Sulfate</td>
<td>Mouse monoclonal</td>
<td>1:100</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>Mouse monoclonal</td>
<td>1:50</td>
</tr>
<tr>
<td>Biglycan</td>
<td>Rabbit polyclonal</td>
<td>1:100</td>
</tr>
<tr>
<td>Decorin</td>
<td>Goat polyclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Lysyl-Oxidase (LOX)</td>
<td>Mouse monoclonal</td>
<td>1:200</td>
</tr>
<tr>
<td>Transglutaminase -2</td>
<td>Rabbit monoclonal</td>
<td>1:50</td>
</tr>
<tr>
<td>Fibrillin-1</td>
<td>Mouse monoclonal</td>
<td>1:100</td>
</tr>
<tr>
<td>TEM-1</td>
<td>Rabbit polyclonal</td>
<td>1:50</td>
</tr>
<tr>
<td>IL-10</td>
<td>Rabbit polyclonal</td>
<td>1:1200</td>
</tr>
<tr>
<td>Tenomodulin</td>
<td>Rabbit polyclonal</td>
<td>1:200</td>
</tr>
</tbody>
</table>
## Table 4.4: Secondary antibody, source and dilution

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlexaFluor®488</td>
<td>Chicken anti Mouse</td>
<td>1:400</td>
</tr>
<tr>
<td>AlexaFluor®488</td>
<td>Chicken anti Rabbit</td>
<td>1:400</td>
</tr>
<tr>
<td>AlexaFluor®488</td>
<td>Chicken anti Goat</td>
<td>1:400</td>
</tr>
<tr>
<td>AlexaFluor®555</td>
<td>Goat anti Mouse</td>
<td>1:400</td>
</tr>
<tr>
<td>AlexaFluor®555</td>
<td>Goat anti-Rabbit</td>
<td>1:400</td>
</tr>
<tr>
<td>Alexa Fluor®568</td>
<td>Donkey anti-Sheep</td>
<td>1:400</td>
</tr>
<tr>
<td>DAPI</td>
<td>NA</td>
<td>1:5000</td>
</tr>
</tbody>
</table>
4.2.15. DAPI Nuclear Staining and Counting

1. Make the 3% paraformaldehyde (PFA) solution.
2. After culture time points remove the medium from the culture.
3. Wash the cell layer 1 times using HBSS.
4. Add the 3% PFA solution to fix the cell layer.
5. Wash the cell layer 1 times (5 minutes) using HBSS.
6. Add pre-chilled (at least overnight at -20° C) methanol, 300\(\mu l\)/well for 24 well plates, incubate for 10 minutes at room temperature.
7. Add 100 \(\mu l\) per well (24 well plate or 4 well chamber slide) DAPI solution (0.5\(\mu g/ml\)) and incubate for 5 minutes at room temperature in dark.
8. Wash the cell layer 3 times (each 5 minutes) using HBSS.
9. Mount with Vectashield mounting media (Vector Laboratories Ltd, Peterborough, UK).
10. Take the images on Olympus IX-81 inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan) using the DAPI filter.
11. Count the DAPI stained nuclei using ImageJ 1.44 (NIH, USA) software.
12. Take the average number of nuclei for each sample from 3 random images.


4.2.16. Gelatin Zymography

1. Gelatin solution: 2.65 mg/ml in water (heat at 65° C degrees to dissolve), sterile filter (can store this for 6 months at 4 degrees).

2. 10% acrylamide gelatin migration gel recipe
   - Gelatin Solution: 8.3ml
   - 1.5 M Tris (Ph8.8): 5.25ml
   - 30% Acrylamide-Bisacrylamide: 7ml
   - 50% Glycerol: 165µl
   - 10% SDS: 165µl
   - TEMED: 10µl
   - 10% APS: 100µl

3. 3% Stacking gel recipe
   - 30% Acrylamide/Bis (37.5:1): 200µl
   - 1.25M Tris-HCl (pH 6.8): 200µl
   - 10% SDS: 33µl
   - ddH2O: 1550µl
   - APS (100mg/ml): 17µl
   - TEMED: 3µl

