CELLULOSE NANOWHISKERS FOR SKELETAL MUSCLE ENGINEERING

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LIST OF ABBREVIATIONS

AB = Antibiotic/Antimycotic preparation
AFM = Atomic Force Microscope
ATP = Adenosine Triphosphate
BC = Bacterial Cellulose
bFGF = basic Fibroblast Growth Factor
bHLH = basic Helix-Loop-Helix
BSA = Bovine Serum Albumin
C2C12 = The C2C12 murine myoblast cell line
CAF = Crocidolite Asbestos Fibre
CBD = Cellulose-Binding Domain
CESA = Cellulose Synthase
CFU = Colony Forming Units
CNC = Cellulose Nanocrystal
CNW = Cellulose Nanowhisker
DAPI = Diamidinophenylindole
DM = Differentiation Medium
DMA = Dynamic Mechanical Analysis
DMEM = Dulbecco’s Modified Eagle’s Medium
DMT = Derjaguin-Muller-Toporov
DNA = Deoxyribonucleic acid
DP = Degree (of) Polymerisation
DTGS = Deuterated L-Alanine Tri-Glycine Sulphate
ECM = Extracellular Matrix
EGF = Epithelial Growth Factor
FA = Focal Adhesion
F-Actin = Filamentous Actin
FAK = Focal Adhesion Kinase
FBS = Fetal Bovine Serum
FITC = Fluorescein Isothiocyanate
FT-IR = Fourier Transform Infrared Spectroscopy
GM = Growth Medium
GRGDS = Glycine-Arginine-Glycine-Aspartic Acid-Serine
HA = Hyaluronic Acid
HEMA = 2-Hydroxyethylmethacrylate
HGF = Hepatic Growth Factor
HMDS = Hexamethyldisilazane
HS = Horse Serum (Adult)
HPTMA = Hydroxypropyltrimethyl ammonium Hydroxide
IGF-1 = Insulin-like Growth Factor 1
IKVAV = Isoleucine-Lysine-Valine-Alanine-Valine
JKR = Johnson-Kendall-Roberts
KASH = (Klarsicht/Anc-1/Syne homology)
KO = Knock-Out
LBL = Layer-By-Layer deposition
LINC = Linkers of Nucleoskeleton and Cytoskeleton
LODP = Levelling-Off Degree of Polymerisation
MHC = Major Histocompatibility Complex
MRF = Myogenic Regulatory Factor
MMP = Matrix metalloproteinase
MSC = Mesenchymal Stem Cell
Mw = Mean molecular weight
MWCNT = Multi-Walled Carbon Nanotube
NBF = Neutral Buffered Formalin
NFC = Nano-Fibrillar Cellulose
NGF = Nerve Growth Factor
NSAID = Non-Steroidal Anti-Inflammatory Drug
PAA = Poly-Acrylic Acid
PAHCl = Poly-(Allylamide) Hydrochloride
PBS = Phosphate Buffered Saline
PDMS = Polydimethylsiloxane
PEI = polyethyleneimine
PEO = Polyethylene Oxide
PEM = Polyelectrolyte Multilayer
PNIPAAm = poly(N-isopropylacrylamide)
PFOS = trichloro(1H,1H,2H,2H-perfluorooctyl)silane
PGA = Polyglycolic Acid
PLL = Poly(L-lysine)
PS = Polystyrene
PSS = poly(sodium-4)-styrenesulfonate
PU = Polyurethane
QCM = Quartz Crystal Microbalance
RGD = Arginine-Glycine-Aspartic Acid
RH = Relative Humidity
RICE = Rest Ice Compression and Elevation
RMS = Root Mean Square
RNA = Ribonucleic acid
RPM = Revolutions Per Minute
SAM = Self-Assembling Monolayer
SELEX = Systematic Evolution of Ligands by EXponential Enrichment
SEM = Scanning Electron Microscope
Shh = Sonic hedgehog
SIEBIMM = Strain-Induced Elastic Buckling Instability for Mechanical Measurement
SFM = Serum-Free Medium
SP = Side Population
SUN = (Sad1/Unc-84)
SUSY = Sucrose Synthase
TC = Terminal Complex
TEM = Transmission Electron Microscope

TEMPO = 2,2,6,6-tetramethylpiperidine-1-oxyl radical

TGA = Thermogravimetry

UCST = Upper Critical Solution Temperature

UDP = Uridine 5’-disphosphate

WNT = Wingless-related integration site

XPS = X-Ray Photoelectron Spectroscopy
ABSTRACT

Prior work has shown that spin-coating tunicin cellulose nanowhiskers onto a glass surface creates a highly oriented surface that supports the adhesion, spreading and proliferation of myotubes.

Building on this work, this project aimed to develop culture surfaces with biologically active topography and tuneable stiffness with the aim of better mimicking native muscle tissue. The ultimate aim is to develop biomaterials that can direct the differentiation of mesenchymal stem cells. Cellulose nanocrystals (CNWs) from *Ascidiella spp* were isolated and characterised.

Polyelectrolyte multilayers (PEMs) are nanocomposite films formed from the sequential deposition of oppositely charged polymers and offer a flexible method of building films with a variety of chemical compositions and physical properties. CNWs were used in combination with chitosan to create PEMs using a combination of two well-established, low-cost and facile production methods, dip-coating and spin-coating.

The resulting PEM was shown to be a nanoporous substrate that was stable under cell culture conditions. It robustly allowed the attachment, alignment and myogenic differentiation of the immortalised C2C12 myoblast cell line. Proteomic analysis of the ECM produced by C2C12 cells in response to the substrate showed that cells cultured on CNW-chitosan PEMs secreted increased fibronectin, tenascin-c, elastins and collagen I, an expression pattern that is consistent with a more developmental, rather than mature, muscle ECM.

The thickness and mechanical stiffness of the PEM films could be tuned by replacing increasing volume fractions of CNWs with poly(4-sodium styrene sulfonate) (PSS). The thickness of the dry films increased with increasing CNW content, increasing from 20 nm for films containing 12 bilayers of PSS and chitosan to 100 nm for films containing 12 bilayers of CNW and chitosan. The compressive stiffness of hydrated films decreased with increasing CNW content, from 1.67 ± 0.73 MPa, to 1.06 ± 0.24 MPa. Unfortunately, PSS-modified PEMs proved to be cytotoxic to cells.

The response of bone marrow stem cells to the substrates showed that mesenchymal stem cells were contact guided by the CNWs, but did so by avoiding the material, thus being better guided by substrates where CNWs were present at a low surface density than substrates where it was present at a high density. When cultured directly on PEMs, MSCs expressed myogenin, a key marker of terminal muscle differentiation, which was suggestive, but not definitive, of a potential of the biomaterial to direct the myogenic differentiation of MSCs.
DECLARATION

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1 INTRODUCTION

Tissue engineering is the artificial creation of tissues from cells and biomaterials for the purpose of implantation or for drug testing. Since emerging as a separate science in the 1960s, tissue engineering has had a role in extending the range of surgical and therapeutic treatments for a range of diseases and injuries. Significant progress has been made\(^1\). Bone is by far the best represented tissue with both many well-established products for improving the integration of joint replacements and fixatives into existing bone, as well as in bone grafting, for example with demineralised bone matrix and injectable substitutes such as Calcibon\(^2\),\(^3\). It is still an area of active ongoing research. A wide range of synthetic and biological materials to supplement autologous and cadaveric skin grafts such as Integra\(^4\), Permacol\(^5\) and Apligraf\(^6\) are commercially available. The importance of innervation for sensation and movement has long been appreciated and products to repair critical-sized nerve gaps are both in commercial application (e.g. NeuraGen\(^7\)) and in academic development\(^8\). Solutions for skeletal muscle repair are not nearly as developed, with no commercial tissue-engineered muscle available. While successful bone repair can save limbs from amputation, the lack of successful muscle repair can lead to long-term disability and disfigurement\(^9\).

The aims of tissue engineering are moving from using materials to replace lost function (although this is still vital) to the development of materials that allow tissues to restore as much of their original function as possible\(^10\). This change in emphasis has several drivers. Non-living materials cannot remodel with the body and wear instead – the average lifespan is increasing and previous once-in-a-lifetime surgeries (such as hip replacements) have to be revised\(^11\). The numbers of people who could potentially benefit from tissue engineered materials is increasing both in absolute numbers and in relative numbers. This is as the consequences of an ageing global population and of so-called “lifestyle diseases” such as obesity and Type II diabetes become increasingly prevalent\(^12\). Additional pressures come from drug-resistant bacteria that more readily colonise non-vital material surfaces. These form a protective biofilm that hinders antibiotic penetration, making such infections difficult to treat\(^13\).
The current emphasis is on ‘instruction’: to effect the regeneration of lost tissue and function by stimulating specific cellular responses at the level of molecular biology. Achieving this aim requires a much more thorough understanding of the interplay of chemical, physical and genetic processes that control the normal development and function of healthy tissue than currently exists.

For skeletal muscle in particular, stem cell therapies have been promising but are limited by the rarity of muscle stem cells. As will be discussed in the literature review, the cells in a tissue are guided and supported by the extracellular matrix which provides the cells constituting a tissue with the appropriate mechanical, topographical and chemical cues. While the full complexity of the extracellular matrix cannot be reproduced, its essential features can be elucidated and mimicked to design.

The hypothesis of this project is that, by controlling the mechanical and topographical properties of a substrate, the differentiation of stem cells can be positively influenced. In particular, this project aimed to use nanoscale topography to guide cell alignment on a compliant matrix. The nanotopographic features would be provided by cellulose nanowhiskers.

Cellulose nanowhiskers (CNWs) are high-aspect ratio cellulose crystals obtained by the selective oxidation of cellulose. As an abundant biopolymer, cellulose is a sustainable, naturally-derived material. Depending on the species they are extracted from, CNWs vary from three to twenty nanometers in diameter and from hundreds to thousands of nanometers in length.

CNWs offer several useful properties for biomaterials. Mechanically, CNWs are very rigid and can serve to strengthen nanocomposites, even at low inclusion percentages. Secondarily, their shape allows anisotropic material properties to be engineered if they are suitably oriented. Cellulose is only slowly degraded in vivo, making CNWs suitable for applications where scaffold strength needs to be maintained for an extended period of time.

CNWs have been shown to be biocompatible and do not provoke an acute inflammatory response nor a chronic foreign body response. Finally, as they
have a large surface area relative to their volume, they can be functionalised with a variety of compounds to stimulate a particular cell response\textsuperscript{17}.

Layer-by-layer construction is a method of depositing successive layers of oppositely charged polymers or particles to create nanocomposites. This technique was used in this work to develop cellulose nanowhisker-based thin nanocomposite films. Within these films, CNWs were used in two different ways. First, they were used to provide contact guidance to cells, building on prior work\textsuperscript{18-19} by offering cells a more mechanically-relevant substrate while preserving cell orientation. CNWs are able to function as contact guides because their size presents cells with topographical features on the same length scale as integrins, the major transmembrane receptors involved in cell-extracellular matrix interactions. Second, they were used to modulate the stiffness of the range of layered nanocomposites. The resulting influence the films have had on the orientation and myogenic differentiation of the C2C12 mouse myoblast cell line and human mesenchymal stem cells (hMSCs) will be discussed.

Cells are normally limited in the number of times they can divide: each cell division is accompanied by the shortening of the DNA strands\textsuperscript{20}. Telomeres, which are regions of DNA that do not code for any genes, exist at the end of DNA strands for the purpose of being gradually shortened with successive divisions and cells become senescent once they reach a critical length. Cells which are able to express telomerase are able to lengthen the cell’s telomeres and thus avoid replicative senescence; these cells are immortalised\textsuperscript{21}.

The murine C2C12 cell line, developed by Yaffe and Saxel from normal mouse tissue in 1977\textsuperscript{22}, is commonly used in\textit{in vitro} skeletal muscle studies. It is a well characterised cell line in terms of growth, differentiation and gene expression that grows readily under\textit{in vitro} conditions. The C2C12 cell line is immortalised, but it is not transformed. While does not undergo replicative senescence, it is otherwise a normal, non-cancerous cell. As a non-transformed cell type, it is able to respond to muscle differentiation signals, exit the cell cycle and form terminally-differentiated muscle fibres\textsuperscript{23}. C2C12s were thus used to
develop and optimise the cellulose-based films before testing mesenchymal stem cells (which are not immortal) on the most promising substrates.

The following literature review will introduce cell responses to the mechanical properties of materials. The structure and developmental origin of skeletal muscle will next be introduced, with an emphasis on the way in which its structure creates the challenge of muscle repair and review extant strategies under development for effecting muscle tissue repair. The final section of the literature review will look at cellulose as a material and at the system of layer-by-layer construction used to present it to cells.
2 LITERATURE REVIEW

2.1 CELL RESPONSE TO THE MECHANICAL PROPERTIES OF BIOMATERIALS

2.1.1 BASIC DEFINITIONS

The mechanical properties of a material are those that describe that material’s ability to receive or exert force. This section will define the key mechanical properties of interest to tissue engineering applications and describe how they relate to soft materials, with an emphasis on the challenge of measuring them in the latter.

Elasticity is the ability of a material to regain its original dimensions after being deformed by an applied force. Elastic deformation is thus distinguished from plastic deformation, where the deformed material does not recover its original dimensions once the load is removed.

The stiffness of a material is its resistance to deformation on the application of an external force (or load) within its elastic deformation regime. The applied load is normally described in terms of stress, $\sigma$, which is the force applied per unit area. It therefore takes on the units of pressure, Pa. The material deformation or strain, $\varepsilon$, is the change in length of a given dimension for a given stress. It has no units.

Because there are many ways stress can be applied to a material, e.g. tensile, compressive, torsional, shear, the measured stiffness needs to be qualified according to what stress has been applied. For a linearly-applied force to an object of uniform cross section, the stress and strain may be described by Equations 2-1 and 2-2 below.

$$\sigma = \frac{\text{Force}, F}{\text{Area}, A}$$

Equation 2-1: Definition of mechanical stress

$$\varepsilon = \frac{\Delta L}{L}$$

Equation 2-2: Definition of mechanical strain
Where \( \sigma \) is the stress, \( F \) is the force applied to the material, \( A \) is its actual cross-sectional area, \( \varepsilon \) is the strain, \( \Delta L \) is the amount by which the length of the object changes and \( L \) is the original length of the object prior to deformation.

The most common measure of linear stiffness is the elastic modulus, \( E \), also known as Young’s modulus of elasticity. Young’s modulus may be either compressive or tensile and is defined by Equation 2-3, which holds true only in cases of uniaxial stress.

\[
E = \frac{\text{Stress, } \sigma}{\text{Strain, } \varepsilon}
\]

Equation 2-3: Definition of Young’s modulus of elasticity

Substituting Equations 2-1 and 2-2 into 2-3, we obtain the relationship

\[
E = \frac{FL}{A\Delta L}
\]

Equation 2-4: Expression of Young’s modulus in terms of material dimensions

In more complex loading situations, where the applied stress is not uniaxial, the elastic modulus is described in terms of the stress applied, e.g. a material’s resistance to shear stress is known as the shear modulus, \( G \). Except where noted, all the mechanical measurements described or performed in this work are as a result of uniaxial stress.

Compliance is the inverse of stiffness and is the strain observed in a material on the application of a given stress. A material with a high elastic modulus will necessarily have a low compliance and vice versa. Compliance is of particular interest in biological tissues such as lung and blood vessels, where the ability to undergo elastic deformation is critical to their normal function.

The strength of a material is defined as the stress a material can withstand before yielding plastically, and therefore deforming permanently. As with stiffness, strength can be defined as tensile or compressive. The stress at which a structure plastically deforms is known as the yield strength. If the material continues being strained, the material response is determined by its ductility, the material’s ability to deform plastically. For brittle materials, such as glasses, ceramics and polymers below their glass transition point, the point at which the material first yields is the same point at which it ruptures. Ductile materials,
such as metals and polymers above their glass transition point, are able to deform plastically before they fail. The stress at the point at which a ductile material undergoing plastic deformation starts to neck, when the cross-sectional area of the structure starts to reduce as the material is pulled thinner, is its ultimate strength. Figure 2-1 below illustrates an idealised stresses-strain curve for a) a ductile material and b) a brittle material.

Figure 2-1: Idealised stress-strain curves for A, a ductile and B, a brittle material. E, the elastic modulus, is the gradient of the stress/strain curve. In a ductile material, the transition between elastic and plastic deformation occurs at the yield strength, while in a brittle material the material undergoes limited if any plastic deformation before failing.
2.1.2 MECHANICAL PROPERTIES OF BIOLOGICAL MATERIALS

A biological tissue such as bone or liver is necessarily a composite, consisting of cells arranged within a complex architecture composed of extracellular matrix (ECM). Notable exceptions exist: epidermis, mature hair and nails are acellular and blood has no solid ECM.

The extracellular matrix provides tissues with their essential structure, giving organs their characteristic shape. It provides mechanical properties, allowing organs to respond appropriately to the stresses imposed on them. ECM is assembled from proteins which form fibrils, which in turn assemble into larger structures, creating a hierarchical structure that provides spatial cues on several length scales. It keeps populations of cells within their correct spatial relationships with one another (over several millimetres to centimetres), it provides individual cells with appropriate spatial cues (over several nanometers to microns)\textsuperscript{24,25}. The constituents of ECM in themselves provide vital cell-signalling cues for the normal proliferation and differentiation of cells.

The ECM serves as a binding site for several essential growth factors, modulating their availability and signalling patterns. When ECM is disrupted by protease cleavage or by injury, concealed sites are exposed in the ECM proteins that act as signals for tissue repair and remodelling mechanisms\textsuperscript{26}.

The core constituents of ECM are collagens, proteoglycans and glycosaminoglycans (GAGs). These are associated with a variety of growth factors, as well as matrix modifying molecules such as metallo-matrix proteases (MMPs).\textsuperscript{27}

As most tissues are composites, their initial response to stress is to undergo a reorganisation of ECM fibres in line with the applied stress before the material itself starts to strain, leading to a quadratic response to stress (see Figure 2-2). The contribution of the various ECM components to stress-strain behaviour has been elucidated using sequential enzyme digestion of these components. Early investigations by Hoffman \textit{et al.}\textsuperscript{28} on bovine nuchal ligament and Sacks \textit{et al.}\textsuperscript{29} on bovine skeletal muscle, using sequential enzyme digestion of collagens and elastins helped to elucidate the relative contributions of these major ECM
components. Samples digested with collagenase become elastomeric, able to strain 100% strain without plastic deformation. Samples digested using elastinase became non-compliant and showed a near-linear stress-strain curve. For tissues rich in glycosaminoglycans (GAGs) such as the eye and cartilage, enzymatic degradation of the GAG content resulted in the tissue becoming stiffer\textsuperscript{30}.

Fibrillar collagens thus provide the main load-bearing component of ECM and GAGs and elastins provide compliance as well as protecting the collagen fibres from over-stretching.\textsuperscript{28,31} Different tissues have differing ratios of the key matrix proteins and this influences the exact stress-strain response they have.

Additionally, non-calcified tissues are soft, highly hydrated materials that exhibit a time-dependent, viscous response to stress in addition to the instantaneous elastic response, a composite behaviour known as viscoelasticity.

Figure 2-2: Stress-strain curve for a compliant biological tissue (such as skin or muscle). The stress-strain curve consists of several regions. The first is a non-linear region of high compliance as the collagen fibres within the tissue align with the direction of main stress followed by an elastic region as the fibres start to stretch. At the yield strength, fibres start to break and the tissue deforms plastically. Adapted from Vegas and Martin del Yerro\textsuperscript{32}.
In keeping with its structural complexity, tissue ECM does not have a single value of stiffness—it rather depends on the length scale on which its mechanical properties are probed. For example in cartilage, the cartilage-forming chondrocytes are surrounded by a pericellular matrix with a compressive Young’s modulus of only 25 kPa, but the surrounding matrix is an order of magnitude stiffer. Stolz et al., using AFM to probe articular cartilage, could relate the progression of osteoarthritis to the progressive disordered and stiffening of the collagen fibrils within the ECM while the bulk cartilage ECM stiffness remained constant at approximately 1.3 MPa.

The most widely used methods for determining biomaterial mechanical properties at the microscale are atomic force microscopy (AFM) and nanoindentation.

**ATOMIC FORCE MICROSCOPY (AFM)**

AFM is a technique that evolved from scanning probe microscopy (SPM) first pioneered by Binnig. Since the earliest commercial instruments were produced in 1988, it has become a powerful method for the analysis of a wide variety of both hard and soft surfaces.

AFM offers the smallest spatial resolutions (lateral spatial resolution limit 0.1nm) and is useful for force mapping structures. It can also be combined with fluorescence microscopy to correlate stiffness to microstructure.

The underlying principle involves the interaction of a cantilever with a sharp tip with the material of interest. The incorporation of a force transducer and piezoelectric crystals allow the position of the cantilever to be more precisely controlled. A laser beam bounces off the reverse of the cantilever and the position of the reflected beam is monitored by a four-part photodiode to produce a map of the cantilever’s movement. Since the distance from the back of the cantilever to the detector is long, small movements of the cantilever are exaggerated, allowing vertical resolutions on the order of one atomic layer, 0.2 nm, to be measured (Figure 2-3).
To determine the compressive stiffness of a material, the tip is indented into the substrate at a specified ramp rate and distance to obtain force curves for both the approach and withdrawal. Further information can be obtained by exchanging the normal cantilever tip for ones functionalised with specific surface chemistries. This is of particular interest as the material can be allowed to attach to the tip and the cantilever withdrawn to obtain either torsional or tensile mechanical properties, for example when probing the unfolding of protein domains.

The complexity of the cantilever's bending modes and of tip-material interactions means that absolute values for stiffness are challenging to determine. AFM mechanical measurements are therefore most appropriate where comparative measurements are being made e.g. evaluating the effect of a treatment on tissue, or where mechanical force measurements are being mapped to a structure.
Nanoindentation refers to a series of related techniques based on indentation developed for measuring the compressive mechanical properties of engineering materials\textsuperscript{42}. It utilises a probe with an indentation tip of defined geometry which is pushed into the material at a controlled rate and load to induce local deformation (Figure 2-4). Nanoindentation is best suited to materials with a flat surface, which has made it most successful in measuring the mechanical properties of calcified tissues, such as bone \textsuperscript{43}. For more compliant tissues, large radius tips, such as flat punches and conospherical tips are used to average out the surface inhomogeneity \textsuperscript{44}. Its minimum lateral resolution is thus larger than that of an AFM, but it is better suited to determining the absolute stiffness of material.

Figure 2-4: Schematic of a nanoindenter system. Adapted from Ebenstein and Pruitt\textsuperscript{42}
PROBE CHOICE

The size and shape of the tip used has a profound effect on the information obtained. Berkovich tips (which are cube corner tips) are commonly used in the measurement of engineering materials and have been used for calcified biological tissues such as enamel. However, with soft materials, spherical tips are often preferred as they cause less plastic deformation of the material. Flat tips give a consistent contact area, but concentrates force at their edges.

Shape wise, small effective contact areas, such as those given by sharp tips, tend to probe the fibres of an ECM matrix and are also useful for intracellular force mapping. Larger ones on the same order of magnitude as that of a cell, give an average value for the stiffness of the matrix and of whole cells. Very large radii are more suited for measuring the overall stiffness of a tissue. Figure 2-5 below shows some common tip geometries and sizes and their relation to a tissue sample.

![Figure 2-5: Effect of tip choice on stiffness measurement of a tissue (visualised here as cells (blue ellipses) in a fibrous matrix). A: tip size approximately the size of a cell. B: tip size smaller than cell. C: tip size much larger than a single cell. Adapted from Ebenstein and Pruitt]({"base_url":null,"path":null})

As illustrated in Figure 2-6, as the probe approaches the surface, it can experience an attractive force and jump down to the surface (1). There is then an increasing force curve vs position until the desired indentation depth (or indentation force) is reached (2). For a perfectly elastic substrate, both the approach and withdrawal curves should be expected to be superimposed on one another. In real materials, there is a generally a visco-elastic component, which creates a notable hysteresis (3). Finally, once the cantilever is withdrawn
from the surface, a dip may be observed where there are attractive forces between the cantilever and the substrate (4).

![Diagram of force distance curves for a compliant material](image)

**Figure 2-6: Force distance curves for a compliant material.**

In air, these attractive forces can be dominated by vicinal water and surface charge, whereas in fluid, charges tend to be shielded and attractive forces tend to be dominated by adhesion. To mitigate the effects of time-dependent behaviour, the manner in which load is applied is often adjusted. For example, a trapezoidal loading profile may be applied, where the maximum load is held for several seconds to allow the material to relax before unloading and so obtain a more accurate unloading curve 47.

While Young’s modulus, $E$, is the gradient of the curve produced by a stress-strain curve within its elastic region, results are rarely reported directly as such. In practice, materials are not perfect – indenters are not perfectly rigid and specimens show some plastic deformation and so a reduced Young’s modulus $E_r$ is determined using Equation 2-5

$$\frac{1}{E_r} = \frac{(1 - \nu^2)}{E} + \frac{(1 - \nu_i^2)}{E_i}$$

**Equation 2-5: Reduced Young’s modulus**
where $E$ – Young’s Modulus of specimen, $\nu$ – Poisson’s Ratio of specimen and $E_i$ and $\nu_i$ are the Young’s Modulus and Poisson’s Ratio of the indenter respectively.

Several models are used to fit the data obtained and so determine material elastic properties. The simplest is the Hertz model applied to the indentation profile, which assumes that the indentation distance of one object into another under a given load may be determined entirely by knowing the spring constants and Poisson ratios of the two materials. Many biological tissues and tissue engineering materials are analysed as thin sections and the Oliver-Pharr method has been successfully adapted to determine their elastic modulus. It uses the gradient of the initial unloading of the film to calculate the stiffness. To allow for the adhesion forces experienced between tip and soft materials, the Johnson-Kendall-Roberts (JKR) and the Derjaguin-Muller-Toporov (DMT) methods have also been developed, which modify the Hertzian model by assuming that an attractive force exists between the two surfaces in contact with one another.
2.1.3 PRINCIPLES OF MECHANOTRANSDUCTION

With the exception of mature blood cells, the cells of the body need to be attached to a substrate in order to survive. Cells need to attach to a matrix, to cohere to one another and to be organised to form functional tissues. Detached cells that cannot find a substrate to attach to soon apoptose, that is undergo programmed cell death, via a process known as anoikis. Cells do not attach directly to materials but rather to the proteins that adsorb onto that material via adhesion molecules.

Animal cells differ from plant cells, as well as from most fungi and bacteria in lacking a cell wall. The fibres and tubules within a cell constitute a cytoskeleton of a cell and serve to give it shape, structure and to enable it to exert force on its environment. The cytoskeleton consists of three types of fibres: filamentous actin, intermediate filaments and microtubules. Filamentous actin consists of monomers of actin and myosin and is the smallest fibre type, with a diameter of approximately 6 nm. Intermediate filaments, which are a collective name given to several proteins that fulfil a structural role such as vimentin and keratin, have a diameter of approximately 10 nm. Microtubules, which consist of hollow tubes of polymerised α and β-tubulin, form the largest fibres, approximately 23 nm in diameter. All of these fibre types are actively remodelled, being assembled, disassembled and transported by carrier proteins as required by the cell. For the purposes of this review, the focus will be on the actin-myosin filamentous fibres as they are the means through which cells exert contractile forces. In skeletal muscle, the actin-myosin microfilament system is heavily modified to enable large-scale movement to occur: this will be discussed specifically in section 2.2.

The nucleus has its own structural elements that form a nucleoskeleton. These consist mainly of fibrous nuclear proteins such as lamins and the nuclear envelope-associated protein emerin. The nucleus is mechanically connected to the cytoskeleton via nuclear envelope attached linker of nucleoskeleton and cytoskeleton (LINC) complexes. Within the LINC complex, the nucleus is mechanically linked to the actin-myosin filamentous fibres through the SUN1/2 proteins which associate with the proteins nesprins 1 and 2 which in turn bind
to filamenous actin via their KASH domains. Other nesprins, nesprins 3 and 4, bind respectively to intermediate filaments and microtubules. Selectively ablating cytoplasmic actin-myosin stress fibres leads to an immediate change in the nuclear shape. The mechanical linkage between the nucleus and the actin-myosin microfilaments is further corroborated by studies which show that selectively depolymerising the actin microfilaments, but not the intermediate filament or the microtubule network resulted in a change in nuclear shape.

Cells adhere to one another and to their matrix using a variety of adhesion molecules, which can be divided into three main groups: the integrins, selectins and cadherins. Within the field of biomaterials, integrins are the most studied adhesion proteins, being important for the initial cell-material interactions.

Integrins are a class of transmembrane proteins with a short cytoplasmic (inside cell membrane) and long extra-cellular domain. 18 α and 8 β integrins are known to occur in mammalian cells and these form αβ heterodimers. 22 combinations of alpha and beta integrins are known, allowing cells to adhere to a wide variety of proteins. When appropriately activated, integrins can bind to an extracellular adaptor protein, such as fibronectin, vitronectin or laminin via a recognition motif, a short sequence of amino acids, on the adaptor proteins. For fibronectin and vitronectin, the most commonly used one is (RGD) – arginine-glycine- l-aspartic acid). Laminin has the motif of (IKVAV) isoleucine-lysine-valine-alanine-valine. Synthesised motifs adhered to biomaterial surfaces suffice to allow integrin-mediated cell attachment.

Integrins are important as initial attachment proteins for a wide variety of processes, such as the implantation of blastocysts to the uterine lining, the templating of the initial muscle anlagen in muscle development, which will be discussed in Section 2.2.3.

Integrin activation can either be outside in, when the recognition sequence of an ECM protein and the availability of divalent ions induces their activation, or inside out, when the cell activates attachment to the ECM. This latter is of particular importance for blood clotting, where the integrins on the platelets
change from a low affinity state to a high affinity state once the platelets have been activated 59.

The β-subunit of the integrin heterodimer has a longer cytoplasmic tail than the α-subunit; this allows adapter proteins such as talin to bind to it 60. Talin is an essential linker protein to the actin cytoskeleton – mutated cells that do not express talin are unable to exert any tractional force on their surroundings 61. Single heterodimers of bound integrin have little effect on cell attachment. However, clusters of integrins allow for the recruitment of focal adhesion kinase (FAK) and vinculin, which act to stabilise actin polymerisation and the talin-integrin bond respectively, making them essential for the formation of stable focal adhesions. The phosphorylation of FAK at Tyr 397 is an essential signal for cell survival and proliferation 62. Once a focal adhesion has formed, α-actinin serves to cross-link the actin microfilaments, further stabilizing them.

Figure 2-8b overleaf gives an overview of the integrin-actin connections.

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Figure 2-7: Model of relationship between substrate elasticity and nucleus. Stiffer substrates (towards the right of the image) allow the formation of larger focal adhesions and stress fibres than softer substrates. Stress fibres are connected to the nucleus via the LINC complex. Boxed regions for focal adhesion and LINC complex are drawn in more detail in Figure 2-8 below. Adapted from Dalby et al. 63
Figure 2-8: Simplified schematic of LINC domain from nucleus to actin microfilaments. Schematic of integrin-actin connections. The longer cytoplasmic tail of the β-integrin subunit provides a binding site for talin, which is anchored by vinculin and PIP-2. Talin provides a linkage from integrin to filamentous actin (the associated myosin is not shown). The Arp2/3 protein complex is responsible for the nucleation and branching of filamentous actin. α-actinin cross-links actin, helping to stabilise it. Adapted from Vincente-Manzanaress et al.

SUBSTRATE ELASTICITY

Focal adhesions as the main way that cells probe and respond to mechanical forces exerted by a solid substrate. The assembly of focal adhesions, the size that they attain and the activation of stress fibres is a dynamic process, depending on the mechanical properties of the substrates. The bulk compressive stiffness of the substrate has been found to be the major determinant of how strongly a cell pulls on its substrate. The stiffer the substrate, the larger the focal adhesions and the stronger the stress fibres that extend from those adhesions. This process of is regulated by the Rho family of signalling molecules, the main ones which are RhoA, Rac1 and Cdc42. RhoA and its downstream effector ROCK induce the assembly of focal adhesions and stress fibres.

Stress fibre size is in turn correlated with several important cell signalling processes. It activates the MAPK-ERK1/2 pathway increasing the proliferation rate of cells. Downstream of this, cell size, measured as the projected cell area, nuclear volume, and nuclear stiffness all increase with substrate stiffness.

Chromosomes are not arranged randomly within the nucleus, but exist in distinct domains known as chromosome territories. Within the nucleus, the part closer to the nuclear envelope tends to consist of more compact, highly methylated chromatin known as heterochromatin, which is transcriptionally
inactive. The less dense, transcriptionally active chromatin, known as euchromatin, occurs preferentially towards the centre of the nucleus. Nuclear size has a profound effect on the relative proportions of heterochromatin to euchromatin. The larger nuclear volumes associated with cells attached to stiff substrates is associated with more transcriptionally-active euchromatin and less heterochromatin. Soft matrices thus tend to inhibit cell proliferation and can keep cells transcriptionally quiescent. Rabineau et al. investigated the molecular basis of cell quiescence by culturing epithelial cells on substrates that ranged from very soft (<<10 kPa) to stiff (200 kPa). They found that very soft matrices could result in cells failing to survive. Cells could be rescued on soft substrates by inhibiting histone deacetylase.

Conversely, stiffer matrices encourage cell proliferation. This is a feature of wound healing, where the increased stresses at the wound edge encourage cell migration into the wound edges. Less positively, this is also a feature of tumorigensis. Tumour ECMs are significantly stiffer than the equivalent ECM of normal tissue. Significantly, softening the ECM by lysl oxidase which reduces the cross-linking density of collagen I can lead to the reduction of tumour size, invasiveness, drug resistance and in some cases, the appropriate redifferentiation of previously invasive cells.

The seminal work on the influence of substrate elasticity on muscle cell differentiation is the 2004 Engler et al. paper researching the influence on substrate stiffness to differentiation of muscle and providing one of the earliest direct ties between in vitro observations of cellular responses to stiff substrates and the formation of functional tissue. Using AFM nanoidentation on both normal and dystrophic murine muscle, he determined the typical physiological elasticity (compressive Young’s modulus) of normal muscle tissue to be 12 ± 4 kPa (which compared favourably with Collinsworth’s finding of 11.5 ± kPa for undifferentiated C2C12 myoblasts). Dystrophic muscle tended to be stiffer, $E_{mdx} = 18 ± 6$ kPa. Myoblasts grown on gels whose elastic modulus was close to that of normal muscle ($E_{muscle}=12 ± 4$ kPa) developed characteristic actin-myosin striations while those on either softer or harder substrates did not. While the study could have been better supplemented with an array analysis examining
the pattern and extent of gene activation, the clear structural differences between normally differentiated muscle and muscle grown on non-physiologically stiff substrates are strongly suggestive of a mechanotransductive effect. Similar work done using cardiomyocytes\textsuperscript{76} provided insight into the disappointing results from stem cell injection into myocardial infarct sites where the stiffer scar tissue worked to inhibit the correct development and spontaneous rhythmic contraction behaviour of cardiomyocytes in more flexible mechanical environments.

Similarly, work by Gilbert et al.\textsuperscript{77} showed that the muscle progenitor cells, satellite cells would rapidly lose their regenerative capacity when cultured on stiff tissue culture plastic, which has a compressive Young's modulus of approximately 1 GPa. However, their capacity to differentiate to form muscle could be maintained if they were cultured on soft hydrogels with a Young's modulus of approximately 12 kPa.

**TOPOGRAPHY**

Skeletal muscle cells need a highly anisotropic substrate in order to form the aligned bundles of myofibers capable of transmitting a net force. Therefore, the topography of substrates has an effect on the spatial organisation of focal adhesions with downstream effects on cell behaviour. Microtopographical features are on the order of 1 to several hundred micrometers in size. These dimensions are of the same order of magnitude as that of a cell and are perceived by the cell as an additional surface.

Clark et al.\textsuperscript{78} performed some of the first systematic studies into the effect grooves of various pitches and depths had on cells, finding that groove depth had a more significant effect than groove pitch on promoting cell alignment. Cell-substrate contact does not necessarily result in alignment: while chick neurones aligned on grooved strata, epithelial cells tended to bridge grooves rather than align.

For myoblasts, orientation along a common axis is a necessary prerequisite to differentiation and muscle progenitor cells are readily oriented on topographically patterned surfaces. In a non-oriented matrix, confluent
myoblast cultures will show short-term orientation to one another, but none of the long term order required for generating an organised tissue. Further work done by Clark et al.\textsuperscript{79} into myoblast fusion showed that cells were only able to fuse end-to-end; where cells aligned to grooves met cells from adjacent grooves at an angle, myoblast fusion was inhibited.

Polyelectrolyte multilayers have been used as a method of deconstructing the relationship between surface chemistry and topography. Monge et al.\textsuperscript{80} investigated the effect of micropatterned PEM grooves on PDMS substrate on myoblast fusion and organisation. The restriction imposed by the 5 µm grooves resulted in the mean nuclear widths of cells restricted to them being 5 µm rather than 6.5 µm. This resulted in the intracellular organisation of the highly restricted myoblasts being inhibited, with the result that myotube maturation was inhibited relative to those not restricted by the substrate.

Palamà et al.\textsuperscript{81} used micropatterning of PEMs to drive the myogenic differentiation of C2C12 cells in culture containing high levels of serum which promotes proliferation at the expense of differentiation.

A material can be considered to be nanoscale when at least one of its critical dimensions is less than 1 µm \textsuperscript{82}. Cells respond to features in this size range by organising focal adhesions around them. Nanotopographical features are able to induce or inhibit cell attachment, contact guidance, stem cell differentiation or phenotype maintenance.

Early pioneers in examining the role of integrin clustering in cell attachment, Massia and Hubbell, found a critical separation of 140 nm to be the maximum distance between RGD units that allowed fibroblasts to successfully spread \textsuperscript{83}. Since then, work by the Spatz group has further. For example, Arnold et al.\textsuperscript{84} investigated the minimum necessary spacing of integrin clustering by presenting cells with RGD-functionalised gold nanoparticles with a diameter of less than 8 nm, which allowed only one integrin heterodimer to bind per particle. By altering the spacing between the gold nanoparticles, they were able to show that a critical distance of 58-73 nm between adjacent nanoparticles was necessary for the appropriate integrin clustering across a wide range of cells.
Stem cells are sensitive to their environment and topography can drive their behaviour. Dalby’s group showed that the nanoscale topography of substrates influenced the behaviour and differentiation of MSCs and could be tuned to direct differentiation (e.g. towards osteogenesis) or to maintain the pluripotency of the cells. Nanofibrous topographies, have been used to maintain iPSCs in a pluripotent state without the use of a feeder layer.

The influence of nanoscale topography on the alignment and differentiation of skeletal muscles has been investigated by several researchers, both on two-dimensional materials as grooves or ridges and on three-dimensional nanofibrous scaffolds. On nano-grooved substrates, cells extend long processes, known as filopodia, to explore the substrate and form focal adhesions preferentially along edges. Fibronectin ‘nanolines’ consisting of features with a mean height of 3.2 nm were sufficient to cause the contact guidance of 3T3 fibroblasts.

Figure 2-9 illustrates the essential difference between microtopography and nanotopography on cell guidance.
Figure 2-9: Schematic of contact guidance of cells via A - microtopography and B - nanotopography. Microtopographical features, such as the grooves illustrated are several micrometres in size and are perceived by the cell as an additional surface. Cells preferentially attach along edges and changes in step height. Nanotopographical features, such as the lines illustrated are several nanometers in size and are detected by the cellular integrins. The shape and spatial arrangement of the focal adhesions they form can in turn align cells.
2.2 SKELETAL MUSCLE

Skeletal muscle comprises approximately 40-50% of the lean body mass of a healthy adult \(^9\) and is one of the three kinds of muscle found in the human body, the other two being smooth and cardiac muscle \(^90\). Most skeletal muscle is connected to bones via tendons in order to supply movement to limbs and body. Its functions are to produce body movement, stabilise position and the heat generated through muscle contractions important to the maintenance of normal body temperature. A figure of the gross structure of skeletal muscle can be seen in Figure 2-10 below.

Figure 2-10: Structure of a skeletal muscle. Bundles of muscle fibres, called fascicles are covered by endomysium. Those fibres are bound in turn by perimysium which forms compartments that are bound by the external epimysium. From \(^91\). Used with a Creative Commons Attribution License 4.0.
2.2.1 STRUCTURAL HIERARCHY

At the macroscopic level, an individual muscle is a fibrous organ that is attached at either end to the surface of bone via tendons. The whole muscle is bound and protected by a dense fibrous sheet of connective tissue called epimysium. The fibres within a muscle, known as fascicles, are bound together in groups of 10 to 100 fibres by perimysium. Within each fascicle, fibres are kept separate from one another by endomysium. The inner surface of the endomysium is covered with basement membrane (an extremely thin layer only about 150 Å thick), to which the cell is attached. All the layers of connective tissue are continuous with the connective tissue attaching skeletal muscle to other structures, such as tendon. Tendons are linked to muscle via the myotendinous junction, where muscle cells interdigitate with the collagen fibres of the tendon.

Each fascicle consists of several bundles of mature muscle fibres, each of which is a syncytical, multi-nucleated cell formed from the fusion of multiple myoblasts. The plasma membrane of a muscle fibre, the sarcolemma, appears reticulated from the outside as tiny invaginations, known as transverse tubules, penetrate the fibre. Their purpose is to enable the efficient transmission of action potentials to all parts of the fibre. Multiple myofibrils, cylindrical structures 1-2 µm in diameter, take up much of the space within a fibre.

The fundamental structural unit of contraction is the sarcomere, which is measured from one z-line to the next. The z-line of the sarcomere traverses the cell and serves as the anchor for the contractile sub-units. Thin filaments, which are actin-rich, extend from either side of the z-line. Thick filaments, consisting of muscle-specific myosin-II bundles partially overlap with the actin filaments. Relaxed muscle appears to have alternating bands, described as I (or isotropic) or A (or anisotropic) bands which are associated with actin and myosin respectively. When the myofibril contracts, the I-band appears to disappear as the sarcomere shortens. Figure 2-11 is a schematic of a sarcomere that emphasises the structural proteins within it.
Figure 2-11: Schematic of a sarcomere, showing the structural proteins in its main elements. The z-disc lies at either end of the sarcomere and consists of thin filaments of actin, cross-linked with α-sarcomeric actinin. Thick filaments of myosin are centred in the sarcomere. The giant protein titin spans the entire sarcomere, serving to stabilise the thick filaments. The thin filaments form the light I bands while the thick filaments form the dark A bands. From Hwang and Sykes95. Reused with permission of © 2015. Nature Publishing Group.

On a molecular basis, this is caused by the myosin heads walking along the actin filaments with the consumption of ATP. The myosin bundle is kept centred in the sarcomere by titin, which connects the myosin to the z-line and consists of globular protein domains known as immunoglobin (IgG) units. These can be reversibly unfolded to provide a restoring force96. Nebulin is associated with the actin filaments and appears to act as a molecular ruler: nebulin KO (knock-out) mice have thin, shortened actin filaments and some of the z-disks are abnormally wide97.
2.2.2 MUSCLE EXTRACELLULAR MATRIX

The ECM of muscle supports and gives structure to the cells, nerves and blood vessels that make it a functional tissue. Skeletal muscle is responsible for voluntary movement and so strong connections between the muscle fibres and ECM are necessary to efficiently transmit the force generated by sarcomeric contractions through to the tendons to create movement. The extracellular matrix that surrounds muscle is very thin, making up less than 5% of the dry tissue by weight. Its influence on muscle is nevertheless profound and currently, there is still much to be understood about its structure, interrelationships and composition.

MUSCLE ECM COMPOSITION AND STRUCTURE

On a compositional basis, the perimysium is rich in collagen I, while the epimysium and endomysium are dominated by a mixture of collagens I and III. The basement membrane is compositionally different from the endomysium to which it is intimately bound, highly-branched collagen IV forming its most abundant protein, with laminins (particularly laminin-2) forming the next most abundant protein. Glycoproteins such as enactin and nidogen are thought to cross-link the collagen and laminin network.

An early study by Purslow and Trotter on the arrangement of the load bearing collagen fibres in bovine muscle is still one of the best studies of its kind. They observed that the collagen fibres in the endomysium formed a meshwork aligned nearly at random. However, the alignment of the fibres varied with the sarcomere length, which varied according to the muscle length at fixation, an example of which can be seen in Figure 2-12. Their study showed that the arrangement of fibres was consistent with contractile force transmission via laminar shear.
Figure 2-12: SEM images and angular distributions of collagen fibrils in the endomysium in muscle at rest length (left) and when highly shortened (right). The muscle fibre direction is parallel to the horizontal axis of the print. From Purslow and Trotter\textsuperscript{102}. Used with permission of Chapman & Hall ©1994.

The perimysium is organised differently; longer bundles of collagen run along the muscle, showing periodicity both longitudinally and transversely. The size of the collagen fibres is variable, varying with the size of the fascicles. They are oriented in a crossed-ply arrangement, with collagen fibres running parallel to one another within sheets but at an angle to the relaxed muscle\textsuperscript{103}. More recent electron microscopy studies have further shown that some cables appear winding in relaxed muscle but appear taut in muscle fixed in a stretched position, suggesting that they play a role in resisting excess stretch\textsuperscript{100}. 

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The epimysium consists of three zones of collagen. The inner and outer layers have coarse sheets of collagen, while the middle portion consists of finer sheets. Fibres in the outer sheet are arranged in parallel, while those of the inner and middle sheets form randomly oriented meshworks\textsuperscript{104}.

**MUSCLE ECM FUNCTION**

The ECM serves as a scaffold that helps to orient and maintain normal cellular function for all the cells within muscle. The basement membrane of muscle appears to be particularly important: its presence helps maintain muscle, it acts as a scaffold for neuromuscular junctions\textsuperscript{92} and blood vessels. Furthermore, it aids in the formation and regeneration of new muscle. Injuries where the basement membrane survives intact see injured muscle regenerated in an orderly fashion, with rapid revascularisation and re-innervation\textsuperscript{105}. Muscle regeneration where the basement membrane has been disrupted results in disorganised myotubes that generate little net force\textsuperscript{101}.

Mechanically, muscle ECM contributes greatly to the elastic modulus of muscle, with the result that bundles of muscle are stiffer than single fibres, generally by a factor of 3:1 where force to strain measurements have been performed. Measuring the role the ECM plays in the stiffness of muscle is challenging as testing skinned muscle fibres directly results in damage. One approach followed by Meyer and Lieber was to perform an indirect test using single muscle fibres and bundles of fibres. Single fibres and bundles of fibres not connected to the ECM showed a linear stress-strain curve. However, once fibre bundles of the same length were linked end to end with ECM, the composite was much stiffer and showed a non-linear stress-strain curve\textsuperscript{106}. Using the rule of mixtures for composites, they were able to elucidate the stiffness of the ECM matrix, obtaining a quadratic elastic modulus of 692 kPa/µm\textsuperscript{2} when its cross-area fraction was assumed to be 5% of the muscle. Gillies et al.\textsuperscript{107} investigated the mechanical properties of muscle ECM directly by decellularising muscle without the use of detergents or proteases. By preserving the glycosaminoglycans that form an integral part of the matrix, its mechanical integrity was preserved with the resulting stress-strain relationship similar to that obtained on intact muscle.
MUSCLE — ECM CONNECTIONS

As reviewed by Clark et al.\textsuperscript{108}, attachment of the cell to the ECM is achieved via costameres, which are transverse plaques that encircle the muscle cell. They are periodically distributed, co-occurring with the z-line and the m-line of the sarcomere. First described by Pardo \textit{et al.} in 1983\textsuperscript{109}, costameres consist of focal adhesion complexes that link to integrins, dystroglycan complexes and the spectrin-based cytoskeleton. Dystroglycans are a group of related transmembrane proteins that are critical for the secure attachment of the muscle-specific actin fibers to the ECM\textsuperscript{110}. Figure 2-18 is a schematic illustrating the links between dystroglycan, the muscle extracellular matrix and the f-actin network within the cell.

Figure 2-13: Schematic of dystroglycan complex. Laminin-2 is an adaptor protein that links the cell to the collagen ECM. Laminin in turn is bound to by dystroglycan, From Straub and Campbell\textsuperscript{111}. Reused with permission of Wolters Kluwer Health, Inc. © 1997.
Integrins are essential to the assembly of myofibrils, templating the network that will eventually form the myosin heavy chains. In mature muscle, β₁ integrin is enriched within costameres. Mice homozygous for null-β₁ in muscle fail to survive, dying in utero with poorly-developed muscle. β₁ integrin’s role in the organisation and development of striated muscle has been demonstrated for cardiac muscle: Pham et al. showed that its overexpression increased the cellular organisation and hypertrophy of cardiac myocytes in vitro, while its blockage prevented hypertrophy. As well as providing physical linkage to ECM proteins, integrins are themselves signalling molecules and stretching induces the phosphorylation of focal adhesion kinase (FAK). FAK is necessary for the successful terminal differentiation of myoblasts: disruption of FAK phosphorylation in C2C12 cells impaired the reduction of cyclin D1 expression and myoblast fusion into myotubes.

αβ₁ binds specifically to laminins; muscle-specific isoforms have a particular affinity for laminins 2 and 4. Laminins appear to be essential to the survival and differentiation of myotubes. It is particularly enriched in the myotendinous junction and Mayer et al. demonstrated that its lack led to the progressive degradation of the junction with associated dysfunction. Noting that αβ₁ expression is enhanced in both Duchenne’s dystrophy and mdx mice, Burkin et al. reported that enhancing its expression in mdx mice led to an amelioration of their phenotype suggesting that αβ₁ can partially compensate for defects in the dystroglycan complex.

A loss of any of the components linking cell to ECM is associated with muscular dystrophies: critical basement membrane components such as the α chain of collagen IV or laminin α2; the transmembrane receptors of laminin integrin α7 and dystroglycan; dystrophin, which links dystroglycan to the cytoskeleton; the sarcoglycans which link to dystroglycan and dystrophin. Several investigators, e.g. Seale have noted that dystrophic muscle is not necessarily related to lower numbers of satellite cells (at least not initially) but rather to their exhaustion through the abnormally high degradation rate of mature myotubes.
2.2.3 MUSCLE DEVELOPMENT

The skeletal muscle of the body, along with skin and bone, is formed from condensations of mesodermal tissue that form on either side of the notochord. These condensations are subsequently divided into segments, known as somites, from head to tail. This is driven by the existence of a morphogenetic field in which the expression of key growth and regulatory factors vary according to location, creating fields with differing gradients and genes are differentially expressed according to where the cells are situated within the field. Somite division is driven by the action of the clock genes Notch and Sonic hedgehog (Shh). The regulatory factor fibroblast growth factor-8 (FGF8) is higher caudally and the gradient it forms, along with the periodic expression of these two genes is able to create a determination front that achieves this segmentation. Figure 2-14 provides a simplified schematic of this process.

Figure 2-14: Simplified schematic of early patterning of embryo, showing relation of somites to notochord. Somites develop in a morphogenetic field from head (rostrum) to tail (caudum).
Within each segment, the inner section of cells migrates to form the structures of the skeleton proper (scelerotome), then the remaining tissue, known as a dermomyotome, splits into two parts with the dermatome being caudal to the myotome. The myotomes split into an epaxial region (epimere) closer to the dorsal surface and a hypaxial region (hypomere) closer to the ventral surface of the embryo. At the limb level, cells additionally migrate from the epithelial surface of the dermomyotome under the influence of the regulatory factor c-met and its ligand hepatic growth factor (HGF) to form buds that undergo further differentiation to form the various muscles of the limbs. Figure 2-15 is a simplified illustration of the cell populations involved in the generation of the various mesodermal tissues from the somites (bone, cartilage, muscle and the inner layers of skin).

Figure 2-15: Schematic of compartments that give rise to mesodermal tissues, visualised as a transverse section through the embryo. The former somite develops into a dermamyotome. Its inner region develops into the scelerotome and the cells delaminate to migrate towards the notochord. They will form the skeleton (bones and cartilage). The myotome forms the trunk muscle. At the limb bud level, cells delaminate from the hypaxial lip of the dermomyotome and migrate into the limb to form the limb muscles. Adapted from 124

Myogenic regulatory factors (MRFs) are a group of four related basic helix-loop-helix transcription factors (bHLH): myogenic differentiation 1 (MyoD),
myogenic factor 5 (Myf5), myf6 (MRF4) and myogenin (MyoG). They function by forming heterodimers with homeobox proteins such as E12 to allow for their binding to myogenic regulatory regions of DNA and activate their transcription of the same. MRFs are found in many tissues of the body, but need a permissive environment (the presence of promoting and the absence of inhibitory regulatory factors) in order to be successfully transcribed. Within the embryo, the regulatory factors Wnt-1, Wnt-3, and Wnt-4\textsuperscript{125}, which are secreted by the dorsal regions of the neural tube, signal cooperatively with Shh, which is secreted by the notochord, to induce MyoD and Myf5 expression\textsuperscript{119}.

While all the myogenic regulatory factors have a similar structure, their effects differ. The myogenic specification factors, MyoD and Myf5, act to specify cells that will become muscle, while myogenin acts to terminally differentiate muscle. It is not able to specify cells. Cells expressing myogenin exit the cell cycle prior to fusion and subsequent maturation\textsuperscript{23}.

Muscle formation proceeds in waves. The first wave of migrating myogenic precursor cells appear early in embryonic development (by 7 weeks in human embryos\textsuperscript{126} and E11-12 in the mouse\textsuperscript{127}) and form a temporary scaffold, known as an anlagen, of primary muscle fibres that serves as a template for future muscle. Later, a second wave of proliferation gives rise to a population of secondary myoblasts that invade the anlagen and give rise to secondary fibres, along with innervation and vascularisation. The reserve population of cells that will promote and repair muscle tissue postnatally appear late in the developmental stage\textsuperscript{128}.

Myotubes are not able to proliferate. They are however, highly plastic and can remodel in response to changes in functional demands or fuel supply\textsuperscript{129}. As multinucleated cells, they are able to recruit additional nuclei to grow larger in response to increased functional demand. This process is known to be mediated by IGF-1\textsuperscript{130} via the P13K/Akt/TOR pathway\textsuperscript{131}. This pathway has been demonstrated to be stretch activated and is one of the pathways by which skeletal muscle respond to mechanical stress\textsuperscript{132}. 

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HEALING IN MUSCLE

As mature skeletal muscle is unable to re-enter the cell cycle, any muscle repair or replacement must require the recruitment and proliferation of myoblasts that will subsequently terminally differentiate to form the mature tissue.

Satellite cells were first described in 1961 by Mauro, who noticed a population of isolated cells, consisting almost entirely of nucleus, wedged between the muscle cell sarcolemma and the basement membrane in frog muscle. He speculated as to the possible role of these satellite cells in muscle regeneration. Contemporaneously with Mauro, Katz detected satellite cells in rat muscle and since then, they have been widely found within vertebrates.

Satellite cells found within the body (but not the head region) are characterised by being Pax7 and Pax3 positive, while those of the head region are only Pax7 positive, reflecting the different embryonic developmental paths undertaken by these cell populations. Regardless of origin, these cells are to be found nestled between the basal lamina and the surface of a muscle fibre and when quiescent, have very little cytoplasm.

Satellite cells have been observed to be able to be stimulated by injury or growth factors to undergo several rounds of division to generate muscle precursors. At the same time, in normal (that is to say non-dystrophic) muscle, the number of satellite cells remains constant. They thus fulfil the two key characteristics of stem cells: self-renewal and the generation of differentiated descendants. Figure 2-16 illustrates the activation and repair process.

In vivo, satellite cells are the major, if not exclusive, source of muscle progenitor cells. Two populations of stem cells are recognised within skeletal muscle: satellite cells and a so-called ‘side-population’ (SP) cells. Gussoni et al. found that SP cells derived from muscle, like those derived from bone marrow, both gave rise to hematopoietic cells and participated in the regeneration of muscle. However, only the former seemed able to generate satellite cells.
Figure 2-16: Simplified schematic of satellite cell activation. Satellite cells are normally nestled between a myofiber and its basal lamina. When stimulated, e.g. by IGF-1, bFGF, they divide to form proliferative myoblasts (not shown is asymmetric division to yield a replacement satellite cell). The myoblasts exit the cell cycle and differentiate to form committed myocytes which orient themselves end-to-end and fuse to form myotubes (note central nuclei). Myotubes in turn mature to form myofibers.

When skeletal muscle is damaged, its healing may be divided into three phases: the destruction, repair and remodelling phases.

In the destruction phase, there is necrosis of the injured regions and a contraction of the ends of the affected myofibers. The injured ends are sealed off with a sarcolemma that demarcates the damaged region and the vacated region is filled with a hematoma which is subsequently invaded by immune cells to engender an inflammatory response. Insulin-like growth factor (IGF-1), basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) as well as several progestins \(^{140}\) appear to be secreted at the injury site, which recruit neutrophils within one hour of injury. These produce free radicals that lyse damaged cell membranes.

In the repair phase, monocytes phagocyte the necrotic tissue. Activated satellite cells migrate to the region – as mentioned above, IGF-1 appears to be a key activating factor in this process \(^{141}\) – where they undergo proliferation to form both satellite cells and myoblasts. Activated satellite cells also appear to be able to chemo-attract macrophages and monocytes, creating a positive feedback loop\(^{142}\). The daughter myoblasts in turn proliferate further and start fusing to form myotubes after about 2 days. These new myotubes replace the old...
necrotised section and start to invade the scar tissue to connect with the stumps of the myofiber. Concomitantly, new blood vessels start to re-vascularise the injury site.

Finally in the remodelling phase, the new myofibers mature to form the contractile apparatus and new axons grow out to them and a neuromuscular junctions. The connective tissue within the injured region is organised to form scar tissue, which forms a persistent bridge between the ends of the repaired myofiber.

The basement membrane is critical to the orderly healing of muscle, providing contact guidance to the differentiating myoblasts and templating the insertion of nerves and blood vessels. In injuries where the basement membrane survives intact, injured muscle is regenerated in an orderly fashion, with rapid revascularisation, re-innervation and no scar tissue formation. The classic model for this type of total repair is acute necrosis produced by means such as crush or freezing injuries in rabbits or rats. Another complete repair model is limb amputation in newts.

Limb regeneration in newts differs from mammalian healing in that terminally-differentiated cells in the former can dedifferentiate to form a blastema that can recapitulate the developmental process, while in mammals and most other vertebrates, regeneration depends on the existence of progenitor cells, which cannot recreate a lost structure.

However, both newt limb regeneration and mammalian necrotic injury models show a change in and a succession of the ECM molecules involved in the repair of muscle. In both, the laminin and collagen VI rich matrix is replaced with a transitional ECM forms that is rich in fibronectin (FN), hyaluronic acid (HA) and tenascin-C (TN). This transitional ECM has been found to encourage the fragmentation of myotubes to myoblasts in newts and the proliferation of C2C12s.

With more extensive injuries, such as a strain injury where not only the myofiber, but its associated basal lamina, myial sheath and blood vessels are disrupted, the muscle heals but fibrosis occurs. More serious injuries still are
those where the ECM is not merely disrupted, but missing altogether: in such volumetric muscle loss (VML) injuries, part of the mass of the muscle is missing. The fibrotic tissue in severe injury may inhibit the re-innervation of the surviving muscle, leading to atrophy of the tissue\textsuperscript{143}.

The fibrosis can extend beyond the immediate injury site: faced with significant injury, ageing, disuse\textsuperscript{147} or muscular dystrophies, the epimysium and perimysium thicken considerably, with more collagens I and III being laid down. This fibrotic matrix itself inhibits the successful differentiation of muscle precursors to form normal tissue, instead encouraging them to produce more collagen I\textsuperscript{148}.

Therapies to treat muscle injuries to promote healing and reduce scarring are largely conservative, consisting of RICE (Rest Ice Compression and Elevation). A review of the use of Non-steroidal anti-inflammatory drugs (NSAIDs) in promoting the healing of muscle found conflicting evidence of their efficacy. While they were found to reduce pain, many reports indicated that their effect on functional repair was somewhere between ineffective (as in it made no clinically significant difference) to counterproductive in delaying the regeneration of muscle\textsuperscript{140}. Glucocorticoid use to limit the inflammatory response with the aim of reducing the quantity of scar tissue has been counterproductive as it increases the time the hematoma persists and reduces the subsequent strength of the repair\textsuperscript{149-150}.

The mechanisms whereby the altered ECM changes the function of muscle precursors is an active area of research. An examination of muscle precursor cells from healthy young and old donors found no difference in the proportion of desmin positive cells (proliferative cells taken from muscle biopsies are a mixture of fibroblasts and myoblasts), doubling time, differentiation capacity or population doublings to senescence\textsuperscript{151} making changes in the ECM one of the likely factors in the well-documented changes in muscle regenerative capacity with age. The increase in collagen within the ECM comes with a concomitant loss of appropriate chemical signals, such as laminin, which are essential to myogenic differentiation\textsuperscript{152-153}. The increase in collagen I has also been found
to directly inhibit the differentiation of C2C12 cells in vitro. The mechanical properties of the stiffer matrix itself also have a profound influence, effects of which will be described in more detail in the next section.
2.3 SKELETAL MUSCLE TISSUE ENGINEERING

2.3.1 CLINICAL NEED AND JUSTIFICATION

Muscle injuries are common: strains and sprains are a part of life. Amongst athletes, they comprise the most common injury, comprising between 10 and 55% of all injuries received\(^\text{143}\). However, the impact of muscle injury on long-term disability is often under-appreciated.

Injuries are a major source of disability worldwide\(^\text{154}\). While much focus in tissue engineering has been given to bone, tendon and nerve repair, the muscle that these serve to support, anchor and motivate respectively has received less attention. The most serious muscle injuries, volumetric muscle loss, tend to happen as a result of high-energy injuries (such as gunshot wounds or traffic accidents\(^\text{155}\)), open fractures, or a side effect of medical treatments such as tumour removal or wound debridement\(^\text{156}\).

The gold standard for treatment of severe muscle injury is the free-muscle transfer. In this process, muscle from a donor site is transferred along with its nerve and blood supply and grafted into the recipient region\(^\text{157}\). However, the size and complexity of the injured site may preclude grafting. There is therefore a need for therapies that can supplement existing strategies.

Tissue engineered methods of repairing muscle fall into three broad groups. The first, the cellular therapy approach, relies on either the introduction of factors to inhibit scarring and/or encourage the proliferation and differentiation of existing cells or the introduction of precursor cells (autologous or donated) to supplement the native cell repair efforts. The second, the acellular therapy approach, involves the introduction of scaffolds to create a microenvironment that the host cells can colonise to form functional muscle. The third is a hybrid approach wherein cells (either autologous or donated) are seeded onto a scaffold and the whole grafted into the lesion to promote healing.
2.3.2 CELL-BASED THERAPIES

In order to deliver a therapeutic effect from cells, several criteria need to be met. The desired characteristics of these cells would be that they are first, readily available, preferably with few ethical concerns in their isolation. Second, that they are readily expandable in vitro, able to be propagated over several population doublings to produce sufficient cells for clinical therapy. Ideally, cells should be able to be propagated in vitro without recourse to serum or feeder cells. Third, that they are readily induced to differentiate myogenically, ideally without the use of transfection agents. Fourth, that they are safe, neither inducing acute immunogenic response nor forming teratomas. And finally, that they survive introduction to the injured environment – or are able to be targeted to it – and contribute to the muscle regeneration in situ.

No single stem cell source meets all these criteria.

SATELLITE CELLS AND PRIMARY MYOBLASTS

Much of the early work was driven by a desire to alleviate muscular dystrophies, in particular Duchenne’s muscular dystrophy. The initial therapeutic approach was to culture human myoblasts as committed precursors and then inject them into muscle. While experimental work on mice had given promising results, only a limited clinical response was seen, with no cases yielding long-term therapeutic improvement.\textsuperscript{158}

Several key issues were identified. First was that myoblasts are not immune-privileged and were attacked by immune system – most were dead within three days post-injection. The compatibility of tissues between a donor and a host is largely determined by the major histocompatibility complex (MHC), a 7 megabase region on chromosome 6 that contains more than 300 genes involved in immune function.\textsuperscript{159} While better MHC I matching between donor and host mitigated the issue, it did not eliminate it. Surviving myoblasts were poorly migratory. Consequently, large numbers of myoblasts would need to be injected in order to provide a therapeutic effect.\textsuperscript{160}
Some progress has been made in promoting the survival and engraftment of primary myoblasts. Muir et al used a ‘prosurvival cocktail’ consisting of Matrigel™, anti-apoptopic factors cyclosporine A, Bcl-XL and benzylxycarboxnyl-Val-Ala-Asp(O-methyl)-fluoromethyl ketone, proliferation-promoting factor IGF-1 and pinacidil to allow cells to better tolerate ischemic stress. They used dermal fibroblasts transfected with MyoD to become myogenic, which the additionally heat-shocked (treated by raising the temperature briefly, which induces heat-shock proteins to be expressed. These protect key enzymes from denaturing) a day before injection. When injected into a dystrophic mouse model, cells survived better and engrafted at a higher percentage than untreated controls or cells exposed only to heat-shock pre-treatment161.

Satellite cells, as the muscle stem cell, have also been investigated as a potential source of muscle repair therapies. Unfortunately, like myoblasts, work on satellite cells have also been met with limited success162. The relative scarcity of satellite cells within tissue and the consequent need to obtain large quantities of muscle to derive sufficient numbers limits its utility as a source of autologous stem cells.

There is a considerable heterogeneity in muscle progenitor cells 163: satellite cells have differing embryonic origins and consequently, display different behaviours. One effect of this issue is that depending on the muscle that they are derived from, isolated satellite cells can replicate a few times or hundreds of times136. Its poor migration capabilities may be related to its niche between basement membrane and sarcolemma 162.

A further challenge of satellite cells is that they appear to age: Cosgrove et al. 164 found that muscle progenitor cells obtained from aged mice were less efficient at engrafting into muscle and producing new myofibers. They also found that inhibiting p38 MAPK α/β signalling – a signalling pathway that is activated when cells undergo stress 165 – resulted in the ‘rejuvenation’ of the cells, with an improvement in their proliferative capacity and contribution to new muscle formation. This effect was enhanced if the cells were also cultured on soft hydrogels with a compressive Young’s modulus of 12 kPa.
OTHER CELL SOURCES

MESENCHYMAL STEM CELLS (MSCs)

As reviewed by Chamberlain 166, MSCs were first isolated from bone marrow by Friedenstein et al. 167 who plated out bone marrow onto plastic culture dishes and noted that a fraction of cells were adherent. Culturing this adherent fraction, they noted that they formed colonies, were fibroblastic in appearance and could differentiate into bone-like or cartilage-like cells.

Castro-Malaspina et al. examined the isolation and properties of the fibroblastic bone marrow colony forming units more systematically to find that these cells were mono-nucleated, non-phagocytic, had a low density of 1.070 g/cm³ (a property used to separate MSCs from other cells in cord blood preparations) and had a low immunogenicity 168.

The ability to adhere directly to tissue culture plastic, to produce colony forming units when plated out at dilute numbers and to differentiate to bone or cartilage continues to form part of the definition of what MSCs are. To better characterise MSCs, several clusters of differentiation (CDs) have been identified. CDs are the collective name for a wide variety of cell surface molecules that can be bound to by antibodies. Specific cell types express a pattern of CDs, making them a convenient target for phenotyping cells as this is compatible with non-destructive methods of sorting and of analysis such as flow cytometry 169.

In a position paper from the International Society for Cell Therapy, Dominici et al. in a proposed the cell culture behaviour along with positive markers for the surface markers CD105, CD73 and CD90, and negative markers for CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules as minimal criteria for the identification of MSCs170.

The ability of MSCs to be cultured directly on tissue culture plastic and to be maintained in relatively simple culture conditions – while specialist, optimised culture media are commercially available, MSCs have been routinely maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum – makes them an attractive source of cells. An additional factor making
them attractive is that unlike embryonic stem cells, they have no tendency to form teratomas and are therefore considered safer to use in clinical applications. MSCs can be isolated from most mesodermal tissues including umbilical cord blood, placenta, bone marrow, adipose tissue, even shed from the body as part of the cellular content of urine or human menstrual blood.

Another readily available source of MSCs are those derived from placenta. Placentally derived mesenchymal stem cells encompass a broad range of cells whose proprieties depend on the tissue type and gestational age from which they are derived. Most studies of these stem cells come from term tissues. Regardless of exact source, placentally-derived MSCs show mesenchymal like properties but are less immunogenic than adult MSCs and are more immunosuppressant. Nazarov et al. have shown chorionic mesenchymal stem cells to be able to differentiate to neuron-like, adipocytes, osteoblasts and hepatocytes. Myogenic differentiation of PSCs has been reported by Kim et al. using human amniotic stem cells, and by Gang et al. using umbilical cord blood.

As may be inferred from the variety of tissues they can be isolated from, MSCs are highly heterogeneous. For example, Jin et al. isolated MSCs from bone marrow, adipose tissue and umbilical cord blood (UCB-MSCs) and noted that the growth characteristics of umbilical cord blood were more favourable than MSCs from the other two sources, with more fibroblastic colony forming units (CFU-F) forming and the cells being able to be passaged for up to 16 passages before entering senescence. UCB-MSCs also showed greater anti-inflammatory effect than the other two stem cell sources tested.

With MSCs being most easily induced to differentiate to osteogenic, chondrogenic and adipogenic lineages, the concept of using them as a resource for myogenic and cardiomyogenic cells appears rather odd at first glance. Their ubiquity and relative ease of expansion render them very tempting as sources for neural (ectodermal) and myogenic (mesodermal) cells. Forced overexpression of MyoD will commit MSCs to myogenic differentiation; this
is however, not a desirable pathway for clinical applications. Furthermore, as will be seen in the paragraphs that follow, while myogenic differentiation has been reported for MSCs, there is no reliably defined pathway to induce this differentiation.

The seminal paper on the myogenic differentiation of MSCs is by Wakitani et al. 183 who were the first to successfully demonstrate the myogenic differentiation of mesenchymal stem cells. MSCs isolated from bone marrow plugs could be induced to undergo myogenesis when treated with the histone disruptor 5-Azacytidine. Qian et al. 184 followed up on these results by elucidating the mechanism via which 5-Azacytidine acted. They demonstrated that it effected myogenic differentiation by ERK phosphorylation. Inhibiting MAPK (and preventing ERK phosphorylation) using U0126 also blocked downstream myogenesis.

A second approach is to use growth factors and specified media to induce myogenesis. No one medium has been shown to definitively promote myogenesis and this is further complicated by differences in MSCs taken from different parts of the body as well as differences in the stem cells between donors. Wakitani et al. 183 found that the addition of basic Fibroblast Growth Factor (bFGF) as well as hydrocortisone helped myogenic differentiation, while Gawronska-Kozak et al. 185 found instead that Epithelial Growth Factor (EGF) supplementation was sufficient to allow the myogenic differentiation of ear MSCs without 5-Azacytidine induction. The most thorough investigation has been done by Stern-Straeter et al. 186 who compared the effect of 5-Azacytidine induction to FGF-supplemented medium and both of those to satellite-cell conditioned media, finding that 5-Azacytidine appeared to be necessary for induction.

A third approach is to use co-culture to induce cell fusion and differentiation in vitro and in vivo. Co-culture of MSCs with C2C12s has been done by several investigators including Dugan 187 and de la Garza Rodea et al. 188. MSCs fuse with the murine myoblasts to form hybrid human/mouse myotubes and the human nuclei can be confirmed using human specific antibodies to nuclear
lamins. In vivo injection of MSCs into muscle has shown that they can survive long-term within the muscle, proliferate (albeit at a low rate) and migrate away from the injection site\textsuperscript{189,188}. Ferrari et al.\textsuperscript{190}, by using cells from a mouse line engineered with a lacZ gene which expressed a nuclear b-galactosidase with a myosin-linked promoter, showed that bone marrow MSCs unambiguously contributed to the repair of cardiotoxin-damaged tibialis anterior muscle in immunodeficient mice.

A final approach is that taken by groups that focus on using the physical microenvironment to drive myogenic differentiation. Both static approaches – utilising substrate stiffness or topography and dynamic approaches using strain have been used, sometimes in combination with other induction methods.

Wang et al.\textsuperscript{191} used grooved polystyrene substrates of varying aspect ratios to investigate the effect of grooved topography on the differentiation of primary rat bone marrow MSCs. Myogenesis was however induced with 24 hours 10µM 5-Azacytidine. They found that grooves improved myogenic differentiation with respect to flat controls. Desmin and F-actin were detected after 2 weeks, with the F-actin going from striated to reticulated after 3 weeks, which also coincided with the appearance of MHC. Deep grooves were associated with more MHC expression. In a rare use of physical cues alone to induce differentiation, Li et al.\textsuperscript{192} used purified bone marrow derived human MSCs from a commercial source and a simple culture medium consisting only of DMEM with 10% FBS. Laser machined grooves in PLLA-PCL substrates over two weeks. Narrower grooves promoted better alignment and upregulated early neuronal and myogenic markers (\textit{Nkx2.5, MAP2, NeuroD1, MyoD, MHC7}) while downregulating osteogenic markers (\textit{ALPL, RUNX2}). MHC was seen in narrow grooved cells at 14 days but not MAP2. Similarly, other methods of micropatterning that introduce a preferred direction, such as microcontact printing of ECM lines\textsuperscript{193} and the use of electrospun scaffolds have served to enhanced the myogenic differentiation of MSCs\textsuperscript{194}.

A particularly interesting approach has been the use of casts of differentiated myogenic cells to act as templates for the differentiation of MSCs. First
demonstrated by Lee et al. 195 induced C2C12 cells to differentiate on fibronectin micropatterned lines. They then cast polyurethane onto the cells to create a cell-patterned relief that was then used to culture MSCs, resulting in the upregulation of myogenic regulatory factors MyoG, MRF4, MyoD and Myh13.

Taken together, these can be seen as a graduated set of approaches – from the purely genomic/chemical method at one end to the purely physical at the other. Pro-myogenic effects such as favourable ECM coating, topography and growth factors appear to have a synergistic effect.

2.3.3 SCAFFOLD-BASED THERAPIES

Tissue engineering approaches seek to understand the factors and pathways regulating myogenic commitment, to develop in vitro constructs allowing the study of muscle and to ultimately create constructs that are suitable for implantation into muscle.

An additional challenge in muscle tissue engineering is that while some tissues, such as cardiac mitral valves and blood vessels, are well-defined by their ECM matrices, making the implantation of decellularised ECM matrices of these tissues viable approaches196, muscle ECM is a scaffold in the truest sense of the term. It is a thin, acellular material that in conjunction with soluble factors provides cells with the physical and chemical cues to build functional tissue. While the ECM of muscle, described in Section 2.3.3., is too complex to replicate in vitro, capturing its essential properties would allow for the reliable regeneration of muscle.