4. After culture time points aspirate the medium and collect them in pre-labeled 1.5 ml tubes. Store at -20° C.

5. Mix the sample (10µg or less) with non reducing 5x SDS sample buffer.

6. Run the gel at 20 mA/gel (see above in SDS-PAGE section).

7. Wash the gels briefly with double distilled water.

8. Incubate the gels in 2.5% Triton X-100 solution in distilled water for 30 minutes. Repeat this step one more time.

9. Incubate the gels for 18 hours at 37° C in a reaction buffer containing 50 mM Tris pH 7.4, 5 mM CaCl$_2$, 1 µM ZnCl$_2$ to support recovery of protease activity.

10. After this incubation stain the gels with 0.5% Coomassie R250 brilliant blue for 30 minutes.

11. Take the images of gels after de-staining with 30% ethanol, 10% acetic acid.

12. Compare the relative expression of MMP2.

13. Use the uncultured medium with various percentages of serum as control.
4.2.17. Development of Cell Sheets

1. Mix the poly(NIPAAM : N-t-BAM) at the ratio of 40µg/ml in alcohol.
2. Mix poly pNIPAAm and pNTBA in anhydrous ethanol in the ratio of 40µg/ml and 20µg/ml.
3. Leave polymer solution overnight with continuous shaking.
4. Pour ~500µl of this polymer solution to the petri-dish (area-3.5 cm²).
5. Spread the polymer uniformly on dish surface.
6. Leave these petri dishes in to the desiccators to dry out the excess of polymer.
7. Leave these petri dishes in to the vacuum oven at 60° C overnight.
8. Leave the petri dishes under the ultraviolet light for 2 hours for the sterilization.
9. Seed the cells at 25,000 cells/ cm² on petri dishes.
10. Change the medium after 24 hours with medium containing crowder.
11. Incubate the culture for next 2days in the humidified chamber at 37° C and 5% CO₂.
12. After culture time point take out the petri dishes from the incubator and leave it at 10° C for 30 minutes.
13. Collect the cell sheet gently with help of tweezers or forceps without breaking it.
4.2.18. Scanning Electron Microscopy (SEM)

1. Harvest the cell sheets as described above.
2. Cut the cell sheet in small pieces of approximately 1 mm³.
3. Fix the cell sheets in 2.5% glutaraldehyde for 1 hour.
4. Post-fix the cell sheets using 1.5% of osmium tetra oxide.
5. Dehydrate the samples using graded ethanol series.
6. Transfer the cell sheets to the adhesive carbon tabs mounted on SEM specimen stubs and then dry.
7. Coat it with gold using an Emitech K550 coating system.
8. Take the SEM images using Hitachi H4700 scanning Electron microscope (SEM) system using a beam voltage of 15kV.
4.2.19. Atomic Force Microscopy (AFM)

1. Seed the cells on 4-well Lab-Tek™ II chamber slides at 50,000 cells/chamber.
2. Incubate the cells in humidified chamber at 37° C and 5% CO₂.
3. Change the medium after 24 hours with medium containing crowder.
4. At the end of culture time point, wash the cell layers with HBSS.
5. Fix the cell layers with 2% PFA at room temperature for 15 minutes.
6. Wash the cell layers three times with PBS.
7. Dehydrate the samples serially dehydrated with 30%, 50%, 70%, 90% and 100% ethanol.
8. Perform the AFM (MFP-3D, Asylum Research) using rectangular Si cantilevers (SSS-NCH, Nanosensors) each having a nominal resonance frequency of 330 kHz and a spring constant of 42 N/m.
9. Record the AFM images using amplitude modulation mode in an ambient environment after drying the sample with nitrogen gas.
4.2.20. Masson’s Trichrome Staining