The concept behind acellular scaffolds is that once implanted into the damaged tissue, they are able to recruit cells from neighbouring tissue and offer the cells a suitable environment to encourage the appropriate differentiation and maturation of functional muscle. Compared to pre-seeding scaffolds with cells, this approach has the advantages of being relatively quick to implement, not requiring autologous cells to be taken and cultured, more easily scalable and presents fewer challenges with sterility. Both natural material derived scaffolds,
such as decellularised porcine small-intestinal mucosa and synthetic, such as electrospun block co-polymers.

On the other hand, few studies of acellular scaffolds have been able to show a clinically relevant increase in the strength of the repaired muscle. It is also the case that with large defects, the number of cells that would be required to invade the scaffold to effect a functional repair is so large as to be impractical.

### 2.3.4 HYBRID THERAPIES

Several investigators have combined scaffolds with populations of cells to create a hybrid system that is more rapidly integrated into the existing host tissue. Hydrogels have been one method of incorporating myogenic precursor cells for delivery into the site. Rossi et al. utilised a hyaluronan-based hydrogel that also contained a photoinitiator to not only deliver cells to site, but also to offer an appropriate mechanical stiffness by curing it once applied to the lesion. Using a murine model, they reported that this method improved not only cell survival but also functional recovery of the treated muscle.

Electrospinning is frequently used as a method to generate 3-dimensional scaffolds. Beachley et al. created 3-D scaffolds by layering successive uniaxially aligned electrospun PCL fibres (mean diameter 700 nm) which had been pre-seeded with C2C12s, using supporting frames in order to keep sheets aligned with one another. They found that a low fibre density (measured by the number of fibres per unit length of a line drawn perpendicular to their orientation) was sufficient to allow the orientation of the cells. By soaking successive scaffold layers in either fibrin of thrombin before apposing them, cell sheets were encapsulated by a fibrin gel. Their goal was to create a handle-able, mechanically strong aligned tissue construct. Implantation into the intravertebral muscle of a mouse (number, breed and gender not given) with retrieval after 1 day showed that the scaffold could be successfully grafted and retrieved intact. No attempt to quantify immune cell response was done, but graft-host cell connections could be observed at that time point.

One way of resolving the challenge of material interactions with the host tissue (acute immunological responses, more chronic fibrosis, material-tissue
mechanical mismatches) is to use the scaffold as a template from which the aligned cells can be released. To that end, a method of releasing cells from culture substrates as entire sheets that can be grafted has been developed. An early study comparing the use of thermally responsive polymers with conventional mechanical or enzymatic methods to recover cells showed that they allowed not only the recovery of cells, but also their associated ECM matrix.

Poly(N-isopropylacrylamide) (PIPAAm), is one such polymer and undergoes a phase transition at 20°C, becoming hydrophilic and water-soluble and allowing the release of entire cell sheets. By culturing human skeletal muscle cells on micropatterned gelatin sheets overlying a PIPAAm layer, Takahashi et al. were able to detach layers of aligned (or random) skeletal myoblasts with their ECM. Apposing successive layers allowed the creation of a multilayered cell construct. Interestingly, cells in the lower layers would rapidly (over an 18 hour period) reorient themselves to match that of the top layer. Terminally-differentiated myotubes did not reorient themselves, but remained within their original orientation if the sheets were apposed post-differentiation.

A similar method was utilised by Villa et al. who developed a poly 2-hydroxyethylmethacrylate (PHEMA) copolymer with PIPAAm to create a series of soft hydrogels which supported the culture and intact detachment of C2C12 cells.

The formation of more mature muscle containing secondary myotubes, vascularisation and innervation remains a goal, is one that cannot readily be achieved in vitro. Innervation appears to be essential to the development of an adult phenotype: Wilson’s aneural muscle model showed a degeneration of secondary myotubes. Perfusion limits the size achievable by in vitro scaffolds: a maximum size of 150 µm is practically achievable without necrosis of cells too far from nutrients to survive.

Addressing these problems, some promising in vivo work on creating implants that can successfully recruit blood vessels and nerves has been done. Dhawan et al. built on earlier work to create muscle scaffolds that were able to create a
vascularised, innervated structure. These consisted of primary myoblast-seeded fibrin gels encapsulated in silicone tubing that was subsequently wrapped around rat femoral arteries. The resected femoral nerve was sutured into the middle of the tube. After four weeks, explanted implants showed neuromuscular junctions and specific forces five times greater than those for implants that were only vascularised. Recently, Williams et al.\textsuperscript{209} have also demonstrated the ability of implanted muscle to recruit blood vessels and generate neuromuscular junctions using laminin constructs. Critical to their work was their suturing the ends of the implant to the tendons of the host muscle so it would experience mechanical stress – and in theory eventually exert useable force, a key goal of any tissue engineering. After 1 week, explants were covered in a fibrous matrix with capillaries extending through the muscle. Relative to controls maintained \textit{in vitro}, \textit{in vivo} explants were larger and generated significantly higher isometric forces (increasing from 192 µN to 549 µN versus \textit{in vitro} controls that increased from 276 µN to 329 µN). Nerve fibres had started to invade the tissue and staining with α-bungarotoxin showed acetylcholine receptor grouping suggestive of a nascent neuromuscular junction.
2.4 BIOMATERIALS

Biomaterials for skeletal muscle repair must offer uniaxial directional contact guidance and be able to provide a tissue-like stiffness. To that end, cellulose nanowhiskers were selected as a method of providing cell guidance. The layer by layer technique was used to create a model nanocomposite material whose components can be easily varied, allowing its mechanical properties, especially its stiffness, to be tuned.

2.4.1 CELLULOSE

STRUCTURE AND ISOLATION

Cellulose is the world’s most abundant biopolymer, with $1 \times 10^{12}$ metric tons produced annually\textsuperscript{210}. It is produced by plants, as well as many species of fungi and bacteria. Cellulose production is less widespread among protozoa. Tunicates are unique among animals in producing cellulose\textsuperscript{211}. Biologically, cellulose fulfils several roles. In green plants, it is a major structural component of the cell wall, lending rigidity to the cell wall. In tunicates, it functions analogously to fibrillar collagen and forms the load-bearing component of its extracellular matrix. Many bacteria that produce cellulose excrete it as part of a biofilm, a protective highly-hydrated mixture of polysaccharides. This serves to allow colonies to cohere, to anchor the colony to host surfaces and to inhibit attack by antibiotics\textsuperscript{212}.

Structurally, it is a linear polymer consisting of units of two $\beta$-1,4-linked anhydro-D-glucose where successive sub-units are rotated 180° in relation to one another\textsuperscript{213} and is illustrated in Figure 2-17. The hydroxyl terminated end of the polymer is known as the non-reducing end and the hemiacetal terminated end as the reducing end. Since cellulose is a large macromolecule, the number of glucose sub-units present in a cellulose chain is known as its degree of polymerisation (DP).
As can be seen from Figure 2-18, the 6 carbon (C₆) is not part of the ring, and its attached hydroxyl is able to participate in hydrogen bonding. The ribbon-like structure of the cellulose fibre is stabilised by intramolecular hydrogen bonding, particularly between the C₆ and C₂ atoms.

There is also extensive intermolecular hydrogen bonding between adjacent chains, which helps chains to self-assemble into fibres. The pattern in which fibres pack into higher structures depends in part on how the hydroxymethyl group is oriented with respect to C₆, which is illustrated in Figure 2-19.
Figure 2-19: Schematic intermolecular hydrogen bonding between cellulose chains. The O₆ oxygen
has been labelled to correspond with the carbon atom it attaches to and the hydrogen atoms have
been omitted from this image for clarity

**BIOSYNTHESIS OF CELLULOSE**

The synthesis of cellulose is catalysed by cellulose synthases (CESA), which
belong to a category of enzymes known as glycosyltransferases. These enzymes
are characterised by their ability to transfer saccharide moieties from an
activated nucleotide sugar to an acceptor molecule\(^{215}\). Plant, bacteria and
tunicates utilise different enzymes and assembly complexes to create cellulose.
Regardless of the exact enzyme, all cellulose synthases use uridine 5’-
disphospho-glucose (UDP-glucose) as a substrate (see Figure 2-20 below).
Uridine is a nucleotide sugar which plays two important roles within cells. First,
it is utilised by RNA (replacing the role played by thymidine in DNA) and is thus
found in the cytoplasm of all cells. Second, uridine is a key carrier molecule for
monosaccharides in several glycosylation processes\(^{216}\). In plants, the addition of
glucose to UDP and the subsequent transfer of UDP-glucose to the CESA
complex is mediated by a plasma-membrane associated enzyme complex
known as sucrose synthase (SUSY).
Cellulose synthases catalyse the transfer of the glucose moiety from the UDP-glucose molecule to the anomeric carbon (the C5 carbon) of an existing β-glycan chain, inverting the glucose molecule with respect to the preceding glycan residue. Structurally, they are trans-membrane proteins with a globular cytoplasmic region that the substrate is thought to bind to and the enzyme forms part of what is termed a terminal complex (TC). As additional glucose residues are added to it, the resulting glucan chain is extruded through a pore in the terminal complex.

Typically, 36 individual cellulose molecules are assembled into protofibrils, which then pack into microfibrils. These microfibrils further associate to form cellulose fibres. The configuration of the TCs controls the thickness and cross sectional shape of the fibrils produced; fibrils so produced range from 2 nm to 20 nm in diameter, depending on the species of origin. The process is illustrated in Figure 2-21 below.
Figure 2-21: Simplified schematic of cellulose synthesis at cell membrane. SUSY catalyses the glycosylation of UDP to form the substrate that is transferred to the CESA enzyme complex of the terminal complexes, where the glucose is transferred to the growing cellulose chain. The extruded cellulose proto-filaments self-assemble to form microfibrils. Adapted from Joshi et al. 219

Most naturally produced cellulose is in the form of cellulose I, a metastable form of cellulose and cellulose II, which is the thermodynamically-stable form. In cellulose I, cellulose chains are ordered parallel to one another. Two slightly different forms exist: cellulose I\(\alpha\) is primarily produced by bacteria and tunicates, while cellulose I\(\beta\) is primarily produced by higher plants 220. In cellulose II, the chains are ordered in anti-parallel form and it is produced when cellulose is regenerated, e.g. dissolution and re-precipitation 213.

Tunicates, also known as urochordates, are the only animals known to produce cellulose221. There are three branches of this family: Ascidiacea (sea squirts), Thaliacea and Appendicularia. All branches of this family are characterised by the production of a tunic, a tough yet flexible protective mantle consisting of cellulose and protein. Tunicates are chordates and possess a notochord, which can be seen in the larval forms, but as adults, most species are sessile filter feeders and lose the notochord. A typical tunicate and its body plan can be seen in Figure 2-22 below222.
Unlike plants, tunicate species studied thus far have linear arrays of terminal complexes, resulting in cellulose fibrils that are either rectangular or square in cross-section\textsuperscript{223}. The successful sequencing of the genome of the tunicate \textit{Ciona intestinalis}\textsuperscript{224} allowed the homology of genes found to be compared. Nakashima \textit{et al.} found evidence for the genes for cellulose synthesis being acquired by tunicates via horizontal gene transfer from bacterial CESA\textsuperscript{225}. This evidence was corroborated by the same group by gene knockdown of cellulose synthesis in \textit{Ciona interstinalis} larvae using an anti-sense bacterial CESA sequence \textsuperscript{226}. 

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure22.png}
\caption{Light microscopic image of a tunicate (a) and a schematic representation of the structural plan of its body (b). Reproduced with permission from Kimura and Itoh\textsuperscript{222}.}
\end{figure}
Tunicate cellulose has two desirable properties. It is highly crystalline, with a degree of crystallinity of between 50 % and 70 % depending on species. Secondly, it is relatively facile to extract, as the cellulose is bound within a proteinaceous ECM. The ECM can be removed by treatment with a strong alkali. In plants, cellulose is tightly associated with several other polysaccharides known as hemi-celluloses and cellulose purification is a complex multi-step process.

2.4.2 CELLULOSE NANOPARTICLES

DISCOVERY

In 1947, Nickerson and Habrle observed that the degradation rate of cellulose boiled in acid reduced to a limit after some time, which they termed the limit of degree of polymerisation (LODP) and ascribed the effect to the rapid oxidation of an amorphous component followed by the slower, steady oxidation of the remaining crystalline component. Rånby followed up on their work and reported on the formation of a viscous colloidal suspension following either acidic or basic oxidation of purified cellulose. He related the colloid particles to x-ray diffraction studies that had deduced the existence of cellulose crystals approximately 6 nm in width and at least 60 nm in length, making him the first author to formally describe nanocrystalline cellulose. Direct confirmation of the needle-like cellulose crystals came from TEM studies by Mukherjee in the 1950s.

The forms of cellulose considered nanoparticulate vary greatly by source and processing conditions. Nanofibrillar cellulose (NFC) consists of disaggregated cellulose fibres that may not be entirely crystalline. Cellulose nanocrystals (CNCs) are crystalline, but may have low aspect ratios when derived from sources of low crystallinity, such as cotton. Only very highly crystalline cellulose with very high aspect ratios (>1:100), such as tunicate cellulose, can be described as cellulose nanowhiskers (CNWs).
PHYSICAL PROPERTIES

The dimensions of cellulose nanocrystals depend on the species that the cellulose has been extracted from, as well as the chemical processing the material has been subjected to. Elazzouzi-Hafraoui et al. studied nanocrystalline cellulose extracted by sulphuric acid hydrolysis from several species and found that the polydispersity (range of polymer sizes) in nanocrystal dimensions increased with length of hydrolysis, consistent with the hydrolysis of the cellulose chain and not simply the disruption of intermolecular bonds between chains. Dimensions also varied by species: the lengths of CNCs from cotton and avicel were approximately 100-300 nm while the diameters ranged between 4 and 6 nm. Numerous lateral aggregations could be found between these CNCs. On the other hand, while the tunicate CNCs could be several micrometres in length and 12 nm in diameter, no lateral aggregation was seen between crystals.

Several groups have measured the elasticity of CNCs. In x-ray diffraction, the change in the lattice spacing of strained cellulose crystals is measured. Using this technique, Sakurada et al. obtained values of 137 GPa for cellulose I, which agreed well with the value of 138 GPa Nishino et al. obtained for the same. In Raman spectroscopy, the strong 1095 cm$^{-1}$ peak of cellulose shifts position on applying a known strain to the material, which is proportional to the elastic modulus of the material. Sturcova et al. used Raman spectroscopy to obtain an elastic modulus of 145 GPa for tunicate cellulose. Iwamoto et al. used an AFM cantilever tip to deform single cellulose microfibrils suspended on a silicon grating to create a three-point bending test. They obtained values of 145.2 ± 31.3 and 150.7 ± 28.8 GPa for tunicate cellulose samples prepared by TEMPO-oxidation and sulphuric acid hydrolysis, respectively.

Fewer studies on the strength of cellulose nanowhiskers have been undertaken. Saito et al. measured the tensile strength of nanocrystals extracted from coniferous wood (Cryptomeria japonica) and tunicate (Halocynthia roretzi) sources obtaining values of 1.6 – 3.0 GPa for the wood nanocrystals and 3 – 6 GPa for the tunicate. The density of crystalline cellulose is 1.5 Mg/m$^3$, which
when normalised to its high elastic modulus, gives it a specific modulus of 92 GPa Mg\(^{-1}\) m\(^3\), higher than those of both aluminium and steel \(^{236}\).

**CHEMICAL MODIFICATION**

Cellulose nanocrystals have abundant hydroxyl groups at their surface, making them amenable to a variety of chemical modifications. It is often desirable to surface modify cellulose nanocrystals as it improves their dispersibility in solvents. As they are highly hydrophilic, the addition of hydrophobic groups facilitates their incorporation into polymers. It can be challenging to functionalise the surface of CNCs without disrupting the internal hydrogen bonding network inside the core of the structure, which results in its disintegration: as a result, several approaches have been taken to achieve this result. \(^{237}\). Modification approaches can be divided into three categories: the derivatisation of surface-accessible hydroxyl groups, the grafting to of polymer chains, and grafting from reactions where an initial surface reaction acts as a nucleus for the growth of polymer chains.

**Oxidation**

Cellulose is hydrolysed by the oxidation of the \(\beta 1,4\) glycosidic bond, most commonly by acids. The reaction mechanism is illustrated in the schematic overleaf in Figure 2-23 and proceeds in three stages. First, the proton from the acid protonates the glycosidic oxygen, creating a net positive charge. Second, the adjacent oxygen donates a lone pair of electrons to form a double bond, and the positive charge is transferred to the C\(_1\) atom, resulting in the cleavage of the glycosidic bond. Finally, the carbonium ion formed in the second stage is attacked by water, which after elimination of the proton, gives a free sugar residue and regenerates the proton. A less favoured reaction (also illustrated) mechanism sees the positive charge transferred from the glycosidic oxygen to the pyranic oxygen before being transferred to the C\(_1\) atom \(^{238}\)
The glycosidic bond that is attacked depends on the environment the chain exists in. Bonds situated at the ends of the chain are preferentially attacked, with the result that instead of obtaining shorter chains of random length, a high yield of glucose molecules are produced as the chains are eroded. When hydrogen bonding between chains is disrupted (e.g. by high temperatures or pressures), the rate at which hydrolysis can proceed increases significantly,
over that that would be expected were the reaction limited by the thermal energy available. Cellulose microfibrils are highly crystalline but are prone to localised chain dislocation defects that create amorphous regions. The degree of crystallinity varies by species, ranging from 20-30% in the Asian fibre plant ramie (Boehmeria nivea) to 90% in the algal species Valonia. The existence of amorphous regions is important in the formation of cellulose nanowhiskers as these regions are preferentially oxidised. Figure 2-24 below shows a schematic of cellulose microfibril with locally disordered sections.

Derivatisation
The most commonly performed derivatives are the substitution of hydroxide groups for either carboxylate groups or sulphate half-esters. Carboxylate groups can be created on the C6 atom by the action of free radical oxidation by 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) oxidation in the presence of sodium bromide and sodium hypochlorite. Under basic conditions, this is catalysed by sodium bromide to the aldehyde, followed by the oxidation of the aldehyde to the corresponding carboxylic acid by hypochlorite. This reaction is selective for the primary hydroxyl groups on the cellulose chain.
Sulphate esters, also known as cellulose sulphate, can be produced by the direct action of sulphuric acid, where it is a by-product of sulphuric acid hydrolysis or via reaction with chlorosulfonic acid\textsuperscript{237}.

The reaction of cellulose with sulphuric acid can be summarised by

\[
\text{Cellulose} - \text{OH} + \text{H}_2\text{SO}_4 \rightarrow \text{Cellulose} - \text{OSO}_3\text{H} + \text{H}_2\text{O}.
\]

A summary schematic for both TEMPO-mediated oxidation and sulphuric acid sulfonation can be seen in Figure 2-25.

**Figure 2-25:** Summarised reactions of cellulose with sulphuric acid and TEMPO to produce sulphate half-esters and carboxylate groups respectively. Adapted from Habibi\textsuperscript{237}.

Sulphate esters are labile and can be exchanged for example for sodium salts by the action of sodium hydroxide\textsuperscript{244}. This can allow for surface functionalisation with other cationic groups such as the ammonium cation in hydroxypropyltrimethylammonium hydroxide (HPTMA)\textsuperscript{244}.

TEMPO-oxidised surfaces may be used as a basis for grafting on functional polymers\textsuperscript{245}, or the carboxylic acid group may be exchanged for other groups,
e.g. by acetylation. Many polymers are hydrophobic, while CNCs are hydrophilic and thus direct incorporation of CNCs into polymer blends does not result in good mechanical linkage. Therefore, acetylation improves the ability of CNCs to be incorporated into polymer composites by reducing its surface energy. Other methods of acetylation include the deposition of vapour-phase trifluoroacetic anhydride and the reaction of vinyl acetate.

**Grafting to reactions**

Grafting on entire functional molecules, such as polymers or proteins, is of great interest for modifying cellulose nanoparticles for direct use and there are a number of approaches. One is to use intermediate molecules which bind specifically to cellulose. Xyloglucan, a polysaccharide, binds to cellulose with great specificity. Xyloglucan can itself be modified with xyloglucan transferase and conjugated xyloglucan transferase has been used to graft on such molecules as the green fluorescent molecule fluorescein isothiocyanate, FITC and the oligopeptide sequence GRGDS.

Another naturally occurring molecule that binds to cellulose with high specificity is the cellulose-binding domain (CBM) found in several carbohydrolases secreted by several species of bacteria. A study utilising recombinant RGD-CBM proteins proved to this to be an efficient way of transferring the tripeptide sequence RGD (\(\text{L-arginine-glycine-L-aspartic acid}\)), to cellulose constructs. This serves as a recognition site for the integrin-mediated binding of cells to substrates. Modified constructs showed improved the adhesion of fibroblasts to the surfaces. Pértile et al. built on this work to create IKVAV (laminin-binding sequence) linked CBM which improved the adhesion and growth of neuroblasts and mesenchymal stem cells to bacterial cellulose pellicles.

The availability of SELEX (Systematic Evolution of Ligands by Exponential enrichment) allows for the creation of DNA aptamers (short DNA sequences) that attain configurations that specifically bind to target molecules under buffered conditions. Sato et al. used this method to create a DNA oligomer that bound specifically to cellulose Iα. Binding polymers to the DNA oligomer allowed for their efficient binding to the surface of cellulose.
Grafting from reactions
An alternative method of modifying cellulose by using the surface of the cellulose nanocrystals as a nucleation site from which controlled polymer chains can be grown. The creation of azide or alkyne groups on the surface of the cellulose nanocrystals allows the highly efficient coupling of complementarily functionalised molecules, the so-called ‘click’ chemistry mechanism \(^\text{255}\). Filpponen et al.\(^\text{256}\) developed a method of physioadsorbing azide or alkyne modified carboxymethyl cellulose to cellulose substrates to create surfaces suitable for click reactions, demonstrated by grafting on bovine serum albumin (BSA).

2.4.3 BIOLOGICAL APPLICATIONS OF NANOCRYSTALLINE CELLULOSE

Cellulose itself is a biocompatible material: it is biologically inert and microcellulose has a long history of usage within the pharmaceutical industry as a pill binder\(^\text{213}\). Cellulosic filters also have a long history of use in haemodialysis\(^\text{257}\).

As most animals lack cellulases, cellulose is degraded only very slowly \textit{in vivo} via by non-enzymatic processes, which can be problematic for applications where quick resorbability is desired. The mechanism of non-enzymatic degradation is via the hydrolysis of the glycosidic bond. It is slow because the primary structure of the cellulose chain acts to sterically inhibit access to the bond. Highly crystalline cellulose secondarily reduces the number of glycosidic bonds available at the surface of the crystal. Therefore, the intermolecular structure of cellulose has an influence on its absorbability. An early study by Miyamoto \textit{et al.}\(^\text{258}\) into how well different forms of cellulose were absorbed or caused an inflammatory response in host tissue by implanting cellulose preparations into the back muscle of dogs. They found that while most forms of cellulose were poorly resorbed, amorphous regenerated cellulose was an exception in being resorbed over a six week period. All forms provoked no to a weak foreign body reaction.

Hart \textit{et al.}\(^\text{259}\) took advantage of the resorbability of amorphous cellulose to develop a blend of milled amorphous cellulose and bovine collagen as a wound dressing for delayed healing, using diabetic mice as a model. The healing rate in
treated mice was significantly accelerated, approaching wild type mice in wound healing. Wound dressings incorporating regenerated oxidised cellulose are commercially available as Promogran™ and Promogran Prisma™ from Systagenix. These have been found to improve the healing rate and reduce scarring of chronic wounds such as diabetic foot ulcers.\textsuperscript{260}

Bacterial cellulose (BC), derived from the interwoven structures secreted by colonies of \textit{Gluconacetobacter xylinum} (\textit{Acetobacter xylinum} in older texts), is the best studied material for its potential as a biomaterial. BC consists of a sponge, known as a pellicle, made from 100 nm diameter cellulose fibres secreted by the bacteria when cultured in nutrient solution. In static solution, it forms a stratified structure with a compact layer at the air-water interface and a porous lower edge. Part of its attractiveness derives from its high level of purity — it is free from compounds such as lignin, pectin and arabian found in wood sources of cellulose\textsuperscript{261} — and the consequent ease of processing. BC has a high wet strength, exceptional water retention and a high permeability to gases, all factors of interest to tissue engineering applications\textsuperscript{262}.

Helenius \textit{et al.}\textsuperscript{263} studied the biocompatibility of purified but otherwise unmodified BC pellicles using a rat model. Subcutaneously implanted pellicles showed no inflammatory or foreign body reactions, neither acutely (1 week) nor chronically (12 weeks). Pellicles were invaded by fibroblasts and blood vessels, particularly where the porous side was apposed to the skin: fibroblasts conformed to the wavy pattern of the scaffold and secreted collagen within it, integrating the scaffold with the surrounding tissue. Commercially, preparations of BC are available as skin wound dressings: initially marketed as ‘Biofill’, FDA approved Dermafill™ for burns and ulcers is marketed by Cellulose Solutions\textsuperscript{264}.

Vascular engineering has seen the greatest development of BC scaffolds. Leitão \textit{et al} \textsuperscript{265} formed prosthetic femoral arteries from purified, sodium dodecyl sulphate (SDS)-leached BC mats by pushing a sharp needle of the requisite size through the mat, before forming and freeze-drying the material. This created a graft with a smooth internal surface that was well-tolerated when grafted onto...
the femoral arteries of pigs. Wippermann et al. performed an in vivo study of vascular implants in the carotid arteries of pigs over a period of three months. Of the eight vascular implants made, one was found to be occluded (blocked) but the implants were otherwise well-tolerated, being colonised by adjacent cells to form vessels showing well-organised intimal and collagenous layers. A longer-term study was performed by Malm et al. who looked at the patency of vascular implants over 13 months, using sheep as the model animal. They found a lower patency rate, with only 50% of implants remaining unoccluded, although surviving implants were well-tolerated and integrated into surrounding tissue.

By contrast, the biological effect of cellulose nanowhiskers is comparatively little-studied. The physical and chemical properties of nanocrystalline cellulose, being very strong, light and amenable to chemical modification make it potentially of interest for tissue engineering applications.

A hydrogel consisting of homogenised beech pulp cellulose nanofibers (cryo-TEM of the material showed individual fibres to be around 7nm wide, but aggregation caused the average fibre bundle width to be 20-30 nm) in aqueous solution formed a weak hydrogel with a storage modulus, $G'$, of 10 Pa. Nanofibrillar cellulose (NFC) hydrogels were found to have a wide range of temperature and pH over which the gel strength remained near constant. Shear thinning behaviour meant that suspensions of NFC hydrogel with cells were injectable, with gelation recurring once shearing stopped. HepG2 cells cultured in NFC hydrogels survived and appeared as mitochondrially active as those cultured in commercial hydrogel preparations such as Puramatrix™, Maxgel™ and Extracel™. NFC hydrogels have also been used to support human pluripotent stem cells, producing spheroids of cells which maintained their stemness and normal karyotype over several passages.

One of the few studies into the direct physical influence of CNWs on cells comes from Dugan et al. Utilising CNWs extracted from two tunicate species Halocynthia roretzi and Asciella aspersa, he found that oriented CNWs could serve as contact guidance templates for C2C12 myoblasts. Given their
small size, 5 nm in width, his work demonstrated the smallest topographical features to act as cell guidance.

Another study making specific use of the scale of CNWs to influence cellular behaviour is by Jia et al.\textsuperscript{270}, who investigated the effect that incorporating both microcrystalline cellulose and cellulose nanowhiskers into electrospun fibres would have on cell growth. Scaffolds of cellulose acetate with both incorporated showed far higher rates of rat smooth vascular muscle cell survival and proliferation than those with either MCC or CNWs alone and the incorporation of either led to an improvement in cell survival and proliferation over cellulose acetate alone. Chemically modifying the surface by deacetylation did not significantly modify cellular behaviour. The authors attributed the observed effects to the creation of multiple roughness scales on the scaffold, with MCC providing attachment sites and CNWs modulating the nature of the cellular attachment so as to permit more proliferative behaviour.

On account of the size of CNCs and, in particular, the high aspect ratio of CNWs, the potential cytotoxicity of cellulose nanocrystals is of concern. There are three main routes through which toxic material can enter the body: via inhalation, oral consumption or through skin contact.

Several studies of nanocrystalline cellulose suspensions on cells have been performed. Moreira et al.\textsuperscript{271} studied the effect of nanowhiskers derived from bacterial cellulose on CHO cells or 3T3 fibroblasts; no adverse effect on cell viability nor genotoxicity was detected. Compared to multi-walled carbon nanotubes, cotton CNCs are far less toxic. Clift et al.\textsuperscript{272} exposed human lung cocultures to dispersions of cotton CNCs, multi-walled carbon nanotubes (MWCNTs)\textsuperscript{1} and crocidolite asbestos fibres (CAFs). While all three materials induced some inflammatory response, pro-inflammatory factors were significantly higher in MWCNTs and CAFs. A more wide-ranging study utilised measurements of the impedance between an electrode and cells grown on its

\textsuperscript{1} Systematic work by several groups have since shown that the reported toxicity of carbon nanotubes and related material is more related to the processing steps undertaken to produce the material associated trace impurities rather than an intrinsic property of the material. The interested reader is referred to Seabra et al. for an excellent review on the topic.\textsuperscript{273}

surface to understand the cytotoxicity of carboxylate-functionalised CNCs derived from various sources. Cells remained viable over the tested concentration range (10 – 200 µg/mL) and the particles had no effect on their doubling time.

In summary, the use of cellulose nanowhiskers for tissue engineering applications is one that is open for further development. Despite their size, they appear to have lower cytotoxicity than other similarly sized nanoparticles. Where not engulfed by cells, their topography appears to influence cellular behaviour both in terms of inducing orientation and in terms of modulating cellular attachment to surfaces.

**Orientation of Cellulose Nanocrystals**

Radial shear in the form of spin-coating has been used by several investigators including Cranston and Gray, Dugan and Kontturi et al. to create layers of uniform thickness. Spin-coating creates uniform coatings but is limited to covering relatively small, planar substrates, up to several centimetres in diameter.

Cranston and Gray took advantage of the ability of cellulose nanocrystals form chiral nematic suspensions which can be magnetically aligned, using the 7T magnetic field from an NMR machine to create aligned deposits on a PAH surface. They initially adsorbed to the surface in a disordered fashion and aligned over 24 hours. A more recent study on magnetic orientation by Pullawan et al. utilised a 1.2T field from an EPR instrument to align solvent cast CNW reinforced nanocomposites over a 3-hour period. The highest mechanical strength and stiffness occurred perpendicular to the applied magnetic field, which corresponds to the direction of the main axis of alignment of CNWs. The obtained strength and stiffness were not as high as would be expected in a fully-aligned film, which is reflective of the slow kinetics of the alignment of CNWs.

A third method of orienting CNWs is via convective and shear forces. When contained in a solution that is drying on the surface, cellulose nanocrystals, being rod-like, tend to be aligned tangentially to the dry-line (as termed by
Mashkour et al.\textsuperscript{279}). Controlling the shape and rate of advancement of this line has been used to create aligned cellulose nanocrystal surfaces\textsuperscript{279}. Hoeger et al.\textsuperscript{280} used a similar method, drawing out the suspension on a mica surface with the help of a glass slide create oriented surfaces. Deposition of CNC multilayers led to an improvement in orientation (from approximately 40% oriented to 70% for multilayers). The degree of orientation of CNWs on surfaces differed by substrate with gold giving the best orientation and mica the worst.

Electric fields are also able to align cellulose nanowhiskers. Csoka et al.\textsuperscript{281} added an external AC electric field to a shear-assembly method obtain near perfect CNW alignment on mica using an AC electric field of 100 cm\textsuperscript{-1} and oscillating at 2 kHz. Chen et al.\textsuperscript{282} used an electric field generated by two parallel electrodes cell to align a CNW-alginate gel using 40V for 1 minute per layer.

Finally, an ingenious method developed by Nyström et al.\textsuperscript{283} uses a PDMS stamp to transfer CNCs to a polyelectrolyte-coated surface. Fine wrinkling is created by stretching the elastomer film. Spin-coating on a solution of CNCs creates a uniform coating aligned within the wrinkles of the stamp which can then be transferred.
2.4.4 CHITOSAN

Chitin is a natural polymer consisting of β-(1-4)-linked D-acetylglucosamine. It is a key structural component of crustacean shells. Chitosan, β-(1-4)-linked D-glucosamine, is a linear polysaccharide formed from the deacetylation of chitin and the structure of both can be seen in Figure 2-26 below.

![Figure 2-26: Structural formula of a) chitin and b) chitosan](image)

Chitosan has a pKa of 6.5 and is thus protonated in acidic and neutral solutions. Chitosan readily forms polyelectrolyte complexes with cellulose nanowhiskers if the latter are negatively charged. Chitosan and cellulose, both being polysaccharides, degrade by the hydrolysis of the β(1-4) linkage between monomers. Rapid hydrolysis, in minutes to hours, requires the use of acids and is enhanced by raised temperatures.

Chitosan has a long history of use in pharmaceutical applications as it is able to adhere to mucous membranes, has a low cytotoxicity and has anti-bacterial properties. It supports the attachment and proliferation of cells. Chitosan and cellulose have been used in a variety of applications, including dye removal,
wound dressings, and controlled drug delivery \(^{285}\). Their potential use as a wound dressing has been of considerable interest. For example, Cao et al. \(^{286}\) solvent cast regenerated cotton cellulose to create a microporous scaffold which was then soaked in chitosan. The resulting material showed several properties desirable in a wound dressing: excellent water retention properties, flexible, anti-bacterial.

Cellulose nanocrystals are also used as a means of improving the mechanical properties, particularly tensile strength of chitosan-based materials. Li et al. \(^{287}\) for example found that incorporating cellulose nanowhiskers into chitosan increased the tensile strength of the composites from 85 to 120 MPa as the whisker content was increased from 0 to 15 wt%.

2.4.5 POLYETHYLENIMINE (PEI) AND POLY(4-SODIUM STYRENESULFONATE) (PSS)

![Figure 2-27: Structural formula of monomer unit of branch-chained polyethylenimine](image)

Polyethylenimine (PEI), is a polymer with a repeating unit consisting of an amine group with aliphatic carbon spacers. It comes both as a linear polymer and as the branched polymer used in this project and illustrated in Figure 2-27 above. Branched PEI has a high cationic charge density at physiological pH and is widely used as a transfection agent within molecular biology. Most biological materials, including cell membranes and DNA, have a net negative charge. Its charge density thus enables micelles or PEI-functionalised nanoparticles to
carry cargoes of DNA $^{288}$ or RNA $^{289}$ and it can force endocytosis into the cell, allowing it to deliver its cargo $^{290}$.

Poly(4-styrenesulfonic acid) sodium salt (PSS) is a strong polyanion. Its structural formula can be seen in Figure 2-28 below.

![Figure 2-28: Structural formula of the monomer of poly(4-styrenesulfonic acid) sodium salt](image)

PSS is classified as non-hazardous according to Regulation (EC) No. 1272/2008 and has an LD$_{50}$ of greater than 8000 mg/kg. It is used medically as a cation-exchange resin for patients experiencing renal failure and who have high circulating levels of potassium. Typical administered doses are 30 g per day and it is frequently combined with sorbitol which has a synergistic effect. However, in this role, there have been concerns about its safety as it is implicated in colonic necrosis of patients $^{291}$.

Cytotoxicity cannot be considered separately from mode of administration. Nanomaterials that are presented as a flat layer are significantly less likely to cause toxicity effects as they cannot be readily internalised by cells $^{82}$. Within this project, PEI was used as an adhesion promoter to ensure the stable adhesion of the cellulose based layer by layer films to the glass substrate, in which role it is widely used $^{292}$. On account of its ability to breach cell membranes, PEI is potentially cytotoxic and indeed there are reports of it inducing cell death via the Akt pathway $^{293}$. However, as the PEI is to be applied as a single layer which is tethered to a solid substrate, not being free to dissolve into solution, the available concentration of PEI was expected to be very low.
PSS has been used to complex with and reduce the cytotoxicity of nanoparticle surfaces. For example, Wan et al. 294 found that coating cetyltrimethylammonium bromide functionalised gold nanoparticles with PSS and/or PAH reduced the cytotoxicity of the particles without compromising their stability. Some investigators have used PSS as part of a layer-by-layer constructs for in vitro cell culture. Palamà et al. 81 for example used micropatterned channels of poly(allylamine hydrochloride) and poly(sodium 4-styrene sulfonate) to constrain, align and promote the myogenic differentiation of C2C12 myoblasts.

In this work, PSS was selected to ensure that the PEM film was always charged to improve its stability under cell culture conditions.
2.4.6 LAYER-BY-LAYER DEPOSITION

DEVELOPMENT

A polyelectrolyte is a polymer that consists of units that are ionized or that are able to be ionized in solution\textsuperscript{295}. Polyelectrolytes are ubiquitous throughout nature: DNA, RNA, and most proteins are polyelectrolytes.

Decher developed the concept of layer-by-layer (LBL) growth using soluble polyelectrolytes\textsuperscript{296}. The concept is simple: on priming a surface with a charged species, an oppositely charged species is adsorbed to the surface by electrostatic attraction until the surface is oppositely charged, illustrated in Figure 2-29. The process is repeated as many times as is desired to build up a multi-layered surface and the structure formed is often called a polyelectrolyte multilayer (PEM).

Figure 2-29: Schematic of the layer-by-layer construction process to build polyelectrolyte multilayer films. A: A charged polyelectrolyte adsorbs to a substrate, creating a substrate with a net charge. B: A polyelectrolyte of the opposite charge then adsorbs onto the substrate. This process can be repeated indefinitely.

Its advantage over chemiabsorption-based methods such as self-assembled monolayers (SAMs) is that it is not dependent on the chemical identity of the
substrate. Whereas thiols form well-organised monolayers only on noble metals, LBL films can be formed on any charged surface. It is less sensitive to the completeness of coverage: charge density is the limiting factor in determining the deposition of material. Unlike Langmuir-Blodgett films, the substrate topography is less important and PEMs can be formed on curved surfaces. Nearly any charged species that can be precipitated from solution can be successfully deposited using LBL growth: polysaccharides, proteins, nanoscale wires or clay platelets.

Traditionally, PEMs are deposited by the dip-coating successive bilayers with or without intermediate rinsing steps. Spin coating, spray deposition and dewetting are all alternative deposition methods in various stages of development.

**MECHANISMS OF LBL GROWTH**

Multilayer formation and the internal structure of PEMs is a result of a complex balance of interactions. Electrostatic interaction is the most obvious of the interactions and was initially thought to be dominant. The charges in a film need to be balanced and this can happen in one of two ways. Complexes can be formed between oppositely charged electrolytes, with small counter-ions being excluded: this form is known as intrinsic compensation. Alternatively, the counterions can interact with the polyelectrolytes to balance the charges in the film, which is known as extrinsic compensation.

The major driving force for PEM formation is the entropy increase due to the release of counterions. The formation of polyionic complexes is favoured over charge compensation by many smaller counterions. The entropic increase enables PEM formation to occur even at high ionic strengths when the thickness of layer growth would be expected to be reduced owing to charge shielding.

**MODES OF GROWTH**

Two growth modes are found in PEMs: linear growth where the thickness increases linearly with the number of bilayers deposited and exponential growth where the thickness of successive layers increases exponentially with
the number of deposited layers. Exponentially grown systems tend to be softer and show more interfacial roughness than linearly grown systems. While exponential growth is more frequently found in natural organic systems (e.g. poly-l-lysine/hyaluronic acid (PLL/HA)) and linear growth is more common in synthetic organic systems (e.g. poly(allylamine) hydrochloride/polystyrene (PAH/PS)), all systems can show exponential growth under appropriate conditions detailed next.

The theoretical model developed by Lavalle et al.\textsuperscript{303} describes exponential growth as occurring when within a multilayer, a mobile species is able to diffuse to the surface and react with incoming polyions to form an additional layer. As the concentration of the mobile species is constant within a deposited layer, with successive layers more free ions are available to react, causing progressively thicker layers to form. This reaction is diffusion-limited and the thickness of new layers reaches a limit as the amount of free polyionic species able to diffuse to the surface becomes limited by its diffusivity, \(d = \sqrt{2Dt}\)

\textbf{Equation 2-7}

where \(d\) is the distance at which free polyion is able to interact with incoming polyions of opposite charge, \(D\) is the diffusion coefficient of free chains, and \(t\) is the contact time with the solution of oppositely charged polyelectrolytes.

Polyelectrolyte film growth and conformation is kinetically complete between 15 and 20 minutes. This has been investigated by grazing angle x-ray reflectivity\textsuperscript{304}. However, a systemic study by Yang et al.\textsuperscript{305} on montmorillonite or laponite platelets intercalated with branched PEI indicated that PEMs can be successfully built using shorted deposition times. By varying the deposition time from 5 seconds to 5 minutes, they found that the deposited thickness of the film was inversely related to the deposition time. Films deposited over a shorter time were less dense and showed a higher surface roughness.

The distance over which polyelectrolye molecules interact depends on the Debye length. As charged particles, polyelectrolyte molecules attract oppositely-
charged ions from solution and form a ‘double-layer’ interaction that electrostatically screens the molecule from the surrounding solution. The effective Debye length depends on the concentration of the polyelectrolytes in solution and the ionic strength of the solution. An investigation by Tadmor et al. 306 using hyaluronic acid with varying concentrations of cationic surfactant allowed them to find effective Debye lengths of between 6.0 and 10.0 nm.

If the multilayer is thicker than the diffusion zone at a given time, then the growth will become linear rather than exponential. In this sense, linearly-growing systems can be seen as diffusion-limited ones and it is possible to cause systems that typically grow linearly to grow exponentially by changing the preparation parameters, such as solution pH or counter-ion concentrations 307.

Once formed, PEMs of strong polyelectrolytes (polyelectrolytes that are charged at all solution pHs) are stable under all ionic solution strengths308, but those of weak polyelectrolytes (whose charge depends on the solution pH, such as proteins) can redissolve into solution at high ionic strengths as counterions stabilise the polyelectrolytes in solution.

A polyelectrolyte chain in solution will tend to form an extended chain as it has a lower free energy due to the self-repulsion of the charges. The addition of counterions shields the charges and allows the chain to form a more folded up morphology. Increasing the ionic strength of the solution polyelectrolytes are dissolved in will, to a limit, allow the creation of create thicker films as successive polymer layers are deposited in a more compact morphology which occupies more space. Above a critical ionic strength, the polyelectrolyte chain is stabilised by the counter ions to such an extent that it is no longer possible to adsorb it onto the surface of an oppositely charged polymer.

The absorption of weak polyelectrolytes can be described by analogy to the phase separation of a binary mixture with an upper critical solution temperature (UCST)309 where the critical solution temperature is replaced by a critical ionic strength, $c_{cr}$. In the figure below, region L is the regime in which a single insoluble phase is formed and is the region the PEM is stable at.
Above the critical salt solution, a single liquid phase exists and any previously deposited multi-layers are redissolved into solution, depicted in the figure as region S. Below it, the system switches from one rich in first one polyelectrolyte, then the other. If both fractions are unstable in solution, as is the case in line 1, then layers will precipitate out of solution as insoluble complexes. In regions L’ and L”, the mole fractions of the polyelectrolytes are such that an insoluble precipitate exists in equilibrium with a soluble phase.

**Characterisation of PEMs**

The pioneers of the use of CNWs in PEMs are Cranston and Gray, starting in 2006. They\textsuperscript{275} initially experimented with the possibility of creating polyelectrolyte multilayers containing cellulose nanocrystals. They used negatively charged crystals formed from cotton (approximately 200 nm in length and 10 nm in diameter) and poly(allylamine hydrochloride) (PAHCl). Spin-coating layers created smoother, thicker coverage than dip-coating bilayers, an effect they attributed to the creation of kinetically entangled as well as thermodynamically favourable coverage. SEM investigation of cross-sectioned layers showed spin-coated layers to be rougher internally. For both methods, they were able to observe three layers: a dense inner layer closest to the substrate, a porous middle layer (a site of active rearrangement) and a low-density outer surface. The electrostatic attraction between the cationic PAHCl
and the anionic cellulose was necessary: using uncharged polyethylene oxide (PEO) as a substrate failed to form PEMs.

The use of cellulose nanocrystals within PEMs continues to be of interest. A search on Web of Science in January 2017 for articles published in English in the last 5 years using the search terms "(nano*) AND (cellulose OR cellubiose) AND (layer-by-layer OR LBL OR polyelectrolyte multilayer)" within the topic field resulted in 152 articles. Most articles were focussed on basic polymer science, followed by applications of cellulose in thin films. Searching for “tissue eng* OR biomat*” within the results only found 25 articles.

Several investigators use quartz crystal microbalance to monitor the build-up of PEMs. Francesko et al. 310 used QCM to verify the successful sequential deposition of aminocellulose nanospheres and hyaluronic acid. Mohanta et al 311 also used QCM to study the mode of growth of nanocrystalline cellulose and chitosan films, finding it to be linear.

The charges on polyelectrolyte components and films is normally determined via zeta potential measurements 312-313. The composition of PEMs is most frequently assessed using Fourier-transform infrared spectroscopy (FT-IR) 310, 314. This is frequently complemented with the use of x-ray diffraction 315.

Cellulose is frequently used as a substrate on which PEMs can be built. Where the PEM has been coated onto a surface, then x-ray photoelectron spectroscopy is used to characterise the chemical composition of the surface and near-surface (up to 10 nm) atoms. An example of this is found in Wu et al. 314 who used electrospun fibres of cellulose acetate as a substrate on which to build chitosan and rectorite PEMs for cell culture applications.

The appearance of films is generally assessed using either AFM or SEM. AFM is preferred when the surface conformation of the film especially when hydrated. SEM is used to measure the thickness and cross-sectional structure of fractured PEMs. Where PEMs have been formed into fibres, such as a study by Zhang et al. 312 on pectin-lysozyme bilayers deposited on a cellulose core, SEM allows for the measurement of fibre diameters.
The mechanical properties of PEMs depend initially on the deposition conditions under which the system forms. Linearly growing systems tend to be stiffer than exponentially grown ones. Secondly, the stiffness can be modulated dramatically by cross-linking. For example, Boudou et al.\textsuperscript{316} experimented with the effect of carbodiimide cross-linking on various exponentially-growing PEMs, showing that the stiffness of a given system could be tuned over 1 to 2 orders of magnitude by the degree of crosslinking. Finally, the stiffness of the PEM depends on the conditions under which the system is measured: Cranston et al. performed direct force measurements of CNC/PAH films using AFM in solutions of varying pH and ionic strengths\textsuperscript{308}. While the PEM was stable under all measured conditions, the surrounding solutions had a profound effect on the swelling behaviour and surface roughness, leading to dramatic differences in adhesion forces and apparent stiffness.

Free-standing films can be measured directly by tensile testing. Free standing cellulose/polymer films were created by Karabulut and Wågberg\textsuperscript{317} where films were created by dip-coating NFC and PEI onto a trichloro(1H,1H,2H,2H-perfluoroctyl)silane (PFOS) functionalised silicon surface, then peeled off using adhesive tape or tweezers. Relatively thick films (>1 µm) needed to be prepared in order to be robust enough to be handled, and dynamic mechanical analysis (DMA) of 150 bilayer films at 30% RH gave a Young’s modulus of 9.37 ± 2.0 GPa. Using this method, it was also possible to determine the strength of the composite, obtaining values of between 63.5 and 80 MPa. More recently, Li et al.\textsuperscript{318} used a similar method to determine the mechanical properties of cellulose nanocrystal-reinforced collagen. They observed no change in Young’s modulus with increasing cellulose content, but did find that the tensile strength increased from approximately 10 MPa for pure collagen films to a maximum of 27 MPa for films containing 7% cellulose nanocrystals by volume.

Testing the physical properties of non free-standing cellulose/polymer PEMs is challenging as the films produced are very thin. Cranston et al.\textsuperscript{319} used strain-induced elastic buckling instability for mechanical measurement (SIEBIMM) to determine the Young’s modulus of nanofibrillar cellulose (NFC)/polyethyleneimine (PEI) films. In this method a rigid, thin film is attached to a
thick, soft underlying substrate such as polydimethylsiloxane (PDMS) and compressed. The wavelength of the ripples formed ($\lambda$) is measured and is related to the Young’s modulus of the film ($E_s$). The wavelength of the ripples is constant regardless of applied force and gives consistent results independent of the film thickness for films over 40 nm thick. The Young’s modulus of the system proved very sensitive to the relative humidity. At 0% RH, (e.g. in an SEM) the Young’s modulus was 17.2 ± 1.2 GPa, reducing to 1.5 ± 0.2 GPa at 50% RH under ambient conditions. Their explanation for this change was that PEI acted as a soft, hygroscopic polymer and would swell to separate the cellulose fibres preventing their interaction and creating a softer than would be expected matrix.

**Biological Applications**

The relative ease with which they can be created, their compositional flexibility and their tuneable mechanical properties of PEMs have resulted in their being used in several studies directly interested in studying the relationship between surface chemistry and substrate stiffness on cell attachment and growth. For example, Grohmann *et al.*320 investigated the response of osteoblasts using either chondroitin sulfate or heparin as the anionic moiety of a Poly-l-lysine (PLL)-based system. Chondroitin sulfate based PEMs proved too soft to sustain cellular attachment and growth; cells responded better to chondroitin-coated PEMs formed from PLL and poly(glycolic acid) (PGA). Heparin-based PEMs did not differ significantly in Young’s modulus from heparin-coated PLL/PGA and sustained cellular spreading and growth. Other examples include measurement of adhesive spreading of cells relative to substrate stiffness using smooth muscle cells321, endothelial cells322 and skeletal muscle cells80-81, 323.

The construction of PEM layers allows for gradients to be built into surfaces allowing the effect of combinations of several physical parameters to be explored. Stiffness gradients can be made in several ways, for example by immersion in a gradient electrolyte to induce a swelling gradient324. A more sophisticated method was to partially immerse substrates in solutions of polyacrylic acid (PAA) and PAHCl. The pH of successive polyelectrolytes was
varied and substrates were rotated by 90° between successive dips to create a film that varied both in thickness and composition\textsuperscript{325}.

PEMs are compatible with spatial patterning and Monge \textit{et al.}\textsuperscript{80} used micropatterned PDMS as a substrate on which to build up micro-patterned PLL/HA PEMs to influence myotube formation. The same group has since developed a photoactive linker enabling the selective crosslinking of PLL/HA polylayers via UV exposure through a photomask\textsuperscript{326}. 


2.5 PROJECT AIMS

Adult derived stem cells, such as mesenchymal stem cells (MSCs), are a promising source of progenitors for tissue engineering and regenerative medicine. This work investigates the ability of a nanotopographical cue to align and promote the myogenesis of cells, using both committed myogenic cells and adult stem cells.

Microtopographical approaches to cell guidance are on the length scale of the cell, whereas nanotopography is on the length scale of the cellular focal adhesions and therefore have the potential to create a more fine-tuned response to material, for example by inducing a change in the integrins used to bind to that surface, with its attendant downstream changes in mechanotransduction.

A second interest of this work is the mechanical properties of the scaffold produced. Creating substrates with defined bulk mechanical properties is relatively straightforward: hydrogels have long fulfilled this need. It is also relatively straightforward to pattern the topography of the substrates. Far fewer studies alter both topography and bulk mechanical properties.

Nonetheless, this may be a useful approach to enhancing the myogenic differentiation of cells as the combination of topographic and mechanical factors may induce myogenic differentiation more efficiently than either alone. It is therefore proposed that careful control of the mechanical and topographical aspects of the cell culture environment may be the ‘missing link’ in the development of a definitive myogenic differentiation protocol. Indeed, it has been shown that under certain circumstances, cell culture substrates of physiological stiffness may lead to spontaneous myogenic differentiation of MSCs.

Using layer-by-layer assembly from a selection of charged synthetic polyelectrolytes and biomacromolecules, methods of modulating the film stiffness and surface topography through cross-linking and incorporating CNWs will be developed. These films will be mechanically characterised using atomic
force microscopy (AFM). The effect of film stiffness, topography and biomolecular constitution on the myogenic differentiation of stem cells, their influence on the make-up of the heterogeneous stem cell population formed by adhesion of mesenchymal cells onto surfaces and the potency, differentiation and proliferation of cells subcultured from isolation through to ultimate senescence on the LBL films.

This project aims to create layer-by-layer based composites using cellulose nanowhiskers from tunicate or cotton sources and to investigate their effect on skeletal muscle myoblasts (C2C12 cells) and also mesenchymal stem cells from various origins. On the basis that the stiffness and topography of substrates affect the phenotype and myogenic differentiation of C2C12 myoblasts as well as the myogenic differentiation of MSCs, the following initial objectives are proposed:

- Produce CNWs from tunicates and cotton and characterise dimensions
- Investigate cellular interaction with CNWs
- Produce films of varying thickness and composition using Layer-by-layer assembly and incorporate CNWs to modulate topography and stiffness.
- Analyse film mechanics by AFM microscopy
- Characterise effects of film stiffness and topography on a) terminal differentiation of C2C12 myoblasts and b) myogenic differentiation of commercially isolated MSCs.
3 METHODS AND MATERIALS

3.1 PRODUCTION OF CELLULOSE NANOCRYSTALS AND LAYER-BY-LAYER FILMS

3.1.1 REAGENTS

CELLULOSE SOURCES

Several kilograms of gutted tunicates from *Ascideilla* spp. were obtained from Glycomar, Oban, Scotland in March 2012 by Dr. Allison Harvey on behalf of the Gough research group and stored at -20 °C. All batches of tunicate cellulose prepared are from sub-samples of this master stock. Cotton cellulose in the form of ashless floc (catalogue number Z752878) was obtained from Sigma-Aldrich, U.K.

LAYER-BY-LAYER MATERIAL SOURCES

Sulphuric acid (H₂SO₄, >95%, ‘Extra Pure’, catalogue number S/9160/PB17), acetic acid (CH₃COOH, glacial, ‘Extra Pure’, catalogue number A/0360/PB17), 18mm x 18 mm coverslips (no.1 thickness, Menzel ‘Best’, catalogue number MNJ-400-010X) and sterile aerosol barrier pipette tips (all Fisherbrand™, 0.1-10 µl, catalogue number 02-707-439, 2 - 20µl, catalogue number 02-707-432, 20-200µl catalogue number 02-707-430, 100-1000µl, catalogue number 02-707-404) were bought from Fisher Scientific, U.K. 13mm diameter coverslips (no.1 thickness, catalogue number ECN 631-1578) were bought from VWR International, U.K. 0.1 M standardised sodium hydroxide solution (catalogue number 319481), 1.0 M standardised sodium hydroxide solution (catalogue number 319511), 0.1 M standardised hydrochloric acid solution (catalogue number 318965) and 1.0 M standardised hydrochloric acid solution (catalogue number 318949) were purchased from Sigma-Aldrich, U.K.

Hydrogen Peroxide (H₂O₂, 30%, Perdrogen™, catalogue number 31642), sodium hydroxide (NaOH, pellets, reagent grade, catalogue number S5881), sodium hypochlorite (NaClO, 4% active Cl, catalogue number 23,930-5) and dialysis
tubing (27mm diameter, MWCO 12-14 kDa, catalogue number D9527) were bought from Sigma-Aldrich, U.K.

Chitosan (Chi, medium molecular weight, \(M_w \approx 190-310\) kDa, 75-85% deacetylated, catalogue number 448877), Poly(sodium 4-styrenesulfonate) solution (PSS, 30% in H2O, \(M_w \approx 70\) kDa, catalogue number 527483), Polyethyleneimine (PEI, branched, \(M_w \approx 25\) kDa, catalogue number 408727) and Poly-(allylamine hydrochloride) (PAHCl, \(M_w \approx 56\) kDa, catalogue number) were all purchased from Sigma-Aldrich, U.K.

**Material Properties Determination**

Mica sheets (75 mm x 25 mm x 0.15 mm thick, catalogue number AGG250-1) and AFM specimen discs (12mm diameter stainless steel, catalogue number AGF7001) were purchased from Agar Scientific Ltd, U.K. 10nm gold nanoparticles (0.1 mM in PBS, catalogue number 752584), poly-L-lysine (0.01%, ‘Bioreagent’, catalogue number P4832), petri dishes (TPP® tissue culture polystyrene, 22.1 cm\(^2\) surface area, catalogue number Z707678), mixed bed ion-exchange resin (‘Amberlite MB 6113’, catalogue number 06791) and hexamethyldisilazane (HMDS, \(\geq 99.0\% ‘GC grade’, catalogue number 52619) were purchased from Sigma-Aldrich, U.K. Ethanol (absolute, analytical reagent grade, catalogue number E/0650DF/17) was purchased from Fisher Scientific U.K.

**3.1.2 NanoCellulose Extraction and Purification**

**Crude Cellulose Extraction**

Cotton cellulose was used as received for nanocrystal extraction. Tunicates were stored at -20 °C, divided between resealable bags which each contained approximately 50 g of raw material. To use, they were defrosted and cleaned under running water to remove debris and adherent organisms, then coarsely chopped with a kitchen knife. Excess water was squeezed out and the material weighed for further processing.

To deproteinize the tunicates, 50 g of material was placed in a beaker with 300 ml water, which was then heated to 80 °C with stirring. 15 g of NaOH as pellets
were then slowly added to the beaker, which was then left to stir overnight. The resulting material was rinsed thoroughly under first tap water, and then deionized water until the pH of the rinsate was approximately 8. If the material appeared to have any surviving gross organic matter such as fine bits of shell or byssus, then the deproteinization step was repeated.

The material was subsequently bleached by suspending in 300 ml deionized water, heating to 60 °C and adding 0.5 ml glacial acetic acid and 1 ml NaClO before stirring for a further 5 hours. After cooling and rinsing in deionized water, the material was pulped using a hand-held blender before being centrifuged to remove the supernatant water. The pulp was frozen to -80 °C and then freeze-dried. The dried material is termed tunicin. A representative sample was always tested for purity by FT-IR (described Section 3.1.4 below) before being used for nanocellulose extraction.

**CELLULOSE NANOCRYSTAL (CNC) EXTRACTION**

The vital parameters to control the production of nanocrystalline cellulose are the concentration of the acid used, the reaction temperature and time. The acid concentration, temperature and time used were developed in the Gray laboratory and are parameters widely used to obtain high-aspect ratio cellulose nanocrystals \(^{329}\). 64% w/w sulphuric acid was prepared from concentrated stock (95%) and the density resulting solution adjusted to 1.5421 g/ml at 20 °C using a specific gravity hydrometer (range 1.480 to 1.550 g/ml, VWR U.K., catalogue number 34627-479).

Samples of cotton or tunicin were gradually added to a round-bottomed flask within an unstirred water bath at 45°C containing sulphuric acid at a ratio of 87.5 ml sulphuric acid per gram of cellulose and stirred with a PTFE stirrer mounted on an overhead stirrer. After 30 minutes, the reaction was stopped by rapidly tipping the contents of the flask into a beaker containing ten times the volume of ice-cold deionized water.

The cellulose was separated by centrifuging 250 ml Nalgene containers at 8000 rpm for 5 minutes at a time, decanting the supernatant and replacing with approximately 100 ml of deionized water. After 2 to 3 centrifugations, the
reduced acid concentration allowed the particles to form a stable colloid that resisted sedimentation. At this point, the mixture was dialysed against deionized water with daily water changes for 10-14 days and was deemed complete when the pH of the surrounding solution had remained stable for 2-3 days.

200 ml aliquots of the material were decanted into a beaker and sonicated using an ultrasonicator (Model 250, Branson) equipped with a flat tip at an amplitude of 30% maximum for ten minutes. The beaker was kept in ice to prevent the suspension temperature rising over 40 °C. Prior to ultrasonication, aggregates could be seen under a low-powered light microscope. No aggregates were visible after sonication.

Tunicate nanowhisker suspensions were decanted into 50 ml polypropylene centrifuge tubes (conical, sterile, VWR U.K., catalogue number 525-0403) and centrifuged at 8000 rpm to sediment any metal particulate matter coming off the sonication probe. Cotton nanocrystals were filtered using a Buchner filter funnel through glass filter paper (Whatman GF/C, Sigma-Aldrich U.K., catalogue number Z242330). Samples were placed into clean, labelled Winchester bottles and kept in the refrigerator. No tendency to sediment was noted, even after several weeks. The stability of the suspensions was confirmed by zeta potential measurements.

A flow chart of the production process can be seen below:

Figure 3-1: Flow-chart of nanocrystalline cellulose extraction process. Tunicin (tunicate cellulose) is produced by cleaning, chopping up and deproteinizing tunicates. The dried product is then treated in the same manner as purified cotton cellulose, nanocrystals being produced through controlled oxidation.
3.1.3 LAYER-BY-LAYER SUBSTRATE PREPARATION

SINGLE BILAYER SUBSTRATES

Substrate cleaning
Glass substrates to be used for alignment studies were made from 18mm x 18 mm borosilicate glass. Using a glass-cutting guide, each square was cut into two 9mm x 18mm rectangles to give a substrate with an aspect ratio of 1:2. Glass substrates for cellular response to CNWs were formed from 13 mm diameter No. 1 coverslips which were used as received. Glass was cleaned via piranha digestion. Approximately 30 glass pieces were placed into a crystallising dish and a 3:7 ratio of 30% hydrogen peroxide/ 95% sulphuric acid was carefully added to the dish. After 20 minutes, the solution was decanted and the glass pieces rinsed in deionized water until the rinsate was at a neutral pH. Substrates were left under deionized water and used within 3 hours of cleaning.

Spin-coating
A Laurell Technologies Spin coater (model WS-650SZ-6NPP-LITE) supplied with nitrogen gas (oxygen-free nitrogen, BOC gases UK) was used to spin coat samples. A three stage process was used to spin-coat samples. The first stage

Cleaned glass pieces would be removed wet from the crystallising dish and individually mounted on a 5 mm diameter vacuum chuck. 200 µl of 0.6% (w/v) PAHCl would be applied at 3000 rpm, followed by rinsing in deionized water. As a polycationic polymer, PAHCl has an excess positive charge to which the negatively charged cellulose nanocrystals are attracted. 200 µl of the appropriate nanocellulose suspension would then be applied, and then rinsed with three aliquots of deionized water. Substrates would be stored at room temperature in individual wells of a 12 or 24 well plate. Small pieces of plastic pipette tips were placed into the bottom of the wells beforehand in order to act as spacers: they facilitated the handling of the substrates.

Single bilayer samples of chitosan/CNW were prepared in a similar manner with 1mg/ml chitosan replacing PAHCl as the polycation. The spin-coating
parameters to produce radially-aligned and randomly-aligned cellulose on substrates are summarised in Table 3-1 and Table 3-2 respectively.

**Table 3-1: Summary of Spin-coating steps to produce aligned CNWs**

<table>
<thead>
<tr>
<th>Step</th>
<th>RPM</th>
<th>Acceleration</th>
<th>Time (s)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3000</td>
<td>6000</td>
<td>30</td>
<td>(200 µl chitosan or PAHCl, x2 500 µl deionized water)</td>
</tr>
<tr>
<td>2</td>
<td>8000</td>
<td>2000</td>
<td>30</td>
<td>200 µl 0.04% CNW</td>
</tr>
<tr>
<td>3</td>
<td>4000</td>
<td>10000</td>
<td>40</td>
<td>x3 500 µl deionized water</td>
</tr>
</tbody>
</table>

**Table 3-2: Spin coater parameters for randomly aligned CNWs**

<table>
<thead>
<tr>
<th>Step</th>
<th>RPM</th>
<th>Acceleration</th>
<th>Time (s)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3000</td>
<td>10000</td>
<td>25</td>
<td>200 µl chitosan or PAHCl, x2 500 µl deionized water</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>10000</td>
<td>20</td>
<td>200 µl CNW suspension</td>
</tr>
<tr>
<td>3</td>
<td>500</td>
<td>10000</td>
<td>30</td>
<td>x3 500 µl deionized water</td>
</tr>
<tr>
<td>4</td>
<td>4000</td>
<td>800</td>
<td>20</td>
<td>Drying step</td>
</tr>
</tbody>
</table>

**MULTIPLE BILAYER SUBSTRATES**

The materials selected for the construction of the PEMs were chosen on the basis of their availability, biocompatibility, low cost and stability. Branched polyethyleneimine (PEI) was used as an adhesion promoter to glass to ensure that built up layers did not prematurely delaminate in solution. Chitosan is a linear polysaccharide formed from the deacetylation of chitin. With a pKa of 6.5, it is protonated in acidic and neutral solutions and serves in this work as a polycation. It is a well-studied biocompatible polyelectrolyte. Poly(4-styrenesulfonic acid) sodium salt (PSS) is a strong polyanion (i.e. negatively charged at all solution pHs) and was chosen to ensure that the PEM film was always charged.

A 10 mg/ml solution of chitosan was prepared by dissolving 1g of chitosan powder in 100 ml of 0.1 M acetic acid in a Winchester bottle and rolling it for 2
days until all the material was dissolved. A working solution of 1 mg/ml was prepared by dilution in deionized water and was adjusted to pH 5 using 1.0 M sodium hydroxide.

All solutions except the cellulose suspension were filtered before use using a 0.45 µm syringe filter (filter material: mixed cellulose esters, Millipore, catalogue number 10460031). Solutions were stored in the refrigerator; they were allowed to come to room temperature before use.

**Dip-Coating**
Glass coverslips were cleaved and cleaned as detailed in the previous single bilayer substrate section. To dip-coat, substrates were loaded into a PTFE coverslip rack (16 at a time), blown dry with nitrogen and dipped into the appropriate solution for 1 minute, followed by rinsing in deionized water three times and drying with nitrogen before repeating with the next solution until all the desired layers are built.

A 5mg/ml solution of PEI at pH 10 was used as an adhesion promoter onto glass. As freshly-cleaned glass has a net negative charge, the polycationic PEI forms a strongly adherent coating. Chitosan was used as the polycationic moiety within the PEM films at 1 mg/ml in 0.01M acetic acid with a pH of 5. It alternated either with a 5mg/ml solution of PSS adjusted to a pH of 5 or with a 0.02% w/v suspension of CNW with a pH of approximately 5.

To investigate the influence of build composition on cellular behaviour, three different compositions were produced, varying in the CNW content of the layers. The total layer count was maintained at 24 layers (with one exception detailed below) and the compositions were labelled according to how many of the layers were CNW suspensions. 0CNW substrates consisted of only alternating PSS and CHI layers. 6CNW substrates had 6 CNW layers, replacing every second PSS substrate. 12CNW substrates consist entirely of alternating layers of chitosan and cellulose nanowhiskers. Figure 3-2 is a schematic summarising the dip-coating process and the resulting theoretical structure assuming no inter-diffusion occurs between successive layers.
Figure 3-2: Schematic of dip-coating process (left) and resulting structure (right).
3.1.4 MATERIAL CHARACTERISATION

YIELD OF CELLULOSE NANOWHISKERS

To assay the concentration of the nanocellulose, 10 g of material was weighed into clean, dry glass vials and dried to constant mass at 80 °C in an oven. This was performed in triplicate.

PURITY ASSAY OF CELLULOSE NANOWHISKERS

FT-IR measures the vibrational modes of molecules with a dipole moment (whether permanent or transient). Molecules with a dipole moment vibrate at frequencies that are characteristic of their structure. It is thus useful for determining the bond structures present in a material, which taken together can provide information about its chemical structure. In this work, attenuated total internal reflection IR (ATR-FT-IR) was used to determine the purity of the cellulose nanowhiskers produced using a Thermo Fisher Nicolet 5700 FT-IR equipped with a diamond window and a KBr beam-splitter. A deuterated L-Alanine triglycine sulphate (DTGS) detector was used to detect peaks. It is a room-temperature detector that detects peaks as thermal energy.

Experimental procedure

Freeze-dried samples of bleached tunicin, cellulose nanowhiskers and dry samples of cotton ashless floc were spread on the plate and scanned between 500 and 4000 cm⁻¹ using 32 scans with a resolution of 4 cm⁻¹. A background sample of air was measured before each sample and corrected for CO₂ and water. The background-subtracted spectra produced were compared to literature values for tunicate cellulose. All scans were performed at room temperature. The detector was not purged.

SUSPENSION STABILITY OF CELLULOSE NANOWHISKERS

Colloidal particles are screened from each other in solution by closely associated counter ions. The zeta potential is the effective electric potential taking into account the counter ion screening. In the case of colloids of like charge, this potential is repulsive and acts to stabilise the suspension with
larger values being more stable. A Zetasizer Nano ZS (Malvern Instruments Ltd) was used to measure the zeta potential of CNW suspensions.

Experimental procedure
The cellulose suspension to be tested was passed through a mixed bed ion-exchange resin to remove any counter-ions present in the solution which would otherwise mask the charge on the particles. A capillary cell was filled with the cellulose suspension and the zeta potential was measured in triplicate at 25 °C, 30 °C and 40 °C. The mean and standard deviation of the values were tabulated.

ELLIPSOMETRIC THICKNESS OF MULTILAYER FILMS

Ellipsometry measures the changes in intensity of parallel and perpendicular polarisations of light in response to interacting with a non-opaque material, by refraction, reflection, absorption and scattering. In theory this is a limited method, requiring a flat, transparent film on a reflective substrate, which is typically silicon, but it is rapid, non-destructive and widely applicable to a variety of thin films.

Typically, a light source polarised at an angle of 45° (and so having an equal vertical and horizontal component) is reflected off a surface at an angle Θ. The polarisation of the reflected light is measured by a detector. The 2 types of polarised light, p-polarised and s-polarised light are measured using two variables, Ψ, the amplitude ratio between the 2 polarisations and Δ, the phase difference between the 2 polarisations. The surface can be modelled as a series of thin sheets each with its own polarisation characteristics. Using a broad range of wavelengths and/or angles of incidence, a numerical solution to the thickness of the layers can be found.

The measured complex effective refractive index of the film is known as an 'Effective Medium Approximation' (EMA). It averages out the material of the whole film and its structure to give the loss and refractive index as if it were a single material. This can give additional information about the composition of the material. At its simplest, the Cauchy model can be used, which is a simple model describing the relationship between the refractive index of materials and the wavelength it is measured. Equations take the form:
\[ n(\lambda) = A + \frac{B}{\lambda^2} + \frac{C}{\lambda^4} + \cdots, \]

where \( n \) is the refractive index of the material, \( \lambda \) is the wavelength of light and \( A, B, C \) are coefficients determined by fitting the equation to the measured refractive indices at each wavelength. Only the first two terms were used in this work.

Light scattering or adsorption is seen as a loss in the polarisation of the reflected light and is known as depolarisation.

A white-light (spectroscopic) ellipsometer (Model M-2000U, J.A. Woollam Co., Lincoln, NE, U.S.A.) running CompleteEASE™ Ellipsometric software was used to make measurements.

**Experimental procedure**

Samples of 0CNW and 12CNW substrates were prepared by dip-coating all 24 layers on sections of piranha-cleaned n-doped silicon wafers. A freshly-cleaned section from the same wafer was prepared as a sample blank immediately before measurements were made.

Measurements were made on 4 samples in Standard Mode with High Accuracy mode selected. Samples were placed on the stage, centred and the polarisation of the reflected light measured at angles of \( 60^\circ, 70^\circ \) and \( 80^\circ \). The resulting \( \Psi \) and \( \Delta \) curves versus the wavelengths were plotted at each angle.

The material thickness, angle offset (uncertainty in the angle incident on the sample), \( A \) and \( B \) were fitted. The goodness of fit (MSE) of the model was also recorded, with lower values indicating a better agreement between the data and the model. Values below 10 were considered acceptable, values below 5 were considered excellent.
**ATOMIC FORCE MICROSCOPY (AFM)**

*Morphology of cellulose nanocrystals*

As the cellulose crystals produced cannot be visualised using light microscopy and being acicular, are not suited to polymer sizing techniques such as dynamic light scattering, which assume that the polymer is spherical, crystal size distributions were measured using Atomic Force Microscopy (AFM).

At its simplest, the cantilever is used in contact mode and the AFM acts like a prolifometer, producing a 3 dimensional map of the surface. A more flexible (and better suited to soft substrates) method is intermittent contact. In this mode, the cantilever is driven (oscillated) close to its resonance frequency that makes intermittent contact with the surface. As the cantilever interacts with the surface, a phase difference between the cantilever position versus the driving frequency can be seen, which gives additional information on the substrate. Modifications of this allow attractive (or repulsive) forces to be measured.

A Bruker Multimode AFM running Nanoscope V and software Research Nanoscope 8.15 (Build R3Sr6 100600, Bruker) was used to measure CNC dimensions using tapping mode in air. Otespa™ (1 Ohm Si, nominal resonant frequency 323-366 kHz, nominal tip radius 7nm) or ScanAsyst-Air™ (Si tip on silicon nitride, nominal resonant frequency 70 kHz, nominal tip radius 2nm) cantilevers were used. For dimensional analysis, 100 µl of suspended CNC was deposited on freshly-cleaved and PAHCl-coated mica, washed and allowed to air-dry. Suspensions were diluted in water if necessary. For orientation, replicate samples were spin-coated onto PAHCl-coated glass at a range of concentrations.

For each sample, 5 µm by 5 µm scans were acquired at a resolution of 512x512 points in three different areas, scanning at 1Hz.

*Topography of layer-by-layer films*

The topography produced was measured in a similar way to the morphology of cellulose nanoswhiskers. As the substrates samples were built on were larger than the Bruker Multimode stage could accommodate, measurements were done in air using the the Asylum 3D MPD AFM using OTESPA™ tips. For each
sample, 5 µm by 5 µm scans were acquired at a resolution of 512x512 points in three different areas, scanning at 1Hz.

Stability of substrates in cell culture medium
An important consideration was the stability of the substrates under cell culture conditions. To check this, 3 each of each substrate type produced were immersed in C2C12 cell growth medium (described in Section 3.2) for up to 28 days. Test substrates were 0, 1, 6 and 12 CNW substrates. Controls were glass only, chitosan-coated glass and a single bilayer of aligned CNWs on chitosan.

Prepared samples were sterilised in labelled 12-well plates and incubated with 1ml of C2C12-GM. The medium was changed weekly. Substrates were removed and dried in graded ethanol (50%, 70%, 90% and 100% ethanol) for AFM analysis after 1 day, 3 days, 7 days, 21 days and 28 days in incubation. For each sample, representative 5µm by 5 µm images were acquired at a resolution of 512x512 points in 2 widely separated areas using a soft tapping tip (Bruker model MPP-12120-10, nominal spring constant 5 N/m, nominal tip radius 8 nm). With three samples per condition, 6 images were thus taken.

Nanoindentation of layer-by-layer films
40 mm diameter TCP Petri dishes were rendered hydrophilic by treatment for 1 minute in an oxygen plasma (PO₂ = 0.2, pressure = 1x10⁻² bar). Working solutions of PEI (5 mg/ml), PSS (5 mg/ml), chitosan (1 mg/ml) and CNW (0.04% w/v) were prepared by dilution in deionized water. All but the CNW suspensions were filtered through a 0.45µm PVDF filter to remove any particulate matter.