1. Seed the cells on 4-well Lab-Tek™ II chamber slides at 50,000 cells/chamber.
2. Incubate the cells in humidified chamber at 37° C and 5% CO₂.
3. Change the medium after 24 hours with medium containing crowder.
4. At the end of culture time point, wash the cell layers with HBSS.
5. Fix the cell layers with 2% PFA at room temperature for 15 minutes.
6. Fix the cell sheets in Bouin’s solution for 1 hour at 56°C.
7. Incubate the samples in Weigert's iron Hematoxylin staining for 10 minutes, followed by rinsing with running tap water.
8. Wash the samples with distilled water, after 10 minutes in warm running tap water.
9. Incubate the samples with Biebrich scarlet-acid fuchsin solution for 10-15 minutes and wash again with the distilled water.
10. Incubate the samples with phosphomolybdic-phosphotungstic acid solution for 15 minutes for differentiation.
11. Transfer the samples directly in aniline blue solution and stained for 10 minutes.
12. Rinse with brief distilled water differentiated again using 1% acetic acid solution for 5 minutes.
13. Dehydrate the samples quickly through 95% ethyl alcohol, absolute ethyl alcohol (these step wipes off Biebrich scarlet-acid fuchsin staining) and cleared in xylene after brief washing in distilled water.
14. Mount the samples and take the images using Olympus BX-51 microscope (Olympus Corporation, Tokyo, Japan).
4.2.21. Picro-Sirius Red Staining

1. Seed the cells on 4-well Lab-Tek™ II chamber slides at 50,000 cells/chamber.
2. Incubate the cells in humidified chamber at 37° C and 5% CO₂.
3. Change the medium after 24 hours with medium containing crowder.
4. At the end of culture time point, wash the cell layers with HBSS.
5. Fix the cell layers with 2% PFA at room temperature for 15 minutes.
6. Quick wash under running water.
7. Incubate the cell sheets with weigert’s Hematoxylin solution for 10 minutes.
8. Rinse briefly under running tap water.
9. Stain with 0.2% phosphomolybdic acid hydrate.
10. Rinse briefly under running tap water.
11. Stain the cell-sheets with Picro-Sirius Red followed by wash in acidified water.
12. Dehydrate the samples through a series of ethanol baths (70%, 80%, 90%, and 100%).
13. Perform the final dehydration in xylene for least 5 minutes.
15. Take the images using Olympus BX-51 microscope (Olympus Corporation, Tokyo, Japan).
4.2.22. Protein Extraction

Extraction of proteins can be performed by using Qproteome™ mammalian protein preparation kit (Qiagen, UK).

1. Seed the cells on 24-well plates (25,000 cells/cm$^2$).
2. Incubate the cells in humidified chamber at 37° C and 5% CO$_2$.
3. Change the medium after 24 hours with medium containing crowder [(75µg/ml CR), 0.5% HS and 100µM L-ascorbic acid phosphate].
4. At the end of culture time point, wash the cell layers with PBS.
5. Aspirate cell-culture medium from culture plate.
6. Wash cells twice with 5ml ice-cold PBS.
7. Add 10ml ice-cold PBS.
8. Remove cells from culture plate by gentle scraping with cell-scaper and transfer cells to a pre-chilled 15ml conical tube.
9. Centrifuge cell suspension for 5 minutes at 450 x g in a centrifuge precooled to 4°C. Discard supernatant. Keep cell pellet on ice.
10. Add 1 U Benzonase® Nuclease and 10µl of Protease Inhibitor (100x) to 1ml Mammalian Lysis Buffer.
11. Resuspend 5–10 x 10$^6$ cells in the Mammalian Lysis Buffer prepared in step 6 and incubate on a rotary shaker for 5 minutes at 4°C.
12. Centrifuge the suspension for 10 minutes at 14,000 x g in a microcentrifuge tube pre-cooled to 4°C.
13. Transfer the supernatant into a into pre-chilled 0.5ml tubes (Protein LoBind Tubes, Eppendorf, UK).
14. For some applications, or if concentration of protein is low: the protein fraction may need to be concentrated. This can be achieved by acetone precipitation.
4.2.23. Acetone Precipitation of Protein Fractions

This protocol is suitable for concentrating and desalting protein samples for downstream applications.

1. Add four volumes of ice-cold acetone to the protein fraction and incubate for 15 minutes on ice.

2. Centrifuge for 10 minutes at 12,000 x g in a pre-cooled microcentrifuge at 4°C. Discard the supernatant and air dry the pellet. Do not over dry the pellet as this may make it difficult to resuspend.