3 each of 0CNW, 6CNW and 12CNW PEMs (described above in Section 3.1.3) were built by pipetting in sufficient solution to cover the base of the Petri dish (~0.5 ml) for 1 minute, followed by 3 rinses in deionised water and drying under a nitrogen gas stream before applying the next layer.

Samples were stored dry overnight before transferring to the BioAFM suite. Before analysis, samples were soaked in PBS at room temperature for 1 hour, taking care to cover the material with sufficient liquid to minimise the effect of water evaporation on the measurements.
A Bruker BioCatalyst running Nanoscope 9.1 and mounted on a Nikon Eclipse 50i inverted fluorescence microscope was used to measure the bulk compressive stiffness of the hydrated PEMs. A fluid cantilever bearing a springboard cantilever with a 2µm diameter polystyrene (PS) ball on the end was used. It had the following parameters (SQU3E, Type CP-FM-PS-A, serial number 2016NM123/1. Material: silicon. Nominal Dimensions: thickness, $T$: 3.0 ± 1 µm; length, $L$: 225 ± 10 µm; width, $W$: 28± 7.5µm. Resonant frequency, $f_0$: 45-115 kHz. Spring constant, $k$: 0.5-9.5 N/m). The nominal dimensions were accepted as given but the resonant frequency and spring constant were determined by calibration.

In mechanical testing, the cantilever is used to probe the compliance of a substrate. To do this, the stiffness of the cantilever as well as the distance it has moved for a given force need to be determined.

Force-distance curves are produced by measuring the deflection per change in voltage (so as to produce accurate force-distance curves). The force-deflection is determined by indenting the cantilever a predetermined distance onto a clean stiff surface, such as glass. The slope of the curve gives the deflection sensitivity, measured in nm/V.

The stiffness of the cantilever can be determined by performing a thermal tune, wherein the small vibrations (on the order of 1nm) that occur thermally in an undriven cantilever are used to find the resonant frequency, where the displacement of the cantilever will reach a maximum. While more accurate methods exist (e.g. the Sader method \(^{330}\)), Cleveland \(^{331}\) showed that the thermal tune alone provides enough information to determine the spring constant of the cantilever if the cantilever material, geometry and 2 of the three dimensions (length and width) are known, using the following equation:

$$k = 2w(\pi f_0)^3 \frac{\rho^3}{E}$$

Equation 3-1: Equation for determination of the spring constant of a cantilever
where \( w \) is the width of the cantilever, \( l \) is its length, \( f_0 \) is the measured resonant frequency, \( \rho \) is the density of the cantilever material and \( E \) is its Young's Modulus. The spring constant has units of N/m.

After calibration of the cantilever, a low-resolution image 10 x10 \( \mu \)m was first acquired to get a qualitative understanding of the evenness of the hydrated surface. On three widely separated areas, 10x10 indentations, spaced 1\( \mu \)m apart were made. Triangular indentation profiles were made at depths of 5 and 20 nm were performed, being deep enough to measure the material, but shallow enough for the resulting force curves to not be affected by the very stiff underlying TCP. The ramp size (distance from initial approach to final stop) was 1.000 \( \mu \)m with a ramp rate of 0.5 Hz. In between indentations, a retracted delay time of 500 ms was imposed. Figure 3-3 shows an SEM image of the type of cantilever used and the indentation profile applied to the samples.

Figure 3-3: A — SEM micrograph of type of spherical tip used in nanoidentation experiments. © 2007 sQube. B – Schematic of indentation profile performed on films.
3.1.5 DATA ANALYSIS

TOPOGRAPHY

AFM micrographs were analysed using Gwyddion (Version 2.45, the Czech Metrology Institute). Topographical micrographs were flattened using a polynomial background and saved as 16 bit greyscale .png files for porosity and orientation analysis using ImageJ (Fiji, Version 1.51d, National Institutes of Health 332).

To obtain a histogram of the directions of the fibres, the ‘Directionality’ module was used, using the Fourier components to determine directions between -90° and +90°. The table was saved. The ‘Dominant Direction’ was determined using the OrientationJ plugin (developed at the Biomedical Image Group (BIG), EPFL, Switzerland originally for measuring bone trabecular structure) which determined both the predominant direction, the spread of that direction and the coherency of the image as a whole. An increase in coherency is the result of the fibres measured becoming better aligned with respect to a common directional axis 333. Results were compiled in GraphPad

STABILITY IN CELL CULTURE MEDIUM

To analyse the images, topography scans were opened in Gwyddion and a linear flattening done using median flattening. If necessary, a descar algorithm was also applied to remove artefacts introduced by contaminants such as dust specks. Any large deposits (protein tended to form globules on the substrate, some of which could grow quite large) were mathematically removed by identifying grains using the ‘Mark Grains by Threshold’ feature which marks features above a certain height and then removing data under the masked feature. It is important to note that Gwyddion does not permanently alter data – manipulated images may be saved but the original is not altered.

The root mean square (RMS) roughness, Rq, of each row (fast scan direction) was measured and the mean and standard deviation measured. Images were then cropped to 5x5µm areas and further flattened for ImageJ using a 4 degree polynomial flatten and handled as for substrate topography described above.
Data was quantitatively assessed as whether a change in roughness and coherency was observed as a function of time immersed and qualitatively as to whether a change in the organisation of the CNWs (such as delamination) was seen. Results were compiled in GraphPad.

NANOMECHANICAL TESTING

To serve as a comparison between substrates, a Hertzian fit was used, assuming a spherical tip, to extract stiffness from the force-distance curves produced. The extension curve was fitted using a least-squares fit of the contact point. Data was exported to Excel as csv files.

Curves with r² values of less than 0.9 were discarded, as were values for the Reduced Young's Modulus, $E_r$, that fell outside two standard deviations of the mean. The remaining data was compiled in Graphpad and one way Anova used to compare the differences between substrates.
3.2 CELL CULTURE

3.2.1 REAGENTS

CELL CULTURE REAGENTS

Fetal bovine serum (FBS, heat-inactivated, sterile-filtered, non-USA origin, catalogue number F9665), adult horse serum (HS, heat-inactivated, sterile-filtered, catalogue number H1138), antibiotic (AB, 10000 units penicillin, 10 mg streptomycin and 25 µg amphotericin per ml, catalogue number A5955), low glucose Dulbecco’s Modified Eagle’s Medium (LG-DMEM, with L-glutamine and sodium pyruvate, catalogue number ), high glucose Dulbecco’s Modified Eagle’s Medium (DMEM, without L-glutamine and sodium pyruvate, catalogue number D5671), L-glutamine (L-glut, 200 mM solution, catalogue number G7513), Dulbecco’s phosphate buffered saline without calcium and magnesium (PBS, sterile-filtered, catalogue number D8537), trypsin/EDTA solution (0.05% trypsin, 0.02% EDTA in Hanks’ balanced salt solution, catalogue number S9417C ), trypan blue solution (0.4%, sterile-filtered, catalogue number T8154), resazurin sodium salt for the Alamar Blue cell viability test (‘BioReagent’, catalogue number R7017), and dimethyl sulfoxide (DMSO, ≥ 99.5% (GC), plant cell culture tested, catalogue number D4540) were all purchased from Sigma-Aldrich, U.K. Minimum essential medium (MEM) non-essential amino acids (NEAA, catalogue number 1140-035) was purchased from Gibco Life Technologies, U.K.

DECELLULARISATION MATERIALS

Molecular biology grade reagents were used. PBS, without Ca or Mg, 5M NaCl (‘BioReagent’ catalogue number S5150), 1 M ammonium hydrochloride (‘BioUltra’, catalogue number 09859), 1 M magnesium chloride (‘BioUltra’, catalogue number 63063), 1 M calcium chloride (‘BioUltra’, catalogue number 211115), 0.5M Ethylenediaminetetraacetic acid solution (EDTA, ‘BioUltra’, catalogue number 03690), 1 M Trizma® hydrochloride buffer solution (‘BioUltra’, pH = 7.4, catalogue number 93313), water (DNAse and RNAse free, sterile filtered, catalogue number W4502), 10% sodium dodecyl sulphate solution in water (SDS, ‘BioUltra’, catalogue number 71736), Triton™ X-100 (for
molecular biology, catalogue number T8787), deoxyribonuclease I from bovine pancreas (DNAse I, as 5 lyophilized vials each with 2000 Kunitz Units, catalogue number D4263) were all purchased from Sigma as sterile solutions and used as received.

**IMMUNOCYTOCHEMISTRY REAGENTS AND ANTIBODIES**

Bovine serum albumin (BSA, ≥96%, ‘BioReagent’, catalogue number A9418), cold water fish skin gelatin (2% in water, ‘BioReagent’, catalogue number G1393), adult goat serum (GS, USA origin, sterile-filtered, catalogue number G6767), 10% neutral buffered formalin solution (NBF, equivalent to 4% formaldehyde, catalogue number HT50-1-2), Triton™ X-100 (catalogue number and antibodies against vinculin (mouse monoclonal to vinculin, catalogue number V9131) were bought from Sigma-Aldrich, U.K. Tween-20 (catalogue number 663684B) was purchased from VWR, UK.

Primary antibodies against myogenin (monoclonal rabbit anti-human, catalogue number ab1835), α-sarcomeric actinin (monoclonal mouse anti-rabbit, catalogue number ab9465), fibronectin (monoclonal mouse anti-cow, catalogue number ab26245) and laminin (polyclonal rabbit anti-mouse, catalogue number ab11575) as well as Cytopainter™ 647-conjugated phalloidin (catalogue number ab176759), were bought from Abcam.

Secondary antibodies Alexa Fluor™ 488 goat anti-mouse (catalogue number A31619), Alexa Fluor™ 488 goat anti-rabbit (catalogue number A31627), Alexa Fluor™ 594 goat anti-rabbit (catalogue number A11012) and Alexa Fluor™ 568 goat anti-mouse (catalogue number A11004) were purchased from Thermo Fisher, as were rhodamine-conjugated phalloidin, (catalogue number R415) and Hoechst 33342 (catalogue number 62249) the latter of which was used as a nuclear counter-stain. Membrane stains HCS CellMask™ deep red (catalogue number H32721) and HCS CellMask™ orange cell membrane stain (catalogue number H32713). Mounting agents were used to affix the stained coverslips to labelled microscope slides and to protect the fluorescent labelling from fading prematurely. Two mounting agents were used: Prolong Gold™ with DAPI (catalogue number P36935) for the initial cell-material compatibility studies,
which was replaced by Prolong Diamond™ without DAPI (catalogue number P36961) as the latter had a lower background fluorescence. Both were purchased from Thermo Fisher U.K.

**PROTEOMIC ANALYSIS REAGENTS**

The Microcon-30kDa Centrifugal Filter Unit with Ultracel-30 membrane (MRCF0R030) and Direct Detect® spectrometer (DDHW00010-WW) were both obtained from Millipore. Ammonium bicarbonate (Ambic, ≥99.5%, ‘BioUltra’, catalogue number 09830), iodoacetamide (IAM; ≥99%, catalogue number I6125), formic acid (FA; Fluka, catalogue number 94318), acetonitrile with 0.1% formic acid (catalogue number 34688) and water with 0.1% formic acid, (catalogue number 34673) were purchased from Sigma Aldrich. Acetonitrile (MeCN, LC-MS grade, catalogue number A955-1) and urea (Fisher, catalogue number U/0500/53) were purchased from Fisher Scientific UK. Trypsin (Promega, catalogue number V528A), LysC MS grade (Wako Chemicals, catalogue number 129-02541), dithiothreitol (DTT, catalogue number MB105) and Tris Base (catalogue number B2005) were purchased from Melford laboratories. POROS R3 beads (catalogue number 1-339-03) and 96-well plates with 0.2 µM PVDF membrane (catalogue number 3504) were purchased from Corning. Microtubes were purchased from Covaris (catalogue number 520145).

**3.2.2 METHODS**

**CELL CULTURE**

**C2C12 MYOBLASTS**

*Source*

C2C12 myoblasts are a well-established immortalised murine cell line first isolated by Yaffe\(^\text{22}\). They are highly proliferative and remain able to exit the cell cycle and differentiate to myotubes under the appropriate stimulation. The cells used in this study had been originally obtained from ATCC and cultured under their recommended conditions before being aliquoted into 1x10\(^6\) units and frozen in liquid nitrogen using a 90% FBS/10%DMSO freezing solution.
Cells were revived in growth media, their viability determined using Trypan Blue dye exclusion test. Briefly, 100 µl of a cell suspension reconstituted in a known volume of medium would be mixed with an equal volume of Trypan Blue dye. Two wells of a hemacytometer (Neubauer improved grid style) would be filled with the suspension and the number of cells contained in ten non-adjacent squares would be counted under a light microscope. Viable cells are able to exclude the dye and appear white against a blue background. As each square has a volume of 1x10^-4 ml, the number of cells contained per unit volume of suspension could be readily calculated. Lots with fewer than 90% of cells alive were discarded. Cells were plated at 10,000 cells/cm² in T75 tissue culture flasks (Nunc™, Thermo Fisher, catalogue number 178905).

Propagation
Cells were fed with growth medium (C2C12-GM, 10% FBS, 1% L-glutamine, 1% AB in DMEM) every other day and passaged at a 1:5 split when they reached 70% confluence. To passage, cells were washed three times in warm PBS- and made to detach by incubation with Trypsin/EDTA solution for 3-5 minutes. The reaction was stopped using C2C12-GM and the cell suspension pooled into a centrifuge tube. After pelleting by centrifuging the cell suspension at 300G for 5 minutes, the pellet was reconstituted in fresh C2C12-GM and the cells counted before plating out or seeding as appropriate.

Differentiation
Two triggers are required for myogenic differentiation. The first is cell-cell contacts and the second is the loss of growth factors. To this end, cells were allowed to reach confluence and differentiation medium (DM, DMEM with 2% HS, 1% L-glutamine, 1% AB) was substituted for growth medium. Cells would be observed to cease proliferating after a day and the first fused myotubes would be observed three days post-treatment.

While C2C12s are an immortalised cell line in the sense that they remain proliferative, the myogenic capacity of these cells is reduced with passage number, a process hastened if they are allowed to become confluent. Cells for differentiation studies were used between P10 and P15, while cells for the alignment and substrate response studies were used between P20 and P30.
Bone Marrow Mesenchymal Stem Cells (BM-MSCs)

Source
BM-MSCs were obtained from Lonza as part of a bone marrow aspirate and their isolation and characterisation was done by Dr. Deepak Kumar following the method outlined in 334. Briefly, fresh bone marrow aspirate was plated out onto uncoated tissue culture plates in low-glucose DMEM with 10% fetal bovine serum, 1% L-glutamine, 1% non-essential amino acids and 1% penicillin-streptomycin. Cells were maintained at 5% CO₂. Medium was changed every third day, which removed non-adherent cells. Adherent cells were cultured to 80% confluence and characterised by flow cytometry. Cells were positive for CD90, CD105, CD73 and negative for CD34, CD11b, CD14, CD19, CD79alpha, CD45 and HLA-DR, which meets the minimum criteria for MSCs as set out by 170.

Cell Culture and Differentiation
Bone marrow MSCs were received at P2 and expanded to P3 in MSC Growth Medium (MSC-GM, low glucose DMEM with 10% v/v FBS, 1% AB, 1% Non-Essential Amino Acids). Aliquots were frozen down at 1x10⁶ cells and revived as needed.

All seeding on substrates for differentiation work was done at P5. Once cells were confluent, half the cells were transitioned to an experimental differentiation media (MSC-DM, low-glucose DMEM with 5% HS, 1%AB, 1% NEAA). This formulation is the standard one for inducing differentiation in primary skeletal satellite cells and has been reported as successful in promoting myogenic differentiation of human MSCs by 335. This is thus the simplest media reported to induce myogenesis in MSCs. Cells were cultured in differentiation medium for up to 14 days.
3.2.3 ANALYTICAL TECHNIQUES USED BY APPLICATION

BRIGHT FIELD MICROSCOPY

A Leica DMIL inverted bright field microscope fitted with a Spot Insight Color camera was used to obtain representative still images of the cells as they grew over time on the various substrates.

ALAMAR BLUE ASSAY (CELL PROLIFERATION RATE)

Cell metabolic activity was measured using the Alamar Blue™ assay after 24 hours and 48 hours. A 10x intermediate stock solution was prepared by dissolving 5 mg of resazurin salt in 40 ml PBS and filtering through a 0.22 µm syringe filter to produce a 5 mM solution. To use, the stock was diluted in the relevant cell culture medium at 1 ml stock to 9 ml culture medium. Medium was removed from the cells to be tested and replaced with 1 ml of working solution and the cells incubated in the dark for two hours. The fluorescence An estimate of the population doubling time was made using the following equation:

$$T_d = \frac{(t_2 - t_1) \cdot \ln(2)}{\ln\left(\frac{f_2}{f_1}\right)}$$

**Equation 3-2: Population Doubling Time**

Where:

- $t_1 =$ Time (hours) at measurement 1
- $t_2 =$ Time (hours) at measurement 2
- $f_1 =$ Fluorescence (background subtracted) at time 1
- $f_2 =$ Fluorescence (background subtracted) at time 2

A fluorescence plate reader (BMG Labtech, model FLUOstar Optima) was used to measure the fluorescence of well plates for Alamar Blue™. Data was gathered by the Optima software package (BMG Labtech, Version 2.20R2, Firmware Version 1.26) and analysed by exporting the data to Excel.
**Fluorescence Microscopy**

Confocal images were acquired on a Leica SP5 inverted confocal microscope (Leica Microsystems CMS GmbH) running LAS AF 2.7.7.12402. Fluorescence images were acquired on a Nikon Eclipse 50i running Lucia GF DXM1200 Version 4.82 (Build 140).

Whole slide scans were imaged using 3D Histech Pannoramic 250 Flash II Slide Scanner at 20x/0.80 Plan Apo, and 40x/0.95 Plan Apo.

**Fixation**

Fixation agents such as formaldehyde are used to rapidly cross-link the proteins within cells, thus ‘fixing’ the membranes and cytostolic structures in place. 4% formaldehyde supplied as 10% neutral buffered formalin (NBF) was used as the fixative. Cells were washed twice in sterile PBS, then sufficient NBF would be added to completely cover the surface for 10 minutes at room temperature. They were subsequently washed three times in non-sterile PBS- and refrigerated under PBS if not to be stained immediately.

**Immunocytochemistry Investigation**

*Background*

To visualise specific structures within a cell, it is necessary to stain or otherwise label them. In fluorescence microscopy, fluorophores, which are compounds that adsorb photons of a specific wavelength and emit photons at a longer wavelength, are conjugated to antibodies that recognise and bind to the antigens of interest. High abundance proteins, such as actin or tubulin can be directly labelled with a fluorescently-tagged antibody or compound. The signal for lower abundance proteins, such as vinculin is amplified by using a two-stage labelling process. The initial antibody, called a primary antibody, is used to label the protein of interest. A fluorophore-tagged second antibody that recognises and binds to the primary antibody is then applied, thus labelling the protein. Several primary antibodies are able to bind to each protein structure and likewise, several secondary antibodies are able to bind per primary antibody, thus amplifying the signal.
**Cytological Structure**
Cells were stained for vinculin, filamentous actin (f-actin), the cell membrane and the nucleus. To do so, fixed washed samples would be blocked and permeabilised at room temperature for 30 minutes using 1% Goat Serum, 0.5% BSA, 0.25% Triton-X100 and 0.025% Tween-20 in PBS at room temperature for 30 minutes. Permeabilisation is necessary to disrupt the phospholipid cell membrane and allow antibodies to enter the cell.

Primary antibody (mouse monoclonal anti-vinculin) was applied at 1:400 dilution for 1 hour at room temperature, followed by secondary antibody (Alexa Fluor™ 488 goat anti mouse) at 1:1000 dilution, which labelled the vinculin green. Rhodamine-bound phalloidin was used at 1:200 to stain for filamentous actin, which was labelled red. The cell membrane was stained using a proprietary membrane stain (HCS Deep Red Cell Mask™) at a dilution of 1:5000. The cells were mounted on glass slides using an antifade reagent (Prolong Gold™ with DAPI), which also counter-stained the nucleus. Coverslips were allowed to cure in the dark at room temperature overnight and then stored in the fridge until required.

**Myogenic Differentiation of Cells**
Fixed cells were permeabilised with 0.5% Triton-X100 and 0.05% Tween-20 in PBS for 5 minutes at room temperature. Samples were then blocked using 2% GS, 1% BSA, 0.1% cold fish skin gelatin for 30 minutes at room temperature. Cells were stained for myogenin using rabbit monoclonal anti-myogenin and for α-sarcomeric actinin using mouse monoclonal anti-α-sarcomeric actinin. Both were diluted 1:200 in the blocking buffer and cells were incubated in the primary antibody solutions for an hour at room temperature. Secondary antibodies were Alexa Fluor 488 Goat anti-rabbit (which labelled the myogenin green) and Alexa Fluor 568 Goat anti-mouse (which labelled the α-sarcomeric actinin red), both diluted 1:1000 in PBS with 0.05% Tween-20. Cells were incubated in the secondary antibody solution for an hour at room temperature in the dark. Additionally, the actin cytoskeleton was stained for using 647-conjugated phalloidin (Abcam, ab176759). This was added to the secondary antibody solution at a dilution of 1:1000.
Finally cells were counter-stained using Hoechst 33342 diluted 1:5000 in PBS for 5 minutes. Samples were washed in deionized water twice to remove excess salt, allowed to partially dry on filter paper and mounted on labelled glass slides using Prolong Diamond™ without DAPI. Samples were stored overnight at room temperature in the dark to allow the mountant to cure, then stored in the fridge until they were analysed.

Negative controls were added. First, unstained C2C12 cells and 3T3 fibroblasts were imaged to monitor and correct for background autofluorescence. Second, to check for non-specific staining, 3T3 fibroblasts were stained for the primary and secondary antibodies to check that the primary antibody staining was specific. C2C12 cells were also labelled with secondary antibodies only to verify that the secondary antibody staining was also specific. Finally, C2C12 cells stained for RUNX-2 to show that cells are not labelled by non-specific antibodies.

**Extracellular matrix (ECM) visualisation**

Fixed cells were not permeabilised, but were only blocked using 2% GS, 1% BSA, 0.1% cold fish skin gelatin for 30 minutes at room temperature.

Cells were stained for fibronectin using mouse monoclonal anti-fibronectin, diluted 1:500 and for laminin using rabbit polyclonal to laminin, diluted 1:300. Cells were incubated in the primary antibody solutions for an hour at room temperature. Secondary antibodies were Alexa Fluor™ 488 Goat anti-rabbit (which labelled the laminin green) and Alexa Fluor™ 568 Goat anti-mouse (which labelled the fibronectin red), both diluted 1:1000 in PBS with 0.05% Tween-20. Cells were incubated in the secondary antibody solution for an hour at room temperature in the dark.

Finally cells were counter-stained using Hoechst 33342 diluted 1:5000 in PBS for 5 minutes. The cell membrane was also labelled using a deep red membrane stain, also diluted 1:5000. Samples were washed in deionized water twice to remove excess salt, allowed to partially dry on filter paper and mounted on labelled glass slides using Prolong Diamond™ without DAPI. Samples were
stored overnight at room temperature in the dark to allow the mountant to
cure, then stored in the fridge until they were analysed.

To process the large numbers of samples produced for the differentiation and
ECM studies, a humid chamber was improvised using two trays and the
atmosphere kept humid using a wetted paper towel. Labelled slides were
wrapped in Parafilm® and the samples placed cell-side up on them. This
allowed 40µl of antibody solution to be used per sample without drying out in
the interim. Washes were done by adding approximately 500 µl of PBS to each
sample followed by careful aspiration.

A summary of the parameters used may be found in Table 3-3, Table 3-4, and
Table 3-5 on the pages that follow:
Table 3-3: Summary of staining used for cytoskeletal structure. Cells are blocked in 2% GS, 1% BSA, 0.1% gelatin in PBS and permeabilized with 0.25% Triton X-100 and 0.025% Tween-20 in PBS

<table>
<thead>
<tr>
<th>Description</th>
<th>Time(s)</th>
<th>Primary Antibody</th>
<th>Manufacturer (cat. no.)</th>
<th>Dilution Factor</th>
<th>Time</th>
<th>Secondary Antibody</th>
<th>Manufacturer (cat. no.)</th>
<th>Dilution Factor</th>
<th>Time</th>
<th>Counterstain</th>
<th>Mountant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common Conditions</td>
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<td>DAPI (contained in</td>
<td>Prolong Gold with</td>
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<td></td>
<td>mountant)</td>
<td>DAPI</td>
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<tr>
<td>Vinculin</td>
<td>1 hour</td>
<td>Mouse monoclonal anti-vinculin</td>
<td>Sigma (V9131-.5ml)</td>
<td>1:300</td>
<td>1</td>
<td>Alexa Fluor 488 Goat anti-mouse</td>
<td>Thermo Fisher (A31619)</td>
<td>1:1000</td>
<td>1</td>
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<td></td>
<td>4 hours</td>
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<td>Filamentous Actin</td>
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<td>Rhodamine-conjugated phalloidin</td>
<td>Thermo Fisher (R415)</td>
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</tbody>
</table>

Abbreviations: BSA – bovine serum albumin, GS – goat serum, PBS – phosphate buffered saline
Table 3-4: Summary of staining parameters used for determination of myogenic differentiation. Cells are blocked in 2% GS, 1% BSA, 0.1% gelatin in PBS and permeabilized with 0.5% Triton X-100 and 0.05% Tween-20 in PBS

<table>
<thead>
<tr>
<th>Description</th>
<th>Time</th>
<th>Primary Antibody</th>
<th>Make (cat. no)</th>
<th>Dilution Factor</th>
<th>Time (hrs)</th>
<th>Secondary Antibody</th>
<th>Make (cat. no)</th>
<th>Dilution Factor</th>
<th>Time (hrs)</th>
<th>Counter-stain</th>
<th>Mountant</th>
</tr>
</thead>
<tbody>
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<td>Common Conditions</td>
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<td></td>
<td>Prolong</td>
<td>Diamond</td>
</tr>
<tr>
<td>Myogenin (early differentiation</td>
<td>1 day</td>
<td>Rabbit monoclonal anti-</td>
<td>Abcam</td>
<td>1:200</td>
<td>1</td>
<td>Alexa Fluor 488 Goat</td>
<td>Thermo Fisher</td>
<td>1:1000</td>
<td>1</td>
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<tr>
<td>marker)</td>
<td>2 days</td>
<td>myogenin</td>
<td>(ab124800)</td>
<td></td>
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<td>anti-rabbit</td>
<td>(A31627)</td>
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<td>3 days</td>
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<td></td>
<td>Abcam</td>
<td>1:200</td>
<td>1</td>
<td>Alexa Fluor 568 Goat</td>
<td>Thermo Fisher</td>
<td>1:1000</td>
<td>1</td>
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<td></td>
<td></td>
<td>Mouse monoclonal anti-</td>
<td>Abcam</td>
<td>1:200</td>
<td>1</td>
<td>anti-mouse</td>
<td>Thermo Fisher</td>
<td>1:1000</td>
<td>1</td>
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<td></td>
<td></td>
<td>α-sarcomeric actinin</td>
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<td>(A11004)</td>
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<tr>
<td>Filamentous Actin</td>
<td>1 day</td>
<td>N/a</td>
<td>647-conjugated</td>
<td>1:1000</td>
<td>1</td>
<td></td>
<td>Abcam</td>
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<td></td>
<td></td>
<td></td>
<td>phalloidin</td>
<td></td>
<td></td>
<td></td>
<td>(ab176759)</td>
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</table>

Abbreviations: BSA – bovine serum albumin, GS – goat serum, hrs – hours, PBS – phosphate buffered saline
Table 3-5: Summary of staining parameters used for determination of ECM production. Cells are blocked in 2% GS, 1% BSA, 0.1% gelatin in PBS but are not permeabilized.

<table>
<thead>
<tr>
<th>Description</th>
<th>Time</th>
<th>Primary Antibody</th>
<th>Make</th>
<th>Dilution Factor</th>
<th>Time</th>
<th>Secondary Antibody</th>
<th>Make</th>
<th>Dilution Factor</th>
<th>Time</th>
<th>Counter-stain</th>
<th>Mountant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Common Conditions</strong></td>
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<tr>
<td></td>
<td>1 day</td>
<td>mouse monoclonal anti-fibronectin</td>
<td>Abcam</td>
<td>1:500</td>
<td>1 hour</td>
<td>Alexa Fluor 568 Goat anti-</td>
<td>Thermo Fisher</td>
<td>1:1000</td>
<td>1 hour</td>
<td></td>
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<tr>
<td></td>
<td>3 days</td>
<td></td>
<td>Abcam (ab26245)</td>
<td></td>
<td></td>
<td>mouse monoclonal anti-fibronectin</td>
<td>Abcam</td>
<td>1:300</td>
<td>1 hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 days</td>
<td></td>
<td>Abcam (ab11575)</td>
<td></td>
<td></td>
<td>Alexa Fluor 488 Goat anti-</td>
<td>Thermo Fisher</td>
<td>1:1000</td>
<td>1 hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 day</td>
<td></td>
<td>Abcam</td>
<td></td>
<td></td>
<td>rabbit polyclonal anti-laminin</td>
<td>Abcam (ab11575)</td>
<td>1:300</td>
<td>1 hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 days</td>
<td></td>
<td>Abcam</td>
<td></td>
<td></td>
<td>rabbit polyclonal anti-laminin</td>
<td>Abcam (ab11575)</td>
<td>1:300</td>
<td>1 hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell Membrane</td>
<td>1 day</td>
<td>N/A</td>
<td></td>
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<td>HCS CellMask Deep Red</td>
<td>Thermo Fisher</td>
<td>1:5000</td>
<td>5 min</td>
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</tr>
</tbody>
</table>

Abbreviations: BSA – bovine serum albumin, GS – goat serum, hrs – hours, PBS – phosphate buffered saline.
3.2.4 DATA ANALYSIS

ORIENTATION

The orientation of a given cell is given as the angle, θ, that the long axis of an ellipse constructed around the object forms with respect to the x-axis (Figure 3-4 below).

![Figure 3-4: Measuring cell orientation. The angle a line segment drawn through the long axis of the cell makes with the x-axis is the angle of orientation θ. It is measured from -90° to +90°.](image)

Images were segmented using CellProfiler (version 2.1.1, The Broad Institute. 336 ) to identify objects of interest and the orientation of the identified objects measured using the ‘MeasureObjectSizeShape’ module. The software was validated for its ability to segment, measure the pixel sizes and orientation of objects. Details of this validation, along with the pipelines used and the images tested may be found in the Appendix.

Briefly, low magnification (x10) images of cells were split by channel with the blue channel being for nuclei which were stained either with DAPI or Hoechst and the red channel for filamentous actin (labelled with rhodamine-conjugated phalloidin). Cells were identified by presence of a nucleus and the cytoplasm was used to ‘construct’ a cell around each nucleus, using a watershed algorithm.
to segment between the cytoplasm and the background. The orientation of both the cell identified and the nucleus were measured for every identified object and exported to Excel as a .csv spreadsheet. Because cells need to be confluent for myogenesis, at times it was not possible to differentiate one cell from another. In that case, the nuclear orientation alone was used as a proxy for cell orientation as the long axis of the nucleus of a cell tends to lie along that of the cell itself for fibroblastic type cells\textsuperscript{337}.

As a radially oriented substrate, simply measuring the direction of cells or fibres over the whole slide will result in an isotropic distribution with all angles being equally likely to be found. However, far (as in 1 mm) away from the centre of rotation, fibres and cells are oriented approximately parallel to one another – this is not perfect as there is a spread derived from the radial orientation. This is illustrated in Figure 3-5.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3-5.png}
\caption{Schematic illustration of radial orientation expected on slide. \textit{a} – view of expected fibre and cell orientation over whole slide. \textit{b} – view of smaller area far from centre of rotation. Fibres (and cells) are expected to be nearly parallel. Some spread from the centre is expected, depending on how far from the rotational centre the image is taken.}
\end{figure}

\textbf{CELL ORGANISATION}

The cytoskeletal structure, cell size and size of focal adhesions (where measured) are critical components of a cell’s response to a material surface. To quantify these, non-scaled images of known magnification and pixel/µm conversion ratio were captured at x20 and x40 magnification and segmented using CellProfiler.
For overall cell size and shape, the number of pixels covered by each object was determined. The surface area covered was calculated by converting the square pixels to a square micrometer value by dividing the pixel area by the square of the conversion ratio pixels/µm.

Focal adhesion size is positively related to the stiffness of the substrate and was measured using x40 images of cells. Focal adhesions were enhanced using the Speckles algorithm which enhances the signal of objects of a given size and masked to more clearly distinguish focal adhesions from the background vinculin staining. Can measure focal adhesion size and size distribution, focal adhesion radial orientation with respect to the cell nucleus – non-oriented cells will be expected to give an even radial distribution while there will be a marked anisotropy with highly aligned cells.

**CELL PROLIFERATION**

The doubling time of cells can be determined from Alamar Blue as described in Section 3.2.3.

**CELL DIFFERENTIATION**

Early differentiation was assessed by the number of myogenic nuclei (nuclei stained positive for myogenin) as a proportion of total nuclei in the field of view. For C2C12 cells, the expression of myogenin was also associated with the upregulation of α-sarcomeric actinin. Later differentiation was assessed by the presence of a high degree of α-sarcomeric actinin and the disappearance of stress fibres. These cells are still myogenin positive, but the myogenin is exported to the cytoplasm of the cell and disappears from the nucleus. Cells expressing α-sarcomeric actinin above a cut-off threshold are identified as primary objects and their size and orientation determined as in the earlier section. To count the number of nuclei per myotube, the cells were treated as parent objects and the nuclei their children and the number of children per parent were determined. α-sarcomeric actinin positive cells containing only one nucleus are known as myocytes.
ECM Organisation

Fibronectin and laminin were assessed separately. As with other parameters, tiff files at x20 magnification were used. The ECM images were split into their respective channels as greyscale images using CellProfiler, and the coherency of the ECM was determined using ImageJ as explained in Section 3.1.5. Biologically, an increase in coherency is associated with an increase in the organisation of the ECM.

3.2.5 Proteomics

Background

Proteomics is a powerful method of studying the proteins actually produced by a cell (and by extension, tissue and organisms). While all somatic cells – other than red blood cells – have the same genome, they differ in the transcription of that genome to mRNA and differ again in how that mRNA is translated into functional proteins. Understanding the proteins produced, along with metabolites generated helps to build a picture of how organisms function and how they respond to changes in state, for example in response to disease.

For this study, the ECM expressed by the cells cultured on control and test substrates was of interest. The ultimate aim is to culture mesenchymal stem cells on CNW-terminated PEMs, with or without pre-templating by C2C12 myoblasts to see if they can be driven to differentiate myogenically. ECM provides physical structure as well as myriad of immediately available and cryptic binding sites that mediate cellular behaviour. It is therefore necessary to understand the nature of the ECM laid down by the myoblasts.

Immunocytochemistry (ICC) provides spatial information on the ECM distribution. While imaging slides stained in the same way under similar conditions allows for relative comparisons to be made between the quantity of ECM expressed, it is not a quantitative method. Furthermore, as the number of fluorophores that can be reliably segregated from one another is limited, it is not suited to give a complete picture of the ECM components secreted.
The protein content of a cell culture consists mostly of cytostolic proteins, which would swamp the signal of any ECM-specific proteins. It is therefore necessary to decellularise samples in order to leave behind the matrix proteins. It is not possible to entirely remove cytostolic proteins, but it is sufficient to enrich the ECM matrix. The method chosen is described in the next section.

Proteomic assays offer a quantitative method for determining the proteins within a matrix, by peptide mass fingerprinting.

To do so, proteins must be reduced to their primary structure. The isolated proteins of interest are first denatured using either urea or SDS. This opens up the protein structure and facilitates the reduction of any -S-S- bridges using reducing agents such as 2-mercaptoethanol or dithiothreitol (DTT). As the exposed thiol groups are highly reactive, it is necessary to modify them to form more stable derivatives, such as by alkylation. This is achieved by reacting the protein mixture with sulphydryl alkylating groups (such as iodoacetamide or acryrlamide). If necessary, post-translational modification may be removed by a process of deglycosylation, which simplifies protein identification at the expense of information. Finally, polypeptides are produced by enzymatic fractionation. Trypsin is normally used as it cleaves arginine-serine bonds, thus creating a polypeptide that will have a net charge.

The LC-MS/MS method used in this work provides a sensitive way to identify and quantify the proteins produced. The polypeptides are fractionated by an HPLC system which separates polypeptides by their mass and their mobility, the latter of which is dependent on the column conditions. Each eluted fraction is then nebulised to form a fine spray, which is then dried and volatilised to form a gas that can be introduced to the MS system.

The MS samples the polypeptides in two ways. First, it scans the according to its mass/charge (m/z) ratio and identifies the largest peak. This peak is then bombarded with ions (normally He) to fragment the polypeptide further. Polypeptides fragment along amino bonds, creating a series of shorter peptides known as a fragmentation tree. The next highest peak is then identified and the process repeated. This takes a finite amount of time and a balance must be
struck between the sharpness of the elution peaks, the frequency of sampling and the m/z range to be scanned over. The fragmentation trees are used to identify the protein they came from using a software package called Mascot (Matrix Science Ltd, London, U.K.). Given the instrument model and running conditions, it models the possible peptide sequences that could correspond to a protein and compares the modelled data against the experimental data to find a match.

Second, the MS sums the entire signal to obtain the area under the curve for each polypeptide. This data is recorded using the Progenesis software package which correlates the information with the elution times of the protein on the LC and the peptide fragmentation tree and consequent identification produced by Mascot to quantify each identified protein in the mixture. A summary of the overall process may be found in Figure 3-6.

![Flow-chart of preparation steps involved in proteomic analysis of cells.](image)
The decellularisation of cells and the preparation of crude ECM protein suspensions were done in-house; the subsequent protein digestion, desalting and analysis were done at the Biomolecular Analysis Core Facility.

**Decellularisation of cell-modified matrices**

To create a scaffold consisting of ECM left behind by cells on the PEM substrates, C2C12 cells were seeded onto control and test substrates and allowed to differentiate into myotubes for 7 days. This time period is long enough to allow myotubes to be established, but short enough to prevent them from contracting off the surface. A modified version of the method used by Brown \(^{339}\) to decellularise pericytes cultured on glass coverslips was used.

**Solutions Prepared**

All solutions prepared in molecular grade water under aseptic conditions. Unless stated otherwise, the resulting solutions were sterile filtered through a 0.22µm filter. As the presence of a phosphate buffer interferes with the decellularisation solution, a wash solution of 0.15 M NaCl was substituted. The decellularisation solution consisted of 0.5% Triton X-100 in 10 mM ammonium hydroxide (NH\(_4\)OH). Reaction buffer (20 mM Tris HCl, 5mM calcium chloride, 2 mM magnesium chloride) was made up as a x10 stock solution. A x10 stock DNAseI solution (2000 U/ml) was made up by dissolving the contents of one vial in 1ml of 0.15M NaCl. This was kept frozen at -80°C until required. EDTA stop solution (5 mM EDTA) was prepared as a x10 stock. Where ECM protein was to be harvested for use in proteomic analysis, a protein dissolution solution of 0.1% SDS with 50 mM Tris-HCl was prepared.

**Procedure**

Medium was removed from the cells and the cells washed gently with PBS, taking care to thoroughly remove as much of it as possible between washes, as PBS interferes with the decellularisation solution. Cells were then washed with 0.15 M NaCl solution. Just enough (50 - 100 µl depending on coverslip size) decellularisation solution was added to cover the substrate and the cells incubated for 10 minutes at 37°C. Some pull-off of ECM was seen, particularly on cells cultured on glass.
The resulting cell debris was removed and coverslips were then washed once in 0.15 M NaCl and once in 1x reaction buffer. A DNAse I working solution was prepared by adding 1ml of 10x DNAse I stock and 1ml of 10x reaction buffer to 8 ml water immediately before use. Sufficient DNAse working solution to cover the material was added and the coverslips incubated for 30 minutes. Excess solution was then removed and an equal volume of x1 EDTA stop solution was added for 5 minutes at room temperature to inactivate any DNAse I. Finally, coverslips were washed in PBS.

If the coverslips were to be reseeded, then they were transferred to fresh well plates using a pair of tweezers sterilized by autoclaving and incubated overnight in the relevant growth medium.

If the ECM was to be harvested for proteomics, then the process was slightly different. No DNAse I was used and coverslips were transferred to fresh well plates containing suspension solution and scraped clean with a cell scraper. ECM was observed to dissolve rapidly. The resulting solutions were transferred into sterile, labelled microfuge tubes and frozen at -80°C until required.

**SAMPLE PREPARATION FOR LC-MS ANALYSIS USING FILTER-AIDED SAMPLE PREPARATION (FASP)**

**Buffers Prepared**
SDS buffer stock = 2% SDS in 50 mM Tris-HCl pH 7.4. UA1 = 8M urea, 0.1M Tris-HCl (pH 8.5). UA2 = 8M urea, 0.1 Tris-HCl (pH 8.5) with 15 mM DTT. UA3 = 75% UA1 buffer + 25% deionised water (=6M Urea). Wet solution = 50% acetonitrile. Wash solution = 0.1% formic acid. Elute solution = 50% acetonitrile, 0.1% formic acid

**Procedure**
The crude ECM solutions were transferred to a 0.5ml eppendorf tube with as little of the suspension buffer (0.05% SDS with 50 mM Tris HCl) as possible. The samples were heated to 95 °C for 10 minutes.

To measure the protein concentration, 2µl of the blank (0.05% SDS with 50mM Tris HCl) was added to a direct detect card. The same volume of protein sample was added to the card in triplicate. The mean protein concentration was used to
determine the volume of each sample needed to obtain 12.5 µg of protein for digestion.

To digest the proteins, 12.5 µg of protein was added to a spin filter tube with 200 µL of UA2 buffer, and then centrifuged at 14000 g at 20 °C for 15 minutes. A further 100 µL of UA2 buffer was added and centrifuged at 14000 g at 20°C for 15 minutes.

To alkylate the samples, 50 µL of UA1 buffer with 0.05 M iodoacetamide was added to the filters and the samples were incubated in darkness at room temperature for 30 minutes. The resultant IAM solution is centrifuged through and then the filters were washed twice with 100 µL of UA2 buffer followed by a further two washes of UA3 buffer.

50 µL of UA3 buffer was added to the filter and the protein was digested using endoproteinase LysC at an enzyme : protein ratio of 1:20 at 37ºC for 2 hours and a fresh collection tube was used for subsequent spins. To prepare the enzyme, lyophilized LysC is dissolved in water with a resistivity of 18 MΩ. Once it is made, the solution is stable at least until the expiration date printed on the label at −80 °C. (Add 6.25 µL of a 100 ng/µL LysC solution, there is 12,500 ng of protein present).

Following this the solution was diluted to 300 µL with the addition of 250 µL of 50 mM Tris-HCl (pH 8.5). This brings the urea concentration down from 6M to 1M. The protein was further digested with trypsin at a protein:enzyme ratio of 1:20 overnight at 37ºC. (Add 6.25 µL of a 100 ng/µL trypsin solution)

After digestion peptides were collected by centrifugation at 4000 g at 20°C for 15 minutes and the filtration units were washed once with 50 µL of UA1 buffer and subsequently with two 50 µL washes of 50 mM ammonium bicarbonate.

Peptides were cleaned up with R3 beads and lyophilized and stored dry at -20°C until analysis.

The resulting peptides required desalting before LC-MS analysis could be performed. A 96-well flow through plate was used to perform this. 1 mg (100 µL of 10 mg/mL stock) of POROS R3 beads was added to each well (label one well
per sample) in a Corning 96 well plate. The plate was centrifuged at 200 g (1400 rpm) for 1 minute (Setting 2 on the Jouan CR3i centrifuge in the BioMS lab).

50 µL of wet solution was added to each well, resuspending gently, and centrifuged at 200 g (1400 rpm) for 1 minute. This process was repeated once. 50 µL of wash solution was added, resuspending gently, and centrifuged at 200 g (1400 rpm) for 1 minute. This process was repeated once.

The flow through was discarded. The filters were removed from the FASP tubes and 100 µL of the protein sample added to the corresponding well, resuspending gently, and centrifuged at 200 g (1400 rpm) for 1 minute. Another 100 µL of sample was added and centrifuged at 200 g (1400 rpm) for 1 minute. This process was repeated until the entire sample was added.

The samples were washed and centrifuged twice using wash solution. The old flow through plate was discarded and replaced with a fresh plate.

50 µL of elution solution was added and centrifuged at 200 g (1400 rpm) for 1 minute. This process was repeated once. The eluted sample was transferred into chromatography sample vials and the samples dried in the Heto SpeedVac for 2 hours.

10 µL of 5% acetonitrile with 0.1% formic acid was added to each vial to resuspend the dried peptides. The samples were vortex mixed and care was taken to ensure that the solution remained at the bottom of the vial with no bubbles present.

Samples were now ready for LC-MS/MS. Samples were diluted if necessary using a solution of 5% acetonitrile and 0.1% formic acid.

**DATA ACQUISITION - MASS SPECTROMETRY**

Digested samples were analysed by LC-MS/MS using an UltiMate® 3000 Rapid Separation LC (RSLC, Dionex Corporation, Sunnyvale, CA) coupled to an Q-exactive HF (Thermo Fisher Scientific, Waltham, MA) mass spectrometer. 300 µg of peptide lysate were loaded onto the column. Peptide mixtures were separated using a gradient from 92% A (0.1% FA in water) and 8% B (0.1% FA
in acetonitrile) to 33% B, in 104 min at 300 nL min⁻¹, using a 75 mm x 250 μm i.d. 1.7 uM CSH C18, analytical column (Waters).

**DATA ANALYSIS**

Data were validated using Scaffold (Proteome Software, Portland, OR). The acquired MS data was analysed using Progenesis LC-MS (v2.0.5556.29015, Nonlinear Dynamics). The retention times in each sample were aligned using one LC-MS run as a reference, then the “Automatic Alignment” algorithm was used to create maximal overlay of the two-dimensional feature maps. Features with charges ≥ +5 were masked and excluded from further analyses, as were features with less than 3 isotope peaks. The resulting peaklists were searched against a combined Uniprot Mouse and Rat database (version 2015111) using Mascot v2.5.1, (Matrix Science). Search parameters included a precursor tolerance of 8 ppm and a fragment tolerance of 0.015 Da. Enzyme specificity was set to trypsin and one missed cleavage was allowed. Carbamidomethyl modification of cysteine was set as a fixed modification while methionine oxidation was set to variable. The Mascot results were imported into Progenesis LC-MS for annotation of peptide peaks.
3.3 STATISTICAL ANALYSES

A commercial statistical package, GraphPad was used to summarise all data and to compare control and test conditions. The average values are presented as mean ± standard deviation. The results were tested for significance ($p < 0.05$) using the t-test, one-way ANOVA or two-way ANOVA as appropriate.
4 RESULTS OF CNW AND POLYELECTROLYTE MULTILAYER PREPARATION AND CHARACTERISATION

While single bilayer substrates (PAHCl and CNWs) have been shown to successfully align C2C12 cells and allow for their myogenic differentiation19, the production of more mechanically-relevant substrates requires a softer substrate. Of particular interest was whether it would be possible to maintain the alignment cues provided by CNWs on a softer matrix.

By considering the interaction of PAHCl and CNW molecules as that of two oppositely charged polyelectrolytes with PAHCl being a polycation and the CNWs as polyanions, it is possible to repeat the process ad infinitum with any oppositely charged polymers to create a composite structure known as a polyelectrolyte multilayer (PEM). PEMs offer advantages over a single bilayer in that they are more stable in culture conditions, offer a more mechanically relevant substrate for cells and can have bioactive molecules more readily incorporated within them. Their porosity is also tuneable340 (although this has not tested in this body of work), potentially allowing for the controlled release of soluble molecules such as growth factors.

It is hypothesised that the production of PEMs instead of a single bilayer will allow the contact guidance of cells along CNWs to be preserved on a softer, more physiologically relevant substrate. This chapter therefore describes the development and characterisation of these films. PEMs used for cell culture in Chapter 5 were characterised to determine their thickness, appearance, roughness and stability under cell culture conditions.

Several PEM formulations were tested, each consisting of 12 bilayers of polyelectrolytes. The PEM consisting only of PSS and chitosan (configuration = PEI/(PSS/CHI)_{11}, also referred to as 0CNW) Where a single layer of CNW has been spin-coated as the terminating layer of this film (configuration = PEI/(PSS/CHI)_{11}/CNW), it is also referred to as 1CNW. PEMs with 12 bilayers consisting of alternating CNW and PSS polyanion layers with Chitosan as the polycation (configuration = (PEI/(PSS/CHI/CNW)/(CHI/PSS/CHI/CNW))_{5} is also
referred to as 6CNW. Finally a PEM consisting only of alternating CNWs and chitosan (configuration = PEI/(CNW/CHI)$_{11}$/CNW) is referred to as 12CNW.

4.1 **CNW YIELD AND SUSPENSION STABILITY**

Yields for tunicin cellulose nanowhiskers were approximately 50%. This is consistent with the reported degree of crystallinity of tunicates. Typical concentrations of dialysed suspensions of tunicin cellulose nanowhiskers (TCNWs) were 0.1% w/w, while those for cotton cellulose nanocrystals (CCNCs) were 0.5% w/w.

Zeta potential measurements of TCNW suspensions (after counter-ions had been removed by passing the suspension through a mixed-bed exchange resin) gave values of -35.4 ± 4.60 mV over a temperature range of 25° to 40°, indicating that suspensions are reasonably thermodynamically stable at room temperature. Suspensions were retested after a year of storage at room temperature and showed no change in their zeta potential (-35.7 ± 1.35 mV) and no flocculation or precipitation was seen.

4.2 **CELLULOSE NANOWHISKER PURITY**

To confirm the identity of the cellulose produced after extraction, FT-IR spectra of bleached tunicin and CNWs extracted from tunicin were compared against cotton ashless floc, which is pure cellulose. The results can be seen in Figure 4-1. The peaks observed are consistent with Type Iβ cellulose (particularly the peaks at 3270 and 710 cm$^{-1}$)\textsuperscript{341}. Overall, the data agrees well with work from Michell’s FT-IR studies on tunicin and Valonia cellulose\textsuperscript{342}. Typical peak shifts are less than 10 cm$^{-1}$ relative to his work. The shift was largest in the 3000-3600 cm$^{-1}$ region, which is associated with hydrogen bonding between cellulose chains. This could be accounted for by slight differences between the samples, the species of origin and preparation method.
Figure 4-1: FT-IR spectra of a) tunicin CNWs, b) tunicin cellulose and c) cotton cellulose
4.3 CNW APPEARANCE AND ALIGNMENT

The cellulose nanocrystals differ in appearance according to their origin. Tunicate-derived nanocrystals are notably longer and have a higher aspect ratios than those derived from cotton. Representative AFM images can be found in Figure 4-2 below. The height of a given tunicate nanocrystal is consistent, suggesting that the crystals produced by *Ascidiella* are rod-like and not twisted.

They also differ in their ability to be aligned by spin-coating. Tunicate-derived CNWs align readily while the cotton-derived CNWs fail to do so. On this basis, they were not used further in this work. The Cotton CNC in Figure 4-2 (d) is representative of the concentration and alignment of surfaces. It is likely that the higher aspect ratio (and the consequent increase in the hydrodynamic drag) of the Tunicate-derived CNWs enabled their alignment.

![AFM height maps of cellulose nanoparticles.](image)

*Figure 4-2: AFM height maps of cellulose nanoparticles. a = 0.02% w/v tunicin CNW, unaligned. b = 0.02% tunicin CNW, aligned by spin-coating at 8000 RPM. c = Cotton CNC, 0.005% unaligned. d = Cotton CNC, 0.5% spin-coated at 8000 RPM, showing no alignment. The cotton suspensions also appear to have spherical particles within them, the origin of which is unclear. Spin-coating dilute solutions resulted in a near-total loss of particles on the surface.*
4.4 **CNW SIZE DISTRIBUTION**

The height distribution data for the tunicin CNWs agrees closely with that measured by Dugan\(^{187}\), which is unsurprising as they are derived from the same source, with a mean value of 4.8nm ± 1.97 nm. The CNW suspension is polydisperse with large range of molecular sizes. The mean length was 0.64µm ± 0.57µm. Shorter fragments had a larger population than longer fragments, resulting in a strong positive skew to the histogram of the CNW length distributions. The dimensional data is summarised in Figure 4-3.

![Figure 4-3: Histograms of Tunicin CNWs from AFM image analysis. a: Height distribution. b: Length distribution](image)

For cotton CNCs, the height (8.1 nm ± 4.0 nm) and length (0.12µm ±0.09µm) data distributions were also in agreement with the literature values. Elazzouzi-Hafraoui et al.\(^{230}\), in a TEM study on sulphuric acid oxidised cellulose nanocrystals, found that cotton nanocrystals had average lengths between 100 and 140 nm, and widths between 12 and 34 nm, depending on the source. Lateral aggregations tended to increase the size of particles, but the lengths of most particles were between 100 and 300 nm. The dimensional data is summarised in Figure 4-4.
Widths measured by AFM were much wider (~20-50 nm) owing to the similarity in size of the crystals (5-8 nm in diameter) and the radius of curvature of the cantilever tip (nominal radius 10 nm). This results in the convolution of the tip and the nanocrystals. There are methods to compensate for the tip convolution by characterising the tip radius using particles of a known radius (e.g. a near monodisperse solution of gold nanoparticles) and eroding the measured crystal width to get the true width. However, the consistent height of the tunicate cellulose nanocrystals, allows a simplifying assumption to be made: that the crystals are approximately cylindrical and that the measured height is equal to the width. This assumption compares well with TEM measurements made of the tunicin cellulose by Dugan187.

4.5 VISUAL APPEARANCE OF PEM FILMS

All samples of PEM films produced were optically clear. Samples consisting only of chitosan and PSS were completely transparent while those containing CNWs are translucent with the optical density increasing with both the layer number and the CNW content. 12 bilayer Chitosan/CNW PEMs show some iridescence on the top layer, showing thin film interference which occurs with transparent layers of subwavelength thickness.

Once hydrated, most substrates are completely clear, but with a paper-like texture can be seen on some 12CNW substrates. None of the substrates are
auto-fluorescent, making them a good low background substrate for fluorescence microscopy studies.
4.6  ELLIPSOLOGY OF LAYER-BY-LAYER FILMS

All films in this section were measured as dry films at ambient temperature and humidity. As the films swell when hydrated, these measurements represent the minimum values for thickness.

4.6.1 SILICON DIOXIDE

As samples were built on silicon, the thickness of the transparent native oxide layer had to be determined in order to obtain a baseline measurement. This thickness was calculated by measuring a cleaned silicon sample, taken from the same wafer and fitting for thickness and angle offset only. As can be seen in Table 4-1, that oxide layer was 1.73nm ±0.002 nm thick. The chart (Figure 4-5) shows excellent agreement between the model and data. This silicon dioxide layer was modelled as being a separate layer at the base of all the multi-layers, which were subsequently built on top of the wafer.

Table 4-1: Fitted Ellipsometric Thickness of base silicon wafer

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Std Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSE</td>
<td>1.681</td>
<td>—</td>
</tr>
<tr>
<td>Native Oxide (nm)</td>
<td>1.73</td>
<td>—</td>
</tr>
<tr>
<td>Angle Offset</td>
<td>0.031</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Figure 4-5: Fitted ellipsometric data for Silicon Dioxide. The dashed line represents the fitting model and the green and red lines are the amplitude shift and phase shift, respectively. The model’s fit to the data is excellent.
4.6.2 0CNW PEM (PSS-CHITOSAN 12 BILAYER FILM)

The samples were modelled fitting the thickness, angle offset and the refractive index coefficients A and B from the Cauchy equation. The ellipsometric data is summarised in Table 4-2 on the basis of the fitted data seen in Figure 4-6. The full fitting agreed well with the data for the PSS-chitosan substrates which behaved like a smooth film.

The only sample with a MSE value above 10 (Sample 3) was also visibly rough. The ellipsometric thickness was found to be approximately 20 nm. The effective medium approximation of the refractive index of the PSS/Chi substrates was 1.455, which is almost exactly halfway between the published values of chitosan (1.52 at 600 nm) and PSS (1.395 at 600 nm). From this information, it is possible to calculate that the layer is composed of 48% PSS and 52% chitosan.

Table 4-2: Summary of Ellipsometric Data for 12 bilayer PSS/Chitosan Films (0CNW)

<table>
<thead>
<tr>
<th>Sample</th>
<th>MSE</th>
<th>Thickness (nm)</th>
<th>Angle Offset (°)</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value</td>
<td>Std. Error</td>
<td>Value</td>
<td>Std. Error</td>
<td>Value</td>
</tr>
<tr>
<td>1</td>
<td>4.931</td>
<td>21.46</td>
<td>0.073</td>
<td>0.138</td>
<td>0.0055</td>
</tr>
<tr>
<td>2</td>
<td>3.33</td>
<td>19.38</td>
<td>0.051</td>
<td>0.101</td>
<td>0.0038</td>
</tr>
<tr>
<td>3</td>
<td>11.302</td>
<td>23.37</td>
<td>0.186</td>
<td>0.337</td>
<td>0.0118</td>
</tr>
<tr>
<td>4</td>
<td>6.247</td>
<td>19.11</td>
<td>0.103</td>
<td>0.162</td>
<td>0.0069</td>
</tr>
</tbody>
</table>

Mean Ellipsometric Thickness (nm) 20.83

Figure 4-6: Chart of fitted data for 12-bilayer PSS/Chitosan Films. The model shows a good fit except at the shortest wavelengths.
4.6.3 12CNW (CNW-CHITOSAN 12 BILAYER FILM)

In contrast to the 0CNW films, modelling the 12CNW substrates was far more challenging. A very poor fit between the model and the data was seen (Figure 4-7). This was due to Rayleigh scattering from the substrate scattering light strongly below 400 nm. Light scattering was measured by the loss of the incident light polarisation upon reflection, and up to 80% of incident light was depolarised (Figure 4-8). For comparison, the maximum depolarisation seen for the 0CNW films was 6%.

To obtain an acceptable model, only wavelengths in the range of 600 to 1000 nm were considered. Even so, the model for the material had only a mediocre fit (MSE > 10) as shown in Table 4-3 on the basis of data fitted in Figure 4-9. The ellipsometric thickness is approximately 100 nm. Due to the quality of the fit, the actual thickness may vary by up to 10 nm, which is acceptable accuracy for this application. The refractive index was measured to be 1.14, which a value that is lower than the refractive index of either polymer and indeed is lower than that of any known polymer. The refractive index of cellulose is 1.54.\textsuperscript{344}

Figure 4-7: Chart of data obtained versus model over full wavelength range. The fit between the model and data is poor and indicates strong adsorption of light at short wavelengths.
Table 4-3: Summary of ellipsometric data for 12 bilayer CNW-Chitosan samples

<table>
<thead>
<tr>
<th>Material</th>
<th>12 bilayer CNW/Chi samples, 12CNW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>MSE</td>
</tr>
<tr>
<td>1</td>
<td>14.27</td>
</tr>
<tr>
<td>2</td>
<td>9.819</td>
</tr>
<tr>
<td>3</td>
<td>15.712</td>
</tr>
<tr>
<td>4</td>
<td>15.056</td>
</tr>
<tr>
<td>Mean Ellipsometric Thickness (nm)</td>
<td>99.73</td>
</tr>
</tbody>
</table>
Fitting the refractive index linearly, for the chitosan/cellulose multilayers suggests that approximately 70% of the volume is air, with an error margin of 3% depending on the refractive index assigned to the solid phases. A more accurate fitting was performed using the Bruggemann model for determining the refractive index of nanoporous materials. This model allows for light scattering from particulate matter (such as cellulose nanowhiskers). It gave an air content of between 65-75%, which was consistent with the linear approximation.

It suggests that successive dips of chitosan form a close conformal layer over the cellulose crystals rather than an intercalating layer, which produced a nanoporous film. This is consistent with the AFM observations in Section 4.7, where layers below the top surface of cellulose nanowhiskers can be probed through the pores in the structure.

As CNWs are invariant in dimension regardless of environment since as rigid rods, they do not swell (~5nm diameter), it suggests that the mean thickness of a dry chitosan film within the Chitosan-CNW layers is ~3nm. As films were measured in their dry form, the thickness cannot be directly correlated to the thickness of the hydrated films.
4.7 ATOMIC FORCE MICROSCOPY TOPOGRAPHICAL STUDY

AFM sharp tips (nominal radius of curvature = 10nm) were used in intermittent contact mode to produce high resolution images while minimising damage to the surface or contamination of the tip from the sample. As the samples tended to foul the tip, a soft-tapping tip with a low spring constant mitigated this issue.

4.7.1 APPEARANCE

Representative AFM micrographs of the various substrates built can be seen in Figure 4-10 below. Glass appears to be a smooth substrate with very small (~ 1nm diameter) pits sparsely but randomly arranged throughout the surface. PAHCl-coated glass appears as a smooth film, while Chitosan-coated glass is notably rougher. On single bilayer films, the CNWs can be clearly visualised as a sub-monolayer of fibres on top of a non-fibrous film.

The PEM consisting of 12 bilayers have a variable appearance. The 0CNW PEM appears to be a smooth film, consistent with ellipsometry data. On 1CNW PEMs, the spin-coated CNW layer appears as an aligned sub-monolayer on a smooth substrate. PEMs with several CNW layers, the 6CNW and the 12CNW PEMs were porous, with several CNW layers visible beneath the top layer.
4.7.2 ORIENTATION

Orientation determination was relatively straightforward in bilayer films and in multilayer films where only the terminating layer contained a sub-monolayer of cellulose: individual fibres. These could be individually measured and tabulated to give a histogram. For multilayer cellulose films, it was not always possible to determine a preferred direction, even when the terminating layer had been spin-coated as the underlying randomly-oriented CNW layers could be detected through gaps between the top layer of fibres. Nonetheless, as will be seen in Chapter 5, these substrates could successfully radially orient cells. Utilising the Amplitude channel from the AFM (which measures the gradient of topography) rather than the topography (which measures the change in height of the surface) made the alignment of the top layer more evident.

Representative histograms of aligned single and multilayer films can be seen in Figure 4-11.

![Histogram of 1 bilayer sample](image)

![Histogram of 6 CNW sample](image)

![Histogram of 12 bilayer Chi-CNW Substrate](image)

Figure 4-11: Representative histograms of CNW alignment on 1, 6 and 12CNW substrates.

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The Poisson’s ratio of the various materials needed to be understood. For the polystyrene ball, it is approximately 0.34. As a first approximation, the hydrated PEMs were modelled as being like hydrogels which have a Poisson’s ratio of approximately 0.5. A Hertzian fit was used assuming a spherical indenter. The limitations of this fit will be discussed in the next section, but it suffices for a relative comparison between the substrates. Indentation studies at 5nm and 20nm were conducted.

At 20nm (Figure 4-12), there appears to be a paradoxical increase in stiffness with reduced cellulose nanowhisker content, with 0CNW and 6CNW substrates appearing stiffer than 12CNW substrates. Values for $E_r$ were $5.56 \pm 3.89$ MPa, $5.77 \pm 2.67$ MPa and $2.47 \pm 1.07$ MPa respectively. Using one-way ANOVA (Kruskal-Wallis test), the differences between these substrates are significant at the $p < 0.05$ level.

The ellipsometry data helped to resolve this discrepancy. With values of approximately 20 nm for the dry 0CNW film but 100 nm for the equivalent 12CNW film, the indentation depth is large relative to the film thickness and a significant contribution from the tissue culture plastic (TCP) substrate the films are cast on is being seen.
When samples were indented by 5nm (Figure 4-13), where less of the film thickness is compressed, the Reduced Young’s Modulus, $E_r$, of all three substrates is reduced, being $1.67 \pm 0.73$ MPa, $1.47 \pm 0.24$ MPa and $1.06 \pm 0.24$ MPa for 0, 6 and 12CNW respectively. Stiffness differences between the various films stiffnesses are also less pronounced with no statistically significant difference seen between films (one-way ANOVA, Kruskal-Wallis test $p > 0.05$).

The change in stiffness is less marked in the 12CNW films, indicating perhaps that the measured stiffness is closer to the true value for this film composition relative to the other two films.

Figure 4-12: Box-whisker plot (whiskers are minimum to maximum value) of reduced Young’s modulus measurements on hydrated PEM films, indented 20 nm. N = 3
In Chapter 5, the process whereby the best PEM to utilise for the culture and myogenic differentiation of C2C12s and MSCs will be examined in detail. Once this was identified, it was desired to know whether the PEM and associated controls were inherently stable under cell culture conditions.

12CNW substrates appeared stable under immersion in cell culture medium with no changes in the distribution of CNWs over the experimental time frame. No areas of delamination were seen. What was seen was the appearance of globular deposits, presumed to be protein, in immersed samples. As of Day 1, a few, scattered deposits could be seen. These tended to grow in size over time, sometimes consolidating to form very large (< 100 µm high) deposits. Deposits made measuring the underlying substrate challenging, but no overall trend was
seen in size, shape and distribution of the CNWs on the upper layer of these films.

Single bilayer Chi-CNW films also appear to be stable under medium conditions with protein aggregates also building up over time. In some cases, protein aggregates appeared to follow the whiskers to form elongated deposits but this was not consistent. Protein deposits were larger on glass and chitosan substrates. However, chitosan is not soluble at pH >6 and is unlikely to have dissolved into the cell culture medium.

Representative images of the substrates at the start (Day 0) and end (Day 28) of the experiment may be seen on Figure 4-14.
Figure 4-14: AFM micrographs of films in cell culture medium. A, C, E, G (left) substrates as produced. B, D, F, H (right) substrates after 28 days immersion in medium. A-B: Glass. C-D: Chitosan. On glass and chitosan films, the protein deposits can be readily seen as globules. E-F: 1 bilayer Chi-CNW. G-H: 12 bilayer Chi-CNW. Scale bar = 2 µm.
The adsorption of protein on substrates could be quantified as an increase in the surface roughness, which is summarised in Figure 4-15.

![Change in Roughness of Substrates with Immersion Time](image)

**Figure 4-15:** Summary graph of the RMS roughness of control and PEM films after immersion in cell culture medium.

Two way ANOVA was performed comparing the effects of substrate type and time elapsed on the roughness of the films. At each timepoint the roughness of the materials differed significantly from each other and immersion time significantly increased the roughness of each film. The changes in roughness with immersion time for a given substrate are most significant for glass and chitosan and the effect of immersion time on the 12CNW substrate is not significant. This data is summarised in Table 4-4 below.

| Table 4-4: Results of a two-factor ANOVA to compare the effect of film identity and immersion time on the roughness of the films. Immersion time (Factor1) and material (Factor2) are both statistically significant in |
|---|---|---|---|
| Factor | Description | p-value | Statistical Difference |
| 1 | Immersion Time | 0.0018 | Yes (p<0.05) |
| 2 | Material | 0.0018 | Yes (p<0.05) |
It can be concluded that all the substrates utilised for C2C12 and MSC culture (See Chapter 5) appear to be stable under cell culture conditions for periods of at least 28 days.

While this shows that topographically, the substrates maintain their condition for at least that period, several limitations in the experiment need to be noted. First, as this is a microscopy test, no inference can be made about whether or not any material is leached out of the layers. Second, no nanomechanical testing was performed so it is not possible to comment on any changes in the stiffness of the substrate. As the samples were scanned in the dried state, it is not possible to make any inferences about the swelling of the substrates under cell culture conditions nor whether this changed as a function of time.

4.10 SUMMARY

This chapter has outlined the isolation and characterisation of CNW from tunicates. The CNWs have been used in layer-by-layer films consisting of chitosan, CNW and PSS in varying proportions by dip-coating.

The ellipsometric thickness of the PEMs was found to vary with the composition of the films, varying from 20 nm for 0CNWs to 100 nm for 12CNW substrates. The effective reduced Young’s modulus of the hydrated films when indented 5 nm was $1.67 \pm 0.73$ MPa, $1.47 \pm 0.24$ MPa and $1.06 \pm 0.24$ MPa for 0, 6 and 12CNW respectively. While these values are significantly stiffer than that of native muscle (~ 12 kPa), they are still less stiff than tissue culture plastic (~1GPa) and may modulate cell behaviour. All films were found to be stable under cell culture conditions for at least 28 days.

A concern was that the uneven surface might interfere with the ability of cells to align on substrates and this is investigated in the next chapter.
5  CELL-SUBSTRATE INTERACTION

Chapter 4 described the production and characterisation of polyelectrolyte multilayer films composed of successive layers of tunicate-derived CNWs, chitosan and PSS in varying proportions, showing them to be very thin, soft films that were stable for at least one month under tissue culture conditions.

This chapter investigates the ability of CNW-containing multilayer films to support the attachment, proliferation, orientation and myogenic differentiation of cells. It was hypothesised that the films would be biocompatible and that the cellular interaction with substrates would be dependent on substrate composition and topography. These same factors were also predicted to affect the cellular orientation and differentiation.

The formation of an appropriate extracellular matrix (ECM) by cells is a key component of successful tissue regeneration. Therefore, the production of ECM proteins by myogenic cells cultured on test and control substrates was investigated in two ways: first, by its spatial organisation, using the abundant ECM proteins fibronectin and laminin and second, compositionally, using LC-MS/MS to identify and quantify ECM proteins found in decellularised digestates.

C2C12 murine myoblasts as a committed muscle-cell line were used to identify the optimal film composition. While these cells have been immortalised and are very fast-growing (having a nominal population doubling time of 14 hours), they are nonetheless capable of responding to differentiation cues, exiting the cell cycle and differentiating to form multi-nucleated myotubes. These cells were also used to perform the ECM production investigations.

Once the optimal film had been identified, human bone marrow MSCs were cultured on the optimised films to assess the ability of MSCs to undergo myogenic differentiation on the films. As a follow up to the production of the ECM by C2C12s, an additional factor was added to the MSC study to see if seeding the MSCs on pre-templated ECM would enhance any myogenic induction over and above that induced by the substrates alone.
5.1 C2C12 ORIENTATION WITH RESPECT TO SERUM PROTEINS

The ability of tunicate cellulose nanowhiskers to orient cells has been established by earlier work \(^{187}\). An initial question was by what mechanism did cells use in order to utilise CNWs as orientation cues. Two key questions were first, did cells attach in the gaps between CNWs or to the nanowhiskers themselves, and second, was it the dimensions of the CNWs themselves or the spatial pattern they imposed on serum proteins that was responsible for aligning cells. A dense nanoparticle surface, which gave cells no alternative but to attach directly to CNWs would provide a suitable initial test.

0.1%w/w CNW suspension was spin-coated onto round glass coverslips, primarily in order to pack CNWs more densely. Plain glass and PAHCl-coated glass were used as controls. For each test condition, cells were also cultured in growth medium as a positive control. Cells were fixed at 1 hour, 4 hours and 24 hours. The cell membrane was stained in order to analyse cells for their relative orientation, length and aspect ratio.

Cells were imaged using the Leica SP5 confocal microscope, first, in order to utilise the far-red filter set required to image the membrane stain. Secondly, the field of view on the microscope could be oriented between -90\(^\circ\) and +90\(^\circ\), allowing the approximate radial orientation of spin-coated CNWs to be aligned so as to run in the vertical direction. All samples were oriented similarly.

Representative images of cell morphology of C2C12 cells grown on various substrates in both serum-containing growth medium and serum-free medium can be seen in Figure 5-1, Figure 5-2, and Figure 5-3 respectively on the pages that follow. 1 hour post-seeding, all cells appeared rounded on the surface and initially, a similar number of cells appear to have attached. At 4 hours, cells had started to spread on all surfaces save the serum-free glass substrate. On the CNW substrates, cell alignment could be seen under both medium conditions. At 24 hours, cells grown on serum-free surfaces were much less densely packed on the substrate surfaces, particularly on glass. PAHCl substrates were partially protective, serum-free surfaces sustaining higher densities of cells that were better-spread than on CNW or glass.
The relative orientation of representative populations of these cells was determined using CellProfiler (detailed in Section 3.3.1), binned and converted into radial histograms in Origin. These are presented alongside the corresponding images on The development of cell orientation over time by substrate and medium condition showed that cells align to the same extent and at a similar rate on CNW-coated surfaces, regardless of serum culture conditions. On glass and PAHCl-coated surfaces, cells do not orient over time. A two-tailed t-test of the effect of culture medium on the orientation of C2C12s on aligned CNW surfaces at 4 hours was not significant (p = 0.91, Mann-Whitney) and barely reaches significance (p = 0.0464, Mann-Whitney) at 24 hours.

Figure 5-1: Confocal micrographs of C2C12 cells on glass, by time and culture medium with corresponding radial histograms. A, C, D are cells cultured in growth medium while B, D, F are cultured in serum-free medium. No alignment is evident at any time point. While cells are sparser on the SFM cultured surfaces, there are considerably fewer ‘bare’ nuclei. Scale bar = 100 μm. Images have been brightened for print contrast.
Figure 5-2: Confocal micrographs of C2C12 cells on PAHCl-coated glass, by time and culture medium. The corresponding radial histograms are on the right. A, C, D are cells cultured in growth medium while B, D, F are cultured in serum-free medium. No alignment is evident at any time point. While cells are sparser on the SFM cultured surfaces, there are considerably fewer 'bare' nuclei. Scale bar = 100 µm. Images have been brightened for print contrast.
Figure 5-3: Confocal micrographs of C2C12s on aligned CNW surfaces by time and culture medium. A, C, D are cells cultured in growth medium while B, D, F are cultured in serum-free medium. Cells are not aligned at the 1 hour time point (images A and B), but alignment is evident after 4 hours (images C and D) and well-developed at 24 hours (E and F). Cells are noticeably sparser on the serum-free surface at 24 hours. The arrows indicate the approximate radial direction of the CNWs. Scale bar = 100 µm. Image brightened for print contrast.

The aspect ratio of the cells, was also calculated and tabulated, the results of which are summarised in Figure 5-4. The aspect ratios of the cells increase with time on all substrates, which is to be expected as cells spread. At a given timepoint, no significant difference was seen in aspect ratio between cells cultured in growth medium or serum free medium for a given substrate. The sole exception to the trend was PAHCl, where at the 24 hour timepoint, cells cultured in serum-free medium had higher aspect ratios than those cultured in growth medium.
The cell culture substrate has a significant effect on aspect ratio. Cells cultured on PAHCl had a significantly higher aspect ratio than those cultured on glass (3.11 ± 1.7 for PAHCl at 24 hours vs 2.49 ± 1.41 for glass at 24 hours, p < 0.05, 2-tailed t-test, Mann Whitney). Likewise, cells cultured on CNW substrates had higher aspect ratios than those cultured on PAHCl.

![Bar chart](image)

**Figure 5-4:** Bar chart (mean and standard deviation) of cells cultured on substrates over time. NS = no significant difference found. ** = p < 0.01. N = 6.