3. Resuspend the pellet in the required sample buffer. (For 2D-PAGE, an extra desalting step may be required).

4. Resuspend the pellet from step 2 in 100μl 8M urea.

5. Desalt the sample using a gel filtration device.

6. Repeat steps 1 to 3.
4.2.24. SDS-PAGE and gel band excision for proteomic analysis

1. Dilute to give total protein desired using 4X NuPAGE® gel loading buffer and water.
2. Make up molecular weight marker.
3. Reduce the protein samples with NuPAGE® reducing agent at 70°C for 10 minutes.
4. Add iodoacetamide to final concentration of 2mM and incubated for 30 minutes at room temperature in the dark.
5. Vortex sample to mix.
6. Separated the samples on 4-12% Bis-Tris Gels (NuPAGE®Novex®, 1.0-mm thick, 10-well, Invitrogen, UK).
7. Run the gels in 1X NuPAGE® MOPS 3-(N-morpholino) propanesulfonic acid SDS running buffer (Invitrogen, UK) containing NuPAGE® antioxidant (Invitrogen, UK) in the upper buffer chambers of XCell SureLock™ Mini-Cell electrophoresis system (Invitrogen, UK).
8. Use5µl of precision plus protein standards (Bio-Rad, UK) as a molecular weight marker with the range of 250kDa to 10kDa.
9. Stain protein bands with Coomassie brilliant blue and destained with destaining solution (40% ethanol, 10% acetic acid and 50% deionised water).
10. Excise the band in a laminar flow cabinet with the help of light box.
11. Excise the gel bands with scalpel and cut each slice into 1mm cubes.
12. Transfer the slices into protein LoBind tubes.
13. The complete proteomics analysis can be carried out at Central Proteomics Facility, Sir William Dunn Pathology School, Oxford University, UK.
4.2.25. Mass Spectroscopy

Mass spectroscopic analysis was carried out at Central Proteomics Facility, Sir William Dunn Pathology School, Oxford University, UK.

4.2.26. Nanoparticle tracking analysis (NTA)

Carried out at Children's Cancer and Blood Foundation Laboratories, Departments of Pediatrics, Cell and Developmental Biology, Weill Cornell Medical College, New York, USA.
4.3. Research Outputs

4.3.1. Patent

4.3.2. Awards and Honours
1. Young Investigator Award - TERMIS-EU 2014 (Tissue Engineering and Regenerative Medicine International Society) for Best Oral Presentation and for demonstrating outstanding achievements within Tissue Engineering and Regenerative Medicine field. 10th - 13th June 2014, Genova, Italy.

4.3.3. Publications


4.3.3. Conferences Presentations


Chapter 4

microenvironment to accelerate extracellular matrix deposition: macromolecular crowding meets tissue engineering by self-assembly, Oral presentation at Matrix Biology Ireland: From Pathophysiology to Therapy, Galway, Ireland, 19th – 21st November 2014.


microenvironment to accelerate extracellular matrix deposition: macromolecular crowding meets tissue engineering by self-assembly, **Oral presentation** at Tissue Engineering and Regenerative Medicine (TERMIS) EU Congress, Genova, Italy, 10th - 13th June 2014.


20. Spanoudes KM, **Satyam A**, Pandit A, Zeugolis DI. Macromolecular crowding maintains tenogenic phenotype *ex vivo*, **Oral presentation** at Tissue Engineering and Regenerative Medicine (TERMIS) EU Congress, Genova, Italy, 10th - 13th June 2014.


32. Kumar P, **Satyam A**, Ritter T, Raghunath M, Pandit A, Zeugolis D. Evaluation of extracellular matrix deposition and organisation by corneal fibroblasts and macromolecular crowding, **Oral presentation** at The 9th World Biomaterials Congress, Chengdu, China, 1st-5th June 2012.

33. Fan X, Nash M, Nosov M, **Satyam A**, Zeugolis D, Rochev Y. Functional tissue engineering thermoresponsive films for improved biocompatibility non-enzymatic multicellular detachment, **Poster presentation** at The 9th World Biomaterials Congress, Chengdu, China, 1st-5th June 2012.


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