It can therefore be concluded that the alignment of C2C12 cells and their aspect ratios are not mediated by the conformation of proteins on the surface of the CNWs, but is rather a direct response to the nanotopographical features presented by the CNWs.
5.2 C2C12 RESPONSE TO PEMS: PRELIMINARY STUDY

5.2.1 SINGLE COMPONENTS

A preliminary study was done to determine the response of cells to the individual chemical components of the different layer by layer films to be built. Chitosan, PAHCl, PEI, PSS and CNW suspensions were spin-coated onto glass coverslips at various concentrations. As polyanions, PSS and CNW suspensions cannot adhere directly to freshly-cleaned glass, which also has a negative net charge: they were spin-coated onto glass with an adhesion layer of PEI. C2C12 cells were seeded onto the various substrates at 10,000 cells/cm² in C2C12-GM. This was performed twice in triplicate.

Cell size was determined by fixing cells at 24 hours and staining for the actin cytoskeleton with nuclear counter-staining. Their size was analysed using CellProfiler. Cell proliferation rates were determined by Alamar Blue assay.

A bar chart of cell sizes after 24 hours measured in µm² can be seen on Figure 5-5. On all substrates, the mean cell size is 1042 µm² The range of cell sizes is large in part because there is always a degree of variability in cell size, and because overlapping cells cannot always be visually separated. In the case of non-separated cells, the cytoplasm is divided up such that each ‘cell’ has only one nucleus. No statistically significant differences can be observed in the cells plated on different substrates.

The doubling times for cells on all substrates was 12 hours ± 2 hours (data not shown). Again, no statistically significant differences could be observed.
5.2.2 12 BILAYER PEMs

12 bilayers are 24 single layers of oppositely charged polymers and represented a compromise between the need to create a film thick enough to mechanically shield the underlying glass support from the cells and to minimise the number of production steps to produce them, necessary as all samples are dipped by hand. The expectation was that layer-by-layer films built with CNWs as the poly-anionic moiety would be firmer and that cells seeded onto such surfaces would spread more readily. On the other hand, films built with PSS as the polyanion would present a softer surface that would inhibit cell spreading. The samples prepared are outline in Table 5-1.
Four controls were used. The first, glass, was a simple negative control presenting a rigid substrate with no topographical cues. Tissue culture plastic (TCP) is the standard substrate cells are cultured on. PAHCl, 0.6%, spin-coated onto glass, which is a polycation known to support the attachment and spreading of C2C12 cells. These three substrates were the negative controls. The fourth control, PAHCl/CNW, was a substrate spin coated with 0.6 w/v% PAHCl and 0.02% CNW. This was a simple positive control as this bilayer film is known to reliably align C2C12 cells radially.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Terminating layer</th>
<th>Role</th>
<th>Cell Alignment hypothesised</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEI/(PSS/Chi)₁₂</td>
<td>Chitosan</td>
<td>Softer PEM</td>
<td>No</td>
</tr>
<tr>
<td>PEI/(PSS/Chi)₁₁/PSS</td>
<td>PSS</td>
<td>Softer PEM</td>
<td>No</td>
</tr>
<tr>
<td>PEI/(CNW/Chi)₁₁/CNW&lt;sub&gt;Random&lt;/sub&gt;</td>
<td>CNW</td>
<td>Firmer PEM</td>
<td>No</td>
</tr>
<tr>
<td>PEI/(CNW/Chi)₁₁/CNW&lt;sub&gt;Aligned&lt;/sub&gt;</td>
<td>CNW</td>
<td>Firmer PEM</td>
<td>Yes</td>
</tr>
<tr>
<td>Glass</td>
<td>None</td>
<td>Negative</td>
<td>No</td>
</tr>
<tr>
<td>TCP</td>
<td>None</td>
<td>Negative</td>
<td>No</td>
</tr>
<tr>
<td>PAHCl</td>
<td>PAHCl</td>
<td>Negative</td>
<td>No</td>
</tr>
<tr>
<td>PAHCl/CNW&lt;sub&gt;Aligned&lt;/sub&gt;</td>
<td>CNW</td>
<td>Positive</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Substrates produced were transferred to labelled 12 well plates within cell culture hood and sterilised by UV exposure for 30 minutes, followed by rinsing in PBS and C2C12 growth medium. Substrates were then incubated in medium overnight before seeding.

C2C12 cells were seeded onto the substrates at 60,000 cells/well. After 24 hours, half the cells were changed from growth medium to differentiation medium to induce myogenesis. As the cells are highly metabolically active, the
medium is changed daily (approximately 75% of medium was withdrawn from each well plate and replaced with fresh medium).

Cells were fixed at 24 hours post seeding, and at 3 days and 7 days post induction of differentiation. Immunocytochemistry was done to identify cellular alignment and markers of differentiation.
CELL ATTACHMENT, SPREADING AND PROLIFERATION

The doubling time of C2C12 cells on the different substrates was assessed using the Alamar Blue assay, which can be seen on Figure 5-6. Cells were cultured in the Resazurin salt solution for two hours at two time points; at 25 hours and 49 hours post-seeding. The resulting change in metabolic activity used to calculate the doubling time of cells on the substrate. This was done using the formula given in Section 3.2.4. Cell doubling times were similar on all surfaces except for those on Chitosan/PSS bilayers. Cell doubling times were increased on those substrates, but the difference did not reach statistical significance (p > 0.05).

![Mean Population Doubling time](chart)

Figure 5-6: Mean and standard deviation of population doubling times of C2C12 myoblasts cultured on control and test PEM film surfaces. While a trend to longer doubling times on the Chitosan-PSS films is observed, this does not reach significance.
Cells were imaged at 5 hours, 24 hours and 3 days post seeding. On all substrates other than the Chi/PSS multilayer films, cells were firmly attached and beginning to spread by the 5 hour timepoint. On the Chi/CNW multilayer film with an aligned CNW terminating layer, cells could be seen to be starting to align. By contrast, on the Chi/PSS multilayer films, cells were still rounded at this time-point.

By the 24 hour timepoint, cells were attached and spreading on all surfaces. Radial cell alignment could be seen on the PAHCl/CNW positive control as well as on the Chi/CNW multilayer films, otherwise cells were not aligned, in line with expectations. Cells on the PSS/Chi multilayer films started to form clumps which grew into aggregates by 48 hours and spread outwards from these to cover the surface of the film. No difference in the shape of cells on either PSS-terminated nor chitosan-terminated surfaces could be observed.

All substrates were confluent by 3 days and the alignment of cells remained stable. Representative bright-field images at 24 hours can be seen in Figure 5-7 of cells cultured on control surface. Figure 5-8 illustrates the surfaces under test.
Figure 5-7: Representative light micrographs of C2C12 myoblasts cultured on control surfaces, from 5 hours post-seeding to 3 days. Double-headed arrows indicated preferred direction of cell growth. A, E, I - Glass. B, F, J - TCP. C, G, K - PAHCl, no CNW. D, H, L. PAHCl spin-coated with CNWs. At the 5 hour timepoint (top row), cells are attached and beginning to spread, but no alignment can be seen on any substrate. At 24 hours (middle row), a weak alignment can be seen on the PAHCl with spin-coated CNWs surface (H). After 3 days, cells are confluent on all substrates cells are well aligned on the PAHCl/CNW surface (L). Scale bar = 200 µm.
Figure 5-8: Representative light micrographs of C2C12 myoblasts cultured on test surfaces, 5 hours post-seeding to 3 days. Double-headed arrows indicated preferred direction of cell growth. A, E, I - Chitosan/PSS PEM, PSS terminated. B, F, J - Chitosan/PSS PEM, chitosan terminated. Cells on these surfaces were slow to attach and formed aggregates by 2 days. C, G, K - Chitosan/CNW PEM, unaligned CNW terminated. D, H, L - Chitosan/CNW PEM, aligned CNW terminated. Scale bar = 200 µm.
CELL ORIENTATION

The orientation of the C2C12 cells was quantified by fixing the cells 72 hours after seeding and staining for the cytoskeletal protein f-actin (pseudo-coloured yellow) and the cell membrane (pseudo-coloured magenta) to aid with cell segmentation. The nucleus was counter-stained with DAPI (blue).

As can be seen from Figure 5-9, the composition and surface termination of the layer by layer films had a significant influence on the orientation of cells. Cells on the PSS/Chi films tended to stay aggregated and show no orientation, whereas cells cultured on the CNW/Chi films grew well-spread and were oriented parallel to one another. The C2C12 cell line is known to self-orient when confluent, so this was not unexpected. However, this self-alignment is short-range.
Figure 5-9: Confocal micrographs of C2C12 cells seeded onto 12 bilayer films of differing composition after 72 hours. The double-headed arrow on A indicates the approximate direction of the CNW layer. Cells grown on CNW/Chi films are well spread (A, B), while those cultured on PSS/Chi films are less spread (C). Cells on CNW/Chi films terminated in aligned CNW (A) show long-range alignment, which is reflected in the histogram of the cell orientation (D). Cells on CNW/Chi films terminated in randomly-aligned CNW (B), show short-range alignment, which is reflected in the histogram of orientation (E). Cells on PSS/Chi films (C) show no alignment, as seen on the histogram (F). Scale bar = 100 µm.
Plotting the orientation angles of the cells on the surfaces shows that cells are oriented over a wider range of angles on the randomly terminated substrates than they are on the aligned substrates. To align cells imaged from different fields of view, the mean orientation angle is calculated for each micrograph and the individual values are subtracted from this mean value to centre the distribution of angles on 0°. The distribution is summarised in Figure 5-10.

The standard deviation for aligned Chi-CNW films was 20.41° while that for unaligned Chi-CNW films was 41.01°. The standard deviation for the Chi-PSS films was 53.45°. 2 tailed t-tests (Mann-Whitney) of aligned and unaligned Chi-CNW films was significant at p<0.05, while comparing aligned Chi-CNW films to PSS-Chi films was significant at p<0.005.

**Spread of Cell Alignment Relative to Mean Orientation Angle**

![Box-whisker plot](image)

Figure 5-10: Box-whisker plot of distribution of cell alignment relative to mean angle of orientation. Whiskers are minimum to maximum values, the centre line is the mean and the box is bounded by 1 standard deviation. N=6.
MYOGENIC DIFFERENTIATION

To assess the ability of the different substrate compositions to induce myogenic differentiation, C2C12 cells were seeded on the various substrates and switched to differentiation medium (DM) after 24 hours. Cells were fixed after 7 days and stained for f-actin and α-sarcomeric actinin, with the nucleus counter-stained with DAPI. Unfortunately cells cultured on PSS/Chi substrates detached after 3-4 days in DM, regardless of terminating layer. It was thus not possible to assess the myogenic differentiation of cells on these substrates.

On the CNW/Chi films, both fused and unfused differentiated cells could be observed on both substrates as evinced in Figure 5-11. The alignment of cells established in the first 24 hours persisted over the entire culture period.

![Figure 5-11: Representative confocal micrographs of C2C12 cells undergoing myogenic differentiation on 12CNW substrates after 7 days in culture. Both fused and unfused cells positive for α-sarcomeric actinin can be seen on both images. On the substrate terminated with aligned CNWs (d), the myotubes have an overall alignment. No overall alignment of myotubes is seen on (e) where the terminating CNW layer is randomly oriented. Scale bar = 250 µm. Cells cultured on other substrates (0CNW) detached after 3 days in culture.](image)

At the time, the reduced spreading of C2C12 cells on PSS/Chi substrates was attributed to the substrate being perceived by cells as being mechanically softer. It was therefore decided to develop a range of 3 component PEMs (consisting of PSS, Chitosan and CNWs) to create films with a range of
compressive elastic moduli to find an optimal composition to support the alignment, growth and myogenic differentiation of C2C12 cells.

5.2.3 C2C12 RESPONSE TO PEMS OF VARYING COMPOSITION

Work in the previous section identified 12 bilayer Chitosan-CNW films (12CNW) as the most promising material for the formation of myotubes. On that basis, a second study was designed to look at the differentiation of cells on that substrate over a longer time period (2 weeks) with glass, chitosan and a single bilayer of chitosan and CNW used as controls.

To reduce the number of samples needed, a shorter study was designed in parallel that examined the effect of varying the proportion of CNWs in the PEMs on C2C12 cells. These other test substrates consisted of 12 layer PSS/Chitosan with and without a terminating CNW layer (0CNW and 1CNW respectively). In addition to this, an intermediate composition consisting of 6 cellulose layers (6CNW) to better understand the influence of film composition on cell orientation and initiation of differentiation.

Table 5-2 overleaf summarises the timepoints and treatment groups per condition.
<table>
<thead>
<tr>
<th>Time Since Seeding</th>
<th>Treatment Groups</th>
<th>Tests done</th>
<th>Predictions</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hours</td>
<td>All Cells in Growth Medium</td>
<td>a. 1st 24 hours for attachment and spreading. &lt;br&gt;b. 2 sets: 1 for F-actin, myogenin, α-sarcomeric actinin, 1 ECM</td>
<td>Myogenin –ve, α-sarcomeric actinin –ve, ECM disorganised</td>
</tr>
<tr>
<td>24 hours</td>
<td>Growth Medium</td>
<td>Differentiation Medium</td>
<td>Split between treatment groups at 24 hours</td>
</tr>
<tr>
<td>48 hours (Treatment Day 1)</td>
<td>Stained for F-actin, myogenin, α-sarcomeric actinin.</td>
<td>Myogenin +ve, α-sarcomeric actinin –ve</td>
<td></td>
</tr>
<tr>
<td>4 days (Treatment Day 3)</td>
<td>Stained for F-actin, myogenin, α-sarcomeric actinin. 2nd set for ECM</td>
<td>Myogenin +ve, α-sarcomeric actinin +ve, ECM organised. Marked difference in myogenic efficiency between growth and differentiation samples.</td>
<td></td>
</tr>
<tr>
<td>8 days (Treatment Day 7)</td>
<td>Only controls and 12 layer Chi/CNW. Stained for F-actin, myogenin, α-sarcomeric actinin.</td>
<td>Myogenin ?, α-sarcomeric actinin +ve, more organised</td>
<td></td>
</tr>
<tr>
<td>15 days (Treatment Day 14)</td>
<td>Stained for F-actin, myogenin, α-sarcomeric actinin. 2nd set for ECM</td>
<td>Myogenin ?, α-sarcomeric actinin +ve, organised. ECM organised</td>
<td></td>
</tr>
</tbody>
</table>
5.2.4 CELL BEHAVIOUR ON PEMs

In order to image the cells and CNW matrix at the same time, C2C12 cells cultured on 12CNW substrates for 24 hours were dried with graded ethanol and HMDS to allow AFM scans to be performed. These showed that cells extended filopodia along the nanowhiskers and a representative image can be seen on Figure 5-12.

![Figure 5-12: Light and AFM micrographs of C2C12 cells cultured on 12CNW substrate for 24 hours and dried using graded ethanol and HMDS. A: a low-magnification view of the AFM cantilever positioned over the cells. The image is 1mm across. The double-headed arrow indicates the preferred orientation of the cells. The yellow box around the cantilever tip is the approximate location of the area imaged in B. B: A single cell on the CNW layer. The blue box outlines the area magnified in C. The scale bar of B is 5µm. C: Image of cell filopodia on the CNWs. The scale bar is 1µm.]

Whole slide scanning allowed the orientation of cells over the whole substrate to be inspected. If the cells were aligned by the terminating layer of CNWs, then the expectation was that the cells would be radially-oriented, following the radial orientation of the CNWs.

Surprisingly, the extent to which cells would align on polyelectrolyte multilayers terminated with aligned CNWs was dependent on the underlying layers.

On the control substrates, glass and chitosan, cells had no overall orientation, but tended to align parallel to each other at short range. This can be seen on Figure 5-13(B) and (b).

Cells cultured on the 1 bilayer Chi-CNW and the 12 CNW substrates were aligned radially in a direction parallel to those of the spin-coated CNW
terminating layer. The cells showed both long range (on the order of several cell lengths) and short range order. This can be seen on Figure 5-13(D) and (d).

By contrast, cells cultured on the 0CNW and 1CNW substrates showed no order at all, neither aligning with respect to one another nor showing long range order. Instead, the cells tended to clump together and also to show no overall orientation. This can be seen on Figure 5-13(A) and (a).

On the 6CNW substrate, an orientation pattern that was intermediate between the aggregates seen on 1CNW substrates and the well-aligned cells seen on 12CNW substrates. Cells tended to aggregate, but cells that grew out from the aggregates oriented themselves parallel to the terminating CNW surface. The cell aggregates in turn aligned perpendicular to the CNW orientation direction, forming a spiral on the slide. This can be seen on Figure 5-13(C) and (c).
Figure 5-13: Fluorescence micrographs of C2C12s cultured on various surfaces, showing orientation of cells. The double-headed arrows give the approximate orientation of the underlying CNWs, where present. A-D: whole slide scans at 0.8x magnification. Scale bar = 2000 µm. a-d: inset areas of slides, at 20x magnification. Scale bar = 100 µm. A and a: No order, seen on 1CNW substrate: cells aggregate randomly and spread out radially with respect to the aggregates. B and b: short-range order, seen on glass: cells are oriented relative to one another in the short range, but no long-range order can be seen. C and c: short-range disorder, seen on 6CNW substrate: cells aggregate, but spread out radially with respect to the underlying CNW orientation. The aggregates themselves are helically arranged. D and d: long range order, seen on 12CNW substrate: cells orient themselves with respect to the underlying CNW orientation and are parallel to one another.
Under growth medium conditions, the cells continued to proliferate and migrate, maintaining long-range order on the Chi-CNW single bilayers as well as the 12 bilayer Chi-CNW substrates. Cells on the 0CNW and 1CNW substrates tend to spread out from their initial aggregates to colonise the entire surface without any preferred direction, coming to resemble the short-range orientation of glass and chitosan substrates. Cells on the 6CNW substrates continued to remain aggregated, with spheroids separated from one another by aligned spread cells. However, as C2C12 cells are not contact-inhibited, they overgrew the surfaces and started to detach as a sheet after 3-4 days. Cells recolonised all substrates, but showed no alignment, indicating that they have detached along with the underlying substrate.

Where cells were transferred to differentiation media after 24 hours, cells continued to proliferate for another day before exiting the cell cycle. Cell seeding numbers were chosen so that the exit from the cell cycle would coincide with their just reaching confluence to avoid the problems of mass cell detachment. Under those conditions, aggregates on the 0CNW, 1CNW and 6CNW substrates persisted as the cells could only spread by migrating outwards from their aggregates.
5.3 MYOGENIC DIFFERENTIATION ON PEMS

After a day of culture in differentiation medium, the first myogenin positive nuclei could be seen on all substrates in differentiation media (Figure 5-14). After three days, the number of myogenin positive cells was increased and the first fused cells started to appear. At the three day time-point, a reduction in the total number of cells could also be seen. Some nuclei are observed to shrivel along with their surrounding cytoplasm and free-floating, presumably dead cells may be collected throughout the test period, although the number of cells stabilized by seven days.

Figure 5-14: Fluorescence micrographs of C2C12s stained for DNA (nuclei), myogenin (green) and α-sarcomeric actinin (red) under differentiation conditions 1 day (left) and 3 days (right) post-treatment. A, B: Glass. C,D: 1 bilayer Chi-CNW. E,F: 1CNW PEM. G,H: 12 bilayer Chi-CNW. At the three day timepoint, myogenin is located in the nuclei and many differentiating cells have upregulated α-sarcomeric actinin, which are much brighter than the background. Images have been brightened for print.
By seven days of treatment, cells appeared to consist of long myotubes with an underlying layer of non-differentiated cells. Not all differentiating cells would fuse – some would remain as single-celled myocytes. Most myotubes grew as long thin tubes, but the occasional branched myotube was observed on the chitosan only control films (an example may be seen in Figure 5-15 and is marked with an asterix *).

![Fluorescence micrographs of differentiating C2C12s on various substrates at seven days.](image)

**Figure 5-15:** Fluorescence micrographs of differentiating C2C12s on various substrates at seven days. The myotubes reach their maximum stable length between days seven and ten. Nuclei (blue), myogenin (green) and α-sarcomeric actinin (red). A: Glass – randomly-aligned myotubes. B: Chitosan – similar to glass in myotube length/orientation * = rare branched myotube, seen only on this substrate. C and D: 1 bilayer Chi-CNW and 12 bilayer Chi-CNW respectively. Myotubes are aligned on these substrates. Double-headed arrow indicates preferred direction of cell alignment. Scale bar = 75 µm.

Myotubes continued to grow in size and length throughout the entire study period of fourteen days, but as they grew longer, they tended to become destabilised on the surface and contract into balls which could then roll off the surface, several of which can be seen on Figure 5-16. The maximum average length that myotubes grew to before detaching occurred between eight and ten
days with 12CNW films tending to stabilise the cells for longer than other substrates. An example of the difference between substrates can be seen in Figure 5-16(D).

Figure 5-16: Fluorescence micrographs of C2C12 myotubes on test and control substrates after 14 days differentiation. The double-headed arrows indicate the preferred orientation of the cells. Many myotubes have contracted and been lost from the surfaces. Nuclei – blue, α-sarcromeric actinin red/yellow due to overlap with myogenin (green) which has been exported from the nucleus into the cytoplasm. A and B: glass and chitosan respectively – note the number of contracted myotubes. Myotube alignment persists on C and D: which are 1 bilayer Chi-CNW and 12 bilayer Chi-CNW respectively. Scale bar = 100 µm.

To quantify the length of the myotubes, cells cultured in differentiation medium for 7 days were measured. 3 random, non-overlapping areas per coverslip were imaged at a 5x magnification to create 9 areas per condition per replicate, using whole slide scans to ensure that the entire myotube could be imaged. The lengths of cells and myotubes that were double-labelled with myogenin and α-sarcomeric actinin cells were measured using CellProfiler. The presence of differentiated but unfused myocytes (which were approximately 50 µm long) provided a strong positive skew to the distribution.
The mean myotube lengths were 167.8 µm ± 66 µm for glass, 196.3 µm ± 86 µm for chitosan only, 188.4 µm ± 85 µm for 1 bilayer Chi-CNW and 148.9 µm ± 54 µm for 12 bilayer Chi-CNW films. Using a one-way ANOVA (ordinary one way ANOVA, multiple comparisons of each substrate against glass, $\alpha = 0.05$), the differences between the lengths according to condition is statistically significant, with $p < 0.05$. The data is summarised in Figure 5-17 as a bar chart with mean and standard deviations of the myotube lengths.

![Figure 5-17: Box-whisker plot of myotube length on test and control substrates after 7 days treatment. * = $p < 0.05$. N= 6.](image-url)
Myogenic differentiation was also associated with a change in cytoskeletal organisation. α-sarcomeric actinin is constitutively expressed by the C2C12 cells and appears to co-localise with the actin microfilaments. The latter formed stress fibres in undifferentiated cells. When cells differentiated, α-sarcomeric actinin became upregulated. This could be qualitatively seen as a brighter signal. It was no longer associated with stress fibres and began to be arranged in plaques. Double-labelling cells for f-actin and α-sarcomeric actinin confirmed that stress fibres disappeared in differentiating cells, with the actinin reorganising to form a punctate pattern. An example of this can be seen on Figure 5-18, where Alexa Fluor 647 conjugated phalloidin has been used to visualise the actin microfilaments as well as its relationship to α-sarcomeric actinin.

Figure 5-18: Confocal micrograph of differentiated and undifferentiated C2C12 cells, illustrating loss of stress fibres. The image is a maximum projection of a z-stack. F-actin (far red, pseudo-coloured purple), α-sarcomeric actinin (green) and nuclei (blue). Differentiated cells ** lose their stress fibres (A) and upregulate expression of α-sarcomeric actinin (B) relative to undifferentiated cells. Scale bar = 50 µm.
Negative controls for the staining were also performed. To show that the antibodies are specific to the proteins of interest, 3T3 fibroblasts were stained for myogenin and α-sarcomeric actinin, which was negative, as expected. C2C12 cells were also stained for RUNX-2 (expressed by differentiating osteoblasts) to show that a non-labelling primary would not result in positive staining. These can be seen in Figure 5-19.

![Figure 5-19: Negative Controls for immunocytochemistry. A: Unlabelled C2C12 cells. B: C2C12s labelled with secondary antibody only. C: C2C12s labelled with RUNX-2. D: 3T3 fibroblasts labelled with myogenin and α-sarcomeric actinin. All cells cultured on glass coverslips for 24 hours. Scale bar = 100 µm.](image-url)
Labelling the extracellular fibronectin and laminin showed that C2C12 myoblasts started to secrete laminin and fibronectin within the first 24 hours. Qualitatively, at 24 hours post-seeding, on all substrates, cells were observed to produce a fibrillar fibronectin in between cells (particularly where cell-cell contacts existed) and a membrane-associated coating of laminin. Representative examples of the ECM production by cells on 12CNW and 6CNW substrates after 24 hours can be seen on Figure 5-20 and Figure 5-21 on the pages immediately following.

On 12CNW surfaces, a non-fibrillar coating of fibronectin could additionally be seen on areas as yet uncolonised by cells. This presumably came from the serum within the culture medium. As the cells were not permeabilised, the intracellular fibronectin could not be detected and the cells were ‘visible’ as dark patches surrounding the counter-stained nucleus. This can be seen on Figure 5-20. This disappeared by day three when the entire surface was covered by cells. For all other substrates (e.g. the 6CNW substrate on Figure 5-21), no such fibronectin coating could be seen: only fibronectin staining in between cells could be observed.
Figure 5-20: Fluorescence Micrograph of ECM formed by C2C12 myoblasts on 12CNW substrate at 24 hours post seeding. A: Overview of image. 12CNW substrates were unique in adsorbing sufficient fibronectin from the cell culture medium to stain the surface. Scale bar = 100 µm. White box is an area of interest, expanded in images B-E below. B: Nuclei of cells (blue, DAPI). C: Laminin (green) produced by cells, outlining cells. D: Fibronectin (red) produced by cells and adsorbed from medium. The signal from the medium adsorbate swamps that of the fibronectin produced by cells and the cells appear as dark outlines against the background. E: Composite image of B-D.
Figure 5-21: Fluorescence Micrograph of ECM formed by C2C12 myoblasts on 6CNW substrate at 24 hours post seeding. A: Overview of image. Cells tend to clump and fibronectin production occurs mainly between the cells. Laminin production occurs at the periphery of cells. Scale bar = 100 µm. White box is an area of interest, expanded in images B-E below. B: Nuclei of cells (blue, DAPI). C: Laminin (green) produced by cells. D: Fibronectin (red) produced by cells. Note that this is more fibrillar than laminin. E: Composite image of B-D.
The organisation of the ECM matrix tended to follow the orientation of the cells. Fibrillar fibronectin can be seen to follow the cell alignment from Day 3 and becomes more organised by Day 14. Where cells were aligned, parallel fibres were formed. Where cells were not aligned, it took on a reticulated nature. Laminin remained closely bound with the cell membrane and occasionally whole cells could be seen outlined by laminin. Figure 5-22 presents a time series of the progression of ECM organisation on 12CNW and Chitosan only substrates.

Figure 5-22: Time series of ECM deposited by C2C12 on 12CNW films (A-C) and chitosan only substrates (D-F). Fibronectin = red, laminin = green, nucleus = blue. A and D: 24 hours post-seeding. B and E: 3 days post-treatment. The cells have now spread over the entire surface and have remodelled both the fibronectin and laminin. The double-headed arrow on the for the 12CNW films, the ECM is oriented along the long axes of the cells, while it has taken on a reticulated pattern on the non-oriented chitosan substrate. C and F: 14 days post-treatment.
To quantify the ECM organisation, the optical coherency of the individual components, laminin and fibronectin were determined using ImageJ and tabulated using GraphPad. Optical coherency gives a measure of the anisotropy of an image, so a well-organised image consisting of parallel lines would be scored 1 while one featuring randomly oriented lines would score 0.

Five areas per slide were imaged to give fifteen areas per sample at each time point. The coherency of all ECM images was low, ranging from 0.05 to 0.2. This is because while cells preferentially deposit the ECM along their long axes, there are also a significant number of fibres that run across the cell to form a meshwork (Error! Reference source not found.). While fibronectin tended to be more coherent than laminin at a given time point, there was considerable overlap between the two and indeed there is no statistically significant difference seen. There is also a trend to greater coherency over time for fibronectin but not for laminin. These data are tabulated in Figure 5-23 as bar charts with the mean and standard deviation presented.
Figure 5-23: Change in ECM protein coherency with time as a function of substrate. Time points are 24 hours, 3 days and 14 days post-seeding. A: Fibronectin, B: Laminin. As can be seen from the graphs above, neither of the ECM proteins is particularly coherent.
5.5 FOLLOW UP INVESTIGATION INTO C2C12 RESPONSE TO PEMS OF VARYING COMPOSITION

In a follow-up investigation into the cellular response to PEMs of varying composition, it was noticed that the extent of cell spreading and contact guidance appeared to be related inversely to the relative PSS content of the PEMs. This had been initially attributed to a reduction in substrate stiffness with the reduction in CNW volume fraction, but measurements of the compressive Young's Modulus (see Section 4.8), which at 1-3 MPa were far stiffer than would be expected if the stiffness was the cause of reduced cell spreading. This made substrate stiffness unlikely to be a sufficient explanation for the experimental observations made. While earlier work had not shown an adverse effect on cells by PSS as a monolayer, it was nevertheless worth investigating.

To investigate whether or not PSS content could be having an effect on cell growth, the Growth medium used to condition the test and control substrates overnight (work done for Section 5.3 above) was pooled by substrate type and C2C12s were seeded onto 24 well plates in substrate-conditioning medium diluted in fresh Growth Medium. Three volume percentage concentrations of conditioning medium were trialled, 10%, 50% and 100% of the total medium.

Substrate-conditioning medium is used to purge the substrate of deleterious material such short-chain polymers that are liable to diffuse out of the bulk material and is normally discarded so as not to affect the cell culture.

The Alamar Blue metabolic assay was done at 24 hours and 48 hours. For the 48 hour timepoint, cells were fed with the same medium they were originally seeded in.
A dramatic effect was seen whereby cells responded differently to the substrate-conditioning medium according to how many PSS layers were present in the substrate that the medium had been conditioned in. From the brightfield images in Figure 5-24, it can be seen that the cell shapes are similar to those of cells cultured on the substrates themselves – comparison images can be seen on Figure 5-7 and Figure 5-8.

Measurements of the cell metabolic activity showed a strong dose-dependent effect seen for the substrate-conditioning medium in substrates containing PSS but not for those containing only chitosan, CNWs or bare glass, which can be seen on Figure 5-25.
Figure 5-25: Effect of medium conditioned in different substrates on cell metabolic activity at 24 and 48 hours post-seeding onto TCP. For each conditioning substrate, cells have been cultured in 10%, 50% or 100% of the conditioned medium diluted in Growth Medium.
To better examine the effect of the leached PSS content, an exposure-response curve was plotted with the data generated from the Alamar Blue testing above. The precise concentration of PSS leached into the medium was not known. However, it was possible to consider the relative concentration of PSS within the leachate. The maximum possible concentration of PSS in the substrate-conditioning medium will come from 0CNW samples which contain 12 layers of PSS. On the other hand, 6CNW substrates contain 6 PSS layers. Therefore a medium with a volume percentage concentration of 100% PSS conditioning medium would be an undiluted conditioning medium from a sample containing 12 PSS layers and lower concentrations are given relative to this.

Several assumptions have been made in this. First, as PSS is freely soluble in water, differences in the actual concentration in solution will not be limited by its solubility. There might be differences in the diffusivity of PSS, owing to the different porosities of the films (as shown in Chapter 4, 0CNW PEMs, consisting of only PSS and Chitosan, are non-porous films while 12CNW PEMs are porous). However, given that all films are very thin and all have been soaked for at least 16 hours, as a first-order approximation, it is reasonable to assume freely diffusing chains have diffused into the substrate conditioning medium. For the 0% PSS data-point, the data from the undiluted 12CNW was used as then all conditions would have the same number of chitosan layers (and any consequent leachate from the chitosan will be common between all the samples). The resulting curve is plotted on Figure 5-26. While there is a small but significant increase in the cell metabolic activity from 0% to 5% PSS, the overall trend is strongly negative.

One-way ANOVA of the exposure-response of C2C12 cells to PSS in conditioned medium using relative PSS concentration showed the effect to be highly significant (Brown-Forsythe test p<0.05, actual value 0.0041; Bartlet’s test p<0.0001).
A separate test was also performed to examine the number of layers necessary to adversely affect cell spreading. A series of layers were built from alternating chitosan and PSS solutions. These ranged from 0 layers (glass) through to 24 layers, using PEI as an initial adhesion layer. Odd-numbered layers were terminated in chitosan while even-numbered layers were terminated in PSS. C2C12 cells were seeded onto these surfaces at 10,000 cells/cm² and fixed after 24 hours. Cells were stained for the cell membrane and nucleus only and then analysed for cell size.

A trend was seen in the mean size of cells with layer number (Figure 5-27). The trend in cell size was negative: while mean cell size was not adversely affected by the presence of two layers, cells cultured on successive layers became smaller. One-way ANOVA of the projected surface areas of C2C12 cells to the number of polyelectrolyte layers showed the effect to be highly significant (Kruskal-Wallis test, approximate p value <0.0001).

The difference between glass substrate and layered substrate became statistically significant at 3 layers (2 tailed t-test, p < 0.001). The chemical identity of the terminating layer (chitosan or PSS) did not have a significant
effect on the size of the cells. The similarity of cell size to the glass control when only two layers are present explains why this effect was not seen in the initial cytotoxicity testing, when only single layers of each polyelectrolyte were used.

Figure 5-27: Chart of mean C2C12 cell size 24 hours after seeding onto Chitosan-PSS films with differing numbers of layers. Mean and 95% CI plotted. The cell size is reduced as the number of layers increases and the cells take on a more rounded morphology. N=6.

On the basis of these tests, the PSS containing substrates were not used for any further work. The work on the proteomic fingerprinting of the ECM produced by C2C12 cells and the response of MSCs to substrates that follows in sections 5.7 and 5.8 respectively is performed using substrates built up with chitosan or CNWs only.
The intention of this work was to better understand the effect that the different substrates have had on the production and expression of ECM proteins.

During the decellularisation process, some ECM was lost from some substrates, particularly on the glass and the Chi-CNW single bilayer substrates, indicating poor adhesion to the underlying glass. The loose ECM would be observed as a thin white sheet floating freely in solution. When this was seen, it would be picked up carefully using a micropipette and pooled into the 0.1% SDS dissolution solution. On the 12CNW substrates, ECM appeared to survive intact and needed considerable scraping to remove from the surface into the dissolution solution. Figure 5-28 shows the appearance of ECM on 12 CNW substrates at different stages in the process. The alignment of the ECM on the substrate was still present after decellularisation.

Figure 5-28: Fluorescence micrographs of decellurisation process for C2C12 cells differentiated on 12 bilayer Chi-CNW substrates for 7 days. The double-headed arrow indicates the preferred cell alignment direction. The cell membrane is stained red, fibronectin is stained green and DNA is stained blue. A: Whole cell preparation, showing alignment of cells. B: Treated with cell lysis buffer (0.5% Triton X-100 in 10 mM NH₄OH). Remnants of the cell membrane and the nuclear contents are seen. C: Treated with DNAse I for 30 minutes at 37 °C. The fibronectin network is much more clearly visible with the removal of most of the DNA. Scale bar = 100 µm.
RESULTS OF PROTEOMIC ANALYSIS

Figure 5-29 shows representative brightfield images on the control (glass) and test substrates (1 bilayer and 12 bilayer Chi-CNW) after 7 days in differentiation media (8 days post-seeding). On all substrates, many cells have fused to form myotubes and these are aligned in a preferential direction on the test substrates.

![Figure 5-29: Brightfield microscopy images of differentiated C2C12 cells on control and test substrates after 7 days differentiation. Double-headed arrows show the preferred direction of the cells on the substrates. A: Glass. B: Chi-CNW (1 bilayer). C: 12CNW (12 bilayers). Scale bar = 50 µm.](image)

Notwithstanding the similar appearance of the cells, the quantity of protein from the decellularised material is markedly different between the substrates as can be seen on Table 5-2. In particular, the cells cultured on the 12 bilayer substrates produced less.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average protein concentration (µg/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>2.2</td>
</tr>
<tr>
<td>1 Chi-CNW Bilayer</td>
<td>2.7</td>
</tr>
<tr>
<td>12 Chi-CNW Bilayers</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Table 5-3: Average protein yield per substrate

The LC-MS/MS scan of the proteins within the decellularised samples identified approximately 3600 proteins. The UniProt database (http://www.uniprot.org), with Mus musculus (house mouse) set as a
taxonomic break was used to identify and give each protein an accession number. Proteins identified by only one peptide or with a low Mascot score, indicating a low confidence in the identity of the protein, were discarded. Proteins that were not quantifiable because they had a very low abundance, as indicated by the size of the area under the curve, were also disregarded.

The raw abundance of each protein for all quantifiable proteins was tabulated and the results normalised to the total amount of protein injected, as there can be small differences in the actual amount of protein injected from replicate to replicate. Results are reported on the basis of the normalised abundances. The ANOVA p-value was calculated based on the difference between the Control (glass) and Treatment (single bilayer and 12 bilayer) samples.

ECM proteins were identified and tabulated. C2C12 cells differentiated on all substrates, forming long, multinucleated tubes (Figure 5-29). However, the ECM proteins expressed differed considerably between cells cultured on glass, 1 bilayer and 12 bilayer Chi-CNW samples.

Table 5-4 expresses the protein population changes of ECM expression relative to the glass control by comparing the abundance of each protein quantified in each of the treatment groups against that found in the control group. Values in italics indicate that the protein was robustly identified and was present at a high abundance, but whose replicate values do not allow them to be differentiated from control at p < 0.05.
Table 5-4: Summary of ECM proteins identified in matrix expressed by C2C12 cells cultured on glass, 1 bilayer Chi-CNW and 12 bilayer Chi-CNW films. The fold changes between the treatment and control groups are colour-coded. N =3

<table>
<thead>
<tr>
<th>Description (synonym)</th>
<th>Gene</th>
<th>1 bilayer</th>
<th>12 bilayers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Collagens</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Collagen α1 (I)</td>
<td>Col1a1</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Collagen α2 (I)</td>
<td>Col1a2</td>
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<td>4.5</td>
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<td>0.1</td>
</tr>
<tr>
<td>Collagen α2 (V)</td>
<td>Col8a1</td>
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<td>0.1</td>
</tr>
<tr>
<td>Collagen α1 (VI)</td>
<td>Col6a3</td>
<td>0.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Collagen α2 (VI)</td>
<td>Col12a1</td>
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<td>1.6</td>
</tr>
<tr>
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</tr>
<tr>
<td>Collagen α1 (XII)</td>
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<td>0.6</td>
</tr>
<tr>
<td>Collagen α1 (XIII)</td>
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<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Collagen α1 (XVIII)</td>
<td>Col13a1</td>
<td>0.9</td>
<td>3</td>
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<tr>
<td><strong>Glycoproteins</strong></td>
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<td>Fibrillin</td>
<td>Fbn1</td>
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<td>0.4</td>
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<td>Nid1</td>
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</tr>
<tr>
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<td>Nid2</td>
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<td>0.9</td>
</tr>
<tr>
<td>Tenascin</td>
<td>Tnc</td>
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<td>2.6</td>
</tr>
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<td>Spock2</td>
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<td>150</td>
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<td>Vcan</td>
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<tr>
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<td>Chondroitin sulfate proteoglycan</td>
<td>Cspg4</td>
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</tr>
<tr>
<td>Basement membrane-specific heparan sulfate proteoglycan core protein</td>
<td>Hspg2</td>
<td>1.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Biglycan</td>
<td>Bgn</td>
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<td>1.8</td>
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<td>Gpl1</td>
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<td>Galectin-3</td>
<td>Lgal3</td>
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<tr>
<td>Adiponectin</td>
<td>Adipoq</td>
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<td>0.5</td>
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<tr>
<td>EGF-containing fibulin-like extracellular matrix protein 2</td>
<td>Efemp2</td>
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</table>
The results indicate that the CNW-containing films have a strong effect on the cells cultured on them, including the composition of the ECM that they produce. It was surprising that cells cultured 12 bilayer Chi-CNW film (12CNW) produced less protein overall.

On both treatment substrates, the most upregulated proteins are the proteoglycans with both absolute and relative increases in abundance. Of these, mimecan (osteoglycin) is the most strongly upregulated in the 1 bilayer films and testican in the 12 bilayer film. One of the collagens identified – collagens XII, is a non-fibrillar collagen termed (fibril-associated collagens with interrupted triple-helices) (FACIT) collagen. This is also a proteoglycan.

Basement membrane proteins such as the laminins and nidogens are upregulated in the treatment groups relative to control. Collagen IV was not identified, which was surprising, but its associated protein, perlecan (HSPG2) is upregulated in both treatment groups. Tenascin C, was also upregulated in the treatment groups, and is more normally associated with tendon, but is also associated with regenerating muscle. This is therefore consistent with muscle development.

The fibrillar collagen I is upregulated. Collagen 6 is expected in muscle but this isn’t particularly upregulated its isoforms being either unchanged or downregulated. In contrast, several minor collagens more typical of developing muscle are upregulated.
5.7 BONE MARROW MESENCHYMAL STEM CELL (BM-MSC) RESPONSE TO PEMS

As 12CNW substrates were shown to be the most successful PEM composition to align and promote differentiation in C2C12s, this was taken forward for the culture of BM-MSCs. For a more complete picture, the 12CNW test substrate was produced both with an aligned CNW terminating layer and a randomly-aligned terminating layer. As a result, the chitosan only control was sacrificed.

An additional consideration was whether the organised ECM produced by C2C12s could have a synergistic effect on any potential myogenic differentiation of MSCs.

Each 9x18mm sample produced was cleaved into two 9x9mm sections to fit into 24 well plates. Briefly, samples were placed upside down in the cutting guide and scored lightly on the reverse side using a diamond scribe. A pair of soft plastic flat-tipped tweezers designed for handling delicate samples (Agar Scientific, Cat. No. AGT5230) was used to grip the free end. Gentle bending resulted in a clean break. The two samples were then placed right side up in labelled 24 well plates for transfer to the cell culture hood.

The substrates were transferred to clean 24-well plates and UV-sterilised for 30 minutes. Half of them were packed into a large autoclave bag for seeding MSCs directly onto. To create a cell conditioned substrate, C2C12s were seeded onto substrates and differentiated for a week, using the method used in Section 5.7. They were subsequently decellularised. The decellularised substrates were washed in PBS and then soaked in MSC-GM for an hour before seeding with MSCs.

MSCs were seeded either directly onto substrates or onto the C2C12-conditioned substrates at 15,000 cells/cm². Once cells reached confluence, half the MSCs were treated with differentiation medium (MSC-DM). Cells were fed with an 80% medium change every other day.

The following conditions were tested:
- The effect of substrate type on MSC behaviour (attachment, alignment, growth, and differentiation).
- The effect of medium (growth vs differentiation) on MSC behaviour.
- The effect of pre-conditioning substrates with ECM secreted by C2C12 myoblasts on MSC behaviour.

MSC myogenic differentiation was assessed at each of 4 timepoints (24 hours after seeding, 24 hours post-treatment, 7 days post treatment and 14 days post treatment). For each timepoint, 48 samples were required consisting of 4 substrates, 2 media (growth vs. differentiation medium) and 2 seeding conditions (pre-conditioned or seeded directly), 3 replicates for each.

For the 24 hour post-seeding samples, half of the cells were stained for cytoskeletal structure (vinculin for focal adhesions, f-actin and a nuclear counterstain). The other half were stained for myogenin, f-actin and a nuclear counterstain. 1 day post-treatment and 7 day post-treatment cells were also stained for early myogenic commitment. 14 day post-treatment cells were stained for both signs of myogenic commitment as well as $\alpha$-sarcomeric actinin, which would be a sign of late myogenic differentiation. A summary of the expected results is detailed in Table 5-5.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Myogenic medium</th>
<th>Non-myogenic medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preconditioned 12 CNW layer PEMs (aligned and random)</td>
<td>Alignment dependent on CNW termination, myogenin+/-</td>
<td>Alignment dependent on CNW termination, myogenin-/+</td>
</tr>
<tr>
<td>Non-conditioned 12 CNW layer PEMs (aligned and random)</td>
<td>Alignment dependent on CNW termination, myogenin-/-</td>
<td>Alignment dependent on CNW termination, myogenin -</td>
</tr>
<tr>
<td>Controls</td>
<td>No alignment, myogenin- to weakly +</td>
<td>No alignment, myogenin-</td>
</tr>
</tbody>
</table>
5.7.1 INITIAL OBSERVATIONS OF BM-MSC RESPONSE

A figure showing the appearance of MSCs on the various substrates three days after seeding can be seen in Figure 5-30. Qualitatively, MSCs appear to be well-spread on the glass control substrate.

The alignment on CNW substrates appears to be negative contact guidance, as the cells appeared to avoid the CNWs. On the 1 bilayer Chi-CNW substrate (which has gaps between fibres – refer to Figure 4-10(C), page 121), cells are well-aligned with large focal adhesions preferentially arranged at the ends of the cells, which is consistent the observed cellular orientation. On the 12CNW substrates, where the gaps between the top layer of CNWs are filled with further layers of CNWs, the cells are smaller, with smaller focal adhesions, which is indicative of poor attachment to the substrate. Cells are better spread on the randomly aligned 12CNW than on the aligned 12CNW substrate. No contact guidance can be seen on the aligned 12CNW substrate.

This interpretation is bolstered by a serendipitous scratch which removed the aligned 12CNW coating and exposed the glass support (Figure 5-30(E)). MSCs can be seen well-attached and spread along the scratch while cells on either side are far smaller and rounded.
Figure 5-30: Fluorescence micrographs of MSC cells cultured for three days on glass (A), 1 bilayer Chi-CN (B), randomly-aligned 12CN (C) and aligned 12CN (D). Cells are well spread with large focal adhesions on glass and 1 bilayer substrates, with the latter showing contact guidance. Double-headed arrow gives preferred orientation of cells. They are less well spread on 12 bilayer substrates (C) and (D). E: aligned, well-spread MSCs on aligned 12CNW substrate that had been accidentally scratched. Nucleus – blue, Vinculin – green and f-actin – red. Scale bar = 100 µm.
5.7.2 MYOGENIC DIFFERENTIATION OF MSCS

MSCs were contact guided by CNW-terminated surfaces with alignment seen on Chi-CNW and on aligned 12CNW substrates. On pre-seeded substrates, where ECM was present on the surface, cells elongated rapidly along it and appeared better aligned than those seeded onto the equivalent fresh substrates. Representative images can be seen in Figure 5-31.

During the preparation of the pre-seeded substrates, it was noticed that the ECM was most stable on the 12CNW substrates, with a tendency tending to peel away partially on the single bilayer Chi-CNW substrates and to lift nearly entirely on the glass substrates. However, even on coverslips where the ECM had partially or entirely detached from the surface, cells still attached rapidly on the surface. This was corroborated with Alamar Blue testing which indicated that more cells attached to the reseeded substrates (even where the ECM had peeled away) than to the freshly-seeded substrates (data not shown).
Figure 5-31: Fluorescence micrographs of human Bone Marrow Mesenchymal Stem Cells (BM-MSCs), 24 hours after seeding onto substrates. Fresh substrates are on the left and pre-conditioned substrates are on the right. Nuclei – blue, f-actin – red. Cells cultured on conditioned substrates elongate themselves along the ECM (where surviving) and are more elongated and aligned than those on the fresh substrates. Scale bar = 100 µm.
MSCs became confluent on the surface of pre-seeded substrates at three days and at five days on freshly-seeded surfaces. Once confluent, half the MSC substrates were switched to differentiation medium (at five days).

After reaching confluence, MSCs started to consolidate themselves on all substrates, forming aggregates of cells that remained attached to the surface. This process was less marked on the pre-seeded substrates and examples of both situations can be seen on Figure 5-32: Fluorescence micrographs of BM-MSCs seeded on fresh substrates, 7 days and 14 days post-treatment. On the 7 day treatment samples, nuclei - blue, f-actin – red, myogenin – green. Cells tend to consolidate themselves on the 12CNW substrates. The occasional cell appears to express myogenin in the cytoplasm, as does the occasional spheroid – all marked with *. Scale bar = 100 µm. On the 14 day treatment samples, nuclei - blue, myogenin - green, α-sarcomeric actinin - red. No α-sarcomeric actinin can be seen on any substrate, but the cells cultured in GM appear to be expressing myogenin where the cells are consolidated into aggregates. Scale bar = 100 µm. Figure 5-32 and Figure 5-33. Cells were observed to grow out from these aggregates to fill the gaps made.

A scattering of myogenin positive cells could be seen at day 7 on all pre-seeded substrates in both growth and differentiation media and also on the non-glass substrates in the freshly-seeded samples. In no instance was the expression of myogenin nuclear – all myogenin positive cells expressed it in the cytoplasm.

At day 14 two distinct populations of cells could be observed: single cells and cell aggregates. For single cells (i.e. cells not part of a tightly consolidated aggregate), few cells expressed myogenin and these occurred apparently at random. Where they did occur, the myogenin positive cells appeared elongated (some examples may be seen on Figure 5-33). None of the cells stained at day 14 expressed a detectable level of α-sarcomeric actinin, which C2C12 cells constitutively express weakly and then strongly once myogenin expression begins (refer to Section 5.4). Representative images may be seen in Figure 5-32 and Figure 5-33.
However, MSCs that were tightly consolidated appeared to express myogenin throughout the aggregate, regardless of the substrate that they were plated on. These tightly consolidated cells appeared preferentially on the freshly-seeded substrates cultured in MSC-GM, although a few did occur on pre-seeded substrates.

![Figure 5-32: Fluorescence micrographs of BM-MSCs seeded on fresh substrates, 7 days and 14 days post-treatment. On the 7 day treatment samples, nuclei – blue, f-actin – red, myogenin – green. Cells tend to consolidate themselves on the 12CNW substrates. The occasional cell appears to express myogenin in the cytoplasm, as does the occasional spheroid – all marked with *. Scale bar = 100 µm. On the 14 day treatment samples, nuclei - blue, myogenin - green, α-sarcomeric actinin - red. No α-sarcomeric actinin can be seen on any substrate, but the cells cultured in GM appear to be expressing myogenin where the cells are consolidated into aggregates. Scale bar = 100 µm.](image-url)
Figure 5-33: Fluorescence micrographs of BM-MSCs cultured on cell-conditioned substrates, 7 and 14 days post-treatment. On 7 day treatment samples, nuclei – blue, f-actin – red, myogenin – green. Cells tend to consolidate themselves on the 12CNW substrates, but less so than on fresh substrates. The occasional cell appears to express myogenin in the cytoplasm, as does the occasional spheroid – all marked with *. Scale bar = 100 µm. On 14 day treatment samples, nuclei - blue, myogenin - green, α-sarcomeric actinin - red Occasional elongated cells appear myogenin positive. Consolidated areas that express myogenin also appear on these substrates (images not shown). Scale bar = 100 µm.

As there were a limited number of MSCs available, it was not possible to perform RT-PCR parallel, which would have given additional information on the differentiation pathways the MSCs were following. This result cannot therefore be considered definitive, only an interesting initial finding to motivate further research.
5.8 SUMMARY

The data in this chapter have shown that myoblasts will align to the topographical cues provided by CNWs. Where substrates did not contain PSS, myoblasts aligned parallel to the local orientation of CNWs, including on multi-layered substrates. PSS interfered with the ability of cells to spread in a dose-dependent manner, which in turn affected the ability of cells to orient on substrates containing PSS.

Myoblasts were able to differentiate and fuse on control and PEM substrates. The multi-layered 12CNW PEMs improved the stability of the myotubes on their surface. The presence of CNWs has an effect on the matrix produced by cells with cells secreting relatively more fibrillar proteins and ECM proteins associated with newly regenerating muscle than those cultured on the glass control substrates. 12CNW substrates

When seeded onto PEMs at a low seeding density of 10,000 cells, Bone marrow derived MSCs attached and spread preferentially on substrates with a single CNW-chitosan bilayer, the only surface on which they showed contact guidance. When seeded onto PEMs at a higher seeding density of 15,000 cells, BM-MSCs preferentially attached to cell-modified substrates, showing a greater tendency to form aggregates on unmodified substrates.

Preliminary immunocytochemical staining for the muscle-specific differentiation marker, myogenin, suggests that PEMs may have the ability to drive the myogenic differentiation of MSCs but this requires more cells from different donors and an independent method of measurement such as Western Blotting or qPCR to confirm.
6 DISCUSSION

The aim of this work was to develop a CNW-based film of controlled topography and bulk mechanical properties to optimise the alignment and myogenic differentiation of C2C12s. The further aim was to promote the differentiation of MSCs without the use of transfection agents or histone disruptors.

Using layer-by-layer deposition of polymers with alternating charges, a range of PEMs that terminate with an aligned CNW top layer for cell contact guidance have been developed. These multilayer substrates have been characterised for thickness, internal structure and compressive stiffness. C2C12 myoblasts have been cultured on the multilayer films to determine the optimal film composition to test the myogenic differentiation potential of MSCs.

As a naturally occurring material, tunicin cellulose needs to be extracted from the organism used (Ascidia spp.) and its purity confirmed. Tunicates of the Ascidia genus are widespread and common in Atlantic waters, being found in coastal waters all along the U.K. coast. Additionally, they are fast-breeding aquafauna, making them a sustainable source of cellulose.

FT-IR spectroscopy analysis of tunicate cellulose and nanowhiskers confirmed that the material consisted of Type Iβ cellulose. The process of selective acid hydrolysis using sulphuric acid (first described by Rånby in 1947) created a suspension of negatively charged cellulose nanowhiskers with a high aspect ratio. This suspension was stable at ambient conditions for at least one year (although the bulk was stored at 4 °C to minimize the risk of it becoming colonised by algae). Given that the density of cellulose is 1.46 g/cm³, it is possible to estimate approximate specific surface areas for tunicin and cotton cellulose nanocrystals, using their mean dimensions. For an average tunicin nanowhisker with dimensions of 5 nm x 5 nm x 0.64 µm, the theoretical specific surface area of CNWs is 276 m²/g.

The swelling ratio of PEM films depends on the film composition, the hydrating medium and the pH of that medium but literature values for aqueous solutions typically range between 100 – 400%.
The initial concept of tuning the stiffness of the layer-by-layer films by changing the volume fraction of cellulose nanowhiskers was challenged by the apparent paradox of the film stiffness being inversely related to the cellulose nanowhisker content. The ellipsometry data resolved this discrepancy. The films swell in water and are inherently soft. They are however very thin and are too thin for the glass support not to exert a dominant effect. Nonetheless, the composite glass-PEM stiffness is still three orders of magnitude less stiff than glass, with a Young’s Modulus being on the order of stiffness between cartilage and bone.

The thickness corrected data would be of interest in finding out how inherently soft the films are, but the uncorrected data is more representative of what the cells experience. Buxboim’s work showed that cells are able to sense the stiffness of their underlying substrate only a short distance relative to the cell size, consistent with integrins being the mechanism through which mechanotransduction occurs. A gel 1µm thick was able to completely shield the cell from the effect of the stiffness of the underlying glass substrate. These materials are considerably thinner than 1µm and so do not mechanically shield the cells from the supporting glass substrate.

The layer numbers used may well have made a sufficient to obtain a film of the requisite thickness. Boudou et al. found 8-10 bilayers of PLL/Chitosan were sufficient to create a substrate soft enough to inhibit the spreading of C2C12 cells unless the layers were stiffened by crosslinking. The method chosen where the samples were dried between coating immersions avoided the exponential growth mode that rapidly creates thick PEMs, thus ensuring that PEM growth would be linear. This choice was made on the basis that films grown with drying steps are more reproducible and of better mechanical quality than PEM films made without drying steps. Doing so however, increased the manufacturing time and the number of handling steps. Concern about the reliability with which successive films could be dried precluded automating the dipping process.

When stored dry, the PEM films produced were stable for months at ambient conditions, being able to be sterilised and used for cell culture without apparent
detriment. Soaking films under cell culture medium in cell culture conditions for periods of up to 4 weeks showed that the films were stable for at least that time. While substrates became covered with protein, which in some cases formed aggregates larger than the dimensions of the whiskers, the material remained with no areas of delamination seen.

While it can be concluded that the films do not visibly degrade in the time frame that they were tested in, high humidity conditions, the physical properties of chitosan are known to degrade. This is particularly relevant for the film mechanical properties, which could have been affected by the environment but this was not tested after stability testing.

**CELL INTERACTION**

The ability of CNWs to be incorporated into a multi-layered film that could provide nanoscale contact guidance on a substrate that was less rigid than glass to myogenic precursor cells was investigated. Seeding C2C12 cells onto films spin-coated with CNW and then cultured in medium with and without and without serum present showed that the contact guidance was provided by the CNWs themselves and not patterning of deposited serum proteins as cell alignment was independent of the presence of serum proteins.

Multilayered films of varying compositions were developed in order to find an optimal film composition to robustly align C2C12s. Effective myogenesis depends on the ability of cells to align end to end in order to fuse to form myotubes. C2C12 myogenesis is not dependent on the substrate they were cultured on, or dependent on the medium conditions. These cells were also able to self-align and fuse over short distances, which were seen on the glass and chitosan control substrates. The resulting myotubes were however unaligned and were formed in a random orientation. On the other hand, where cells were contact guided by CNWs, they stayed in-plane and differentiated in-plane, forming myotubes that were well aligned and well-organised.

C2C12 cells were observed to align more reliably on multi-layer than on single-bilayer substrates. Muscle cells when differentiated were laterally restrained, growing thinner than they do on unrestrained substrates. The ECM was better
supported, evidenced by being less inclined to detach, which allowed for the reseeding of MSCs onto ECM conditioned substrates. Both cell-seeded and acellular substrates supported cell alignment for at least one month.

Additionally to orienting the cells, 12CNW substrates also appeared to constrain and compress the myotubes laterally, which was particularly notable when compared to those on the unconstrained glass and chitosan substrates. The single bilayer Chi-CNW substrates were intermediate in effect between the 12CNW and unconstrained substrates, showing the influence of the underlying layers.

The tendency of myotubes to detach from the surface is a well-described phenomenon. As myotubes are highly contractile, they spontaneously contract and detach from rigid substrates. Seeding cells onto a more elastic substrate, such as that provided by a fibroblast feeder layer, other myoblasts or gels with a muscle tissue-like stiffness has been reported to significantly improve the stability and maturation of myotubes.

As highly contractile cells, C2C12s appeared able to pull the hydrated PEM substrates away from the glass, evinced by their recolonising the surface in a random orientation. On the other hand, the less contractile MSCs are able to consolidate themselves but do not pull the substrate away.

The multilayer film RMS roughness of the 12CNW films was 10 nm, which is larger than the mean diameter of CNWs (at 4.8 nm). This roughness is isotropic, showing no preferential direction. Nevertheless, this more topographically complex substrate supports C2C12 cell alignment robustly.

There is great value for cellulose nanowhiskers in composites and as part of gels, with investigations in many fields inside and outside of medical applications, because of the unique mechanical and biocompatible properties of CNWs. It is also of interest to align these fibres, and several techniques have been developed, including spin-coating, magnetic field alignment, and the use of electrical fields. Dugan’s work on using spin-coated CNWs on glass represented at the time the smallest feature sizes shown to contact guide cells. Aside from its mechanical properties, glass presents a smooth
substrate, a property no biological substrate possesses. Thus, this work, in demonstrating that the alignment of CNWs on top of a rough film can produce a template-free film with contact guidance on the nanoscale level is novel. The closest technology in the same size regime to produce contact guidance in cells in a complex topography are electrospun nanofibers. Depending on the investigator and the method of production, the mean reported diameters of electrospun nanofibers range between 300 nm $^{358, 359}$ and 700 nm $^{360}$. These are an order of magnitude larger than the feature size presented by the CNWs.

The cytotoxic effect of PSS has been attributed to the effect of short-chain ionic impurities present. Sen et al. $^{361}$ compared the effect of dialysis and ion exchange resins in purifying commercially purchased PSS (Mw ~ 70 kDA, purchased from Sigma-Aldrich). They found that attempting to remove ionic species by cation-exchange resins served instead to increase the number present. 10-12 dialysis cycles were necessary for the UV absorbance and conductivity of the dialysate to match that of the distilled water.
**EXTRACELLULAR MATRIX PRODUCTION**

Proteomic fingerprinting was undertaken to evaluate changes in ECM. Care is required in interpreting the results of proteomic fingerprinting as proteins have several functions depending on their spatial location and what other proteins they interact with. An additional caveat is that the results represent a single time point: as protein expression is a dynamic process, no firm conclusion as to which, or whether any of the test substrates is ‘better’ at promoting typical muscle ECM can be drawn.

The method chosen to decellularise the test and control substrates, while non-proteolytic, used the detergent Triton X-100 to lyse cells and may have solubilized some proteins, particularly heavily glycosylated short-chain proteins. Gilles et al developed a detergent- and proteolytic-free method of decellularisation of skeletal muscle tissue, but their method was better suited to sections of tissue rather than the fragile thin-substrate cultures used here. Nevertheless, some inferences can be made.

The substrates are able to induce cells to change the ECM they secrete, both in composition and by ratio. C2C12 cells on 12 bilayer films secreted less total ECM than those on glass or single bilayer films but the ECM produced had enhanced levels of several proteins associated with matrix remodelling.

Compared to native muscle, several proteins are either absent or present at very low concentrations: e.g. the GAG decorin is nearly absent and no agrin was detected. The latter being a key component of the neuromuscular junction, it may not yet be expressed at this stage of differentiation.

The type of collagens expressed is also different from that found in native tissue. Unlike mature muscle, collagen type IV was not found and collagen type VI, which is a feature of muscle ECM was only slightly upregulated on treatment surfaces relative to the glass control surface. A lack of collagen IV is a feature of regenerating muscle where collagen IV expression disappears. Rather, several minor collagens associated with development and repair were upregulated. Collagen type VIII is important for endothelial cell migration.
Collagen type XIII is actually a transmembrane collagen and is important in formation of neuromuscular joints.

Several of the ECM proteins upregulated relative to glass are associated with the formation of load-bearing fibrillar structures. Tenascin-c is classically found in tendon, but is a key component of regenerating muscle, where it associates with fibronectin to form a temporary scaffold for nascent myotubes. Fibulin7 is enriched in cartilage and bone and tenascin-c is classically found in tendon. Mimecan is most strongly upregulated – it is associated with ectopic bone formation but also with cardiac hypertrophy.

Versican (cell guidance through negative adhesion) and vitronectin (cell positive adhesion) are both upregulated, all pointing to an ECM that is being actively remodelled by its resident cells. Several proteoglycans are vital to skeletal muscle development such as biglycan and glypican-1 are transiently upregulated during muscle repair: both of these are upregulated on the treatment substrates terminated with aligned CNWs.

The roles that the less frequently reported matrix proteins matrilin and testican (SPOCK) play in myogenesis is not as clear-cut but what information is available is interesting.

Matrilins are known to be adaptor proteins widely expressed in the extracellular matrix, but their precise function is not entirely clear. Matrilin-2 has been associated with nerve regeneration. Malin et al. found that it is expressed by pre-myelinated Schwann cells during development and that when substrates are coated with it, it promotes the migration of neuronal cells. However, matrilin-2 has recently been found to be a key protein controlling the timing of the myogenic programme. Déak et al. used a rat muscle injury model to show that matrilin-2 was transiently upregulated within healing muscle beginning 4 days after the injury, at the point when satellite cells have been recruited to the injury site, have proliferated and are beginning to differentiate. They confirmed this in an in vitro study of C2 myoblasts. Differentiating C2 cells deposited matrilin-2, starting at 2 days after differentiation induction. Repressing matrilin-2 expression with TGF-β served
to inhibit myoblast differentiation and fusion, a finding corroborated in matrilin-2 knock out mice. Muscle injuries in these mice healed much more slowly in these mice compared to wild-type mice – biopsy revealed that myoblasts within the injured muscle proliferated, but could not commit to terminal differentiation. These findings are indicative of early muscle development and potentially the creation of a permissive environment that encourages innervation.

SPOCK is normally associated with the developing central nervous system and attracts research interest for its ability to induce cell migration which is of particular relevance in cancer. Cifuentes-Diaz et al. did however report that it templates the future neuromuscular junction in embryos, becoming associated with agrin.

These changes may be partially due to the mechanical properties of the films as the 12 bilayer film is softer mechanically than bare glass and is functionally more distinct from either the 1bilayer or glass substrates are from one another. The surface chemistry of the films may also provide a partial explanation of the changes seen, unlike the glass control, both the single layer and the 12 bilayer Chi-CNW films were made of the same material. The topography was significantly different between the films with the 12 bilayer film providing a rough, nanoporous surface against the relatively smooth, aligned surface of the single bilayer film. Another difference was the films’ ability to adsorb proteins from the medium. The 12 bilayer substrate was able to adsorb sufficient fibronectin (and by inference other serum proteins) to form a bright immunostain. How these factors interact to produce the observed difference is not as yet fully understood.

The nature of the changes suggests that the presence of even a single CNW layer enhances the myogenic development of C2C12 cells. It would have been desirable to conduct the cell differentiation studies over a longer period of time in order to observe changes in the ECM over time, as a key question is whether the matrix formed would mature with time and become more typical of mature muscle. However, the cost of the experiment made that prohibitive. The
The objective of the study was to understand if the substrates had an effect on the cells and that was confirmed, to a significant degree.

While there are studies on the response of myoblasts, including C2C12s to ECM, both natural decellularised tissue matrix \(^{373}\) and coatings of ECM\(^ {374}\), I am aware of few studies into the ECM produced by C2C12 cells on any substrate. More importantly, understanding what ECM cells lay down helps in the rational design of an ECM enriched substrate to modulate cell development. The potential of ECM-based hydrogels for example is of great interest, but currently, whole decelluarised muscle tissue (normally porcine) is used\(^ {375}\). This is a complex system and a method whereby the needs of the cells can be understood could allow for a far simpler, more scalable and reproducible tissue engineering system to be designed.

**MSC INITIAL STUDY**

The CNW containing films were able to align MSC cells and allow them to proliferate. The presence of ECM enhanced cell adhesion and orientation.

On account of having been produced using sulphuric acid hydrolysis, the CNWs are decorated with sulphate esters, which the MSCs avoided. This led to robust contact guidance on the single bilayer Chi-CNW films where the CNW surface density is low relative to that in the 12 bilayer films.

The formation of consolidated cells and in some cases spheroids on unconditioned, fresh substrates was not expected. MSCs interacted more strongly with the substrates when they had been pre-conditioned by C2C12 cells, consolidating less and consequently forming fewer spheroids. The significance of these spheroids is not fully understood, although cells within these appeared to structures expressed myogenin, a marker of terminal myogenic differentiation. In some cases, myogenin positive cells appeared to be migrating out of these into the surrounding culture. All the myogenin expressed by MSCs wherever the cells occurred was cytoplasmic. This is consistent with the expression pattern seen by other investigators, for example in Lee et al. \(^ {195}\).
The presence of myogenin positive cells on both freshly-seeded and pre-seeded substrates would suggest that the cells seen are not contaminating murine cells. The morphology of the myogenin-positive cells does not resemble that of differentiating C2C12s, nor do they co-express alpha-sarcomeric actinin, which C2C12s constitutively express at a low level. Furthermore, C2C12 cells have a doubling time of between 12 and 16 hours: as the MSC-seeded substrates had been maintained in growth medium conditions for a week before switching to differentiation medium, even a low concentration of contaminating cells would have proliferated to a notable extent in that time.

That the cells appeared to differentiate best in the growth medium used to maintain and proliferate cells reflects one of the challenges of inducing the myogenic differentiation of mesenchymal stem cells. As discussed in the literature review, no given medium condition has been definitively shown to promote myogenic differentiation. It was not possible to investigate and optimise the effects of various growth factors and steroids on cell differentiation could be performed. Therefore a very simple medium, where the only change was a reduction in the serum available, was deliberately chosen as a baseline against which the effect if any of the substrates could be measured.

There are several crucial limitations that render this strictly a preliminary study. Most critical amongst these is that all cells came from a single donor and there were a limited number available. Practically, this meant that cells needed to be expanded to P5 for use, which limits their ‘stemness’ and their differentiation potential. It also precluded having sufficient cells to perform RT-PCR on which would have given a more complete picture of the differentiation pathways followed. As there is considerable heterogeneity between people, no single source of stem cells can be said to give a definitive answer. In order for this study to be more comprehensive, cells from at least two other donors would need to be available. RT-PCR would need to be performed in parallel with the cell-material work.
7 CONCLUSIONS

Negatively charged CNWs with a mean diameter of 4.8 nm were produced from *Ascidiella spp.* These were used to create chitosan-based polyelectrolyte multilayers using a combination of two well-established, low-cost and facile production methods, dip-coating and spin-coating. Spin-coating a final layer of CNWs on top of a dip-coated PEM allowed the formation of an aligned terminating layer on a relatively rough, uneven substrate.

The resulting PEM was shown to be a complex, nanoporous substrate that robustly allow the attachment and alignment of the immortalised C2C12 myoblast cell line. It also allowed the subsequent myogenic differentiation of C2C12 cells. Proteomic analysis of the ECM produced by C2C12 cells in response to the substrates showed that the material altered the expression patterns of these proteins towards an expression that is consistent with a more developmental, rather than mature, muscle ECM.

An attempt was made to tune the mechanical stiffness of the PEM by replacing increasing volume fractions of CNWs with PSS, but this was unsuccessful owing to the cytotoxicity of the latter polymer in multiple (but not single) layers.

The response of bone marrow stem cells to the substrates showed that MSCs preferred to avoid the CNWs, thus showing contact guidance with 1 bilayer but not 12 bilayer substrates. When cultured on substrates as produced and pre-conditioned by C2C12 cells, MSCs could undergo myogenic differentiation in a manner that was promising, but not definitive.
8  FUTURE WORK

Tune the surface properties of CNWs by for instance, extracting samples using
different acids to create different terminating functional groups in order to
optimise the surface chemistry and the response of target cells to CNW-based
substrates. In conjunction with the chemical modification, it would be very
desirable to quantify the protein adsorption onto the CNW substrates.

Develop complementary methods of monitoring cell differentiation, such as
Western Blotting of expressed proteins and RT-PCR of differentiating cells,
particularly stem cells.

Measure produced films in situ for mechanical properties, e.g. using acoustic
scanning microscopy for films hydrated in cell culture media, both with and
without cells.

Produce free-standing films in order to allow the creation of 3D scaffolds that
can serve as a tissue engineering scaffold.
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9.1.1 MAGNIFICATION CONVERSION FACTORS

Pixel to micron conversion ratios for each magnification used on the Leica SP5 confocal microscope and the Nikon Eclispe 50i fluorescence microscope are to be found in Table 9-1: Conversion ratio for Leica confocal microscope and Table 9-2: Conversion ratio of Nikon fluorescence microscope. These are taken from the instrument calibration files.

**Table 9-1: Conversion ratio for Leica confocal microscope**

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<th>Pixels Measured (px)</th>
<th>microns/pixel (µm/px)</th>
<th>Ratio as a proportion of 10x magnification</th>
<th>Effective magnification compared to 10x magnification</th>
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**Table 9-2: Conversion ratio of Nikon fluorescence microscope**

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</tbody>
</table>
9.1.2 VALIDATION OF CELLPROFILER ORIENTATION PIPELINE

METHOD

In order to validate the ability of the programme to correctly identify objects, measure their dimensions and orientation, test images were produced in Inkscape, a freeware vector drawing programme (software version 0.91 r13725, Inkscape.org). The sizes chosen for the circles are similar to those seen in microscopy at either low magnification images or with small cells. The shapes produced are summarised in Table 9-3.

Images were saved as black and white .png files. Portable network graphics (PNG) files are compressed via a lossless data algorithm.

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Shape</th>
<th>Size (pixels)</th>
<th>Aspect Ratio</th>
<th>Orientation (long axis with respect to x-axis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orientation</td>
<td>Circles</td>
<td>10, 20, 40</td>
<td>1:1</td>
<td>None</td>
</tr>
<tr>
<td>Orientation</td>
<td>Ellipses</td>
<td>10, 20, 40</td>
<td>1:1.01</td>
<td>90°</td>
</tr>
<tr>
<td>Orientation</td>
<td>Ellipses</td>
<td>10, 20, 40</td>
<td>1:1.02</td>
<td>90°</td>
</tr>
</tbody>
</table>

Images were run in CellProfiler version 2.1.1 (rev 6c2d896) using the modules ‘IdentifyPrimaryObjects’ to separate the circles from the background and the ‘MeasureObjectSizeShape’ to make measurements on the identified objects. The data was compiled in GraphPad and the alignment of the identified objects plotted as a histogram.

RESULT

127 circles were drawn for each image. All 127 objects were successfully segmented. For circles, CellProfiler was unable to determine a preferred direction, albeit with a preference for selecting 0° (parallel to the x-axis). With a 1% or 2% difference in aspect ratio, a preferred direction (90°) is seen. The histograms can be seen in Figure 9-1.
The mean measured aspect ratios for the circles, 1:1.01 aspect ratio ellipse and 1:1.02 aspect ratio ellipses were $1.016 \pm 0.054$, $1.02 \pm 0.05$ and $1.04 \pm 0.099$. For nearly circular objects, the accuracy of the aspect ratio is low.

To further investigate the aspect ratio, squares were used as a second shape. Squares with dimensions of 2x2 pixels, 5x5 pixels, 10x10 pixels and 20x20 pixels were drawn in Inkscape and analysed by CellProfiler. Figure 9-2 shows the differences between the shapes as drawn and the shapes identified. The intensity of the edges of shapes are averaged with the background and the programme selects the boundary according to the gradient of the change of intensity from object to background. As a result, the very small shapes, only 2 pixels across were not identified. Additionally, sharp features, such as the square vertices were also rounded off.

Figure 9-1: Directional histograms of circles and near-circular objects identified in CellProfiler.
Figure 9-2: Image of squares as drawn in Inkscape (A) and after segmentation by CellProfiler (B). The ellipses highlight the location of the smallest squares, which are not identified by CellProfiler. C is a closer view of (B) showing the rounding-off of the vertices of the squares.
There is more than one method of measuring the orientation of a square -- parallel to either edge and along its diagonal. This ambiguity is reflected in the directional histogram in Figure 9-3. There are local maxima of direction at 0°, 90° and ±45°. However, the rounding off of the edges creates a range of errors and there are additional maxima at \( \sim \pm 20° \) and \( \pm 70° \). Consistent with the object rounding off error, the mean aspect ratio of the squares was 1.044 ± 0.045.

Figure 9-3: Histogram of orientation of squares.
Table 9-4 looks at the impact of the segmentation algorithm on the measured surface area. The mean measured area is underestimated for all squares, consistent with the rounding off of edges. However, the relative error in area measurement, defined as the difference between the measured and theoretical areas as a percentage of the theoretical area, falls from 12.6% for 5x5 pixel squares to 0.41% for 20x20 pixel squares.

Table 9-4: Theoretical versus measured object areas in validation images tested using CellProfiler.

<table>
<thead>
<tr>
<th>Square size (pixels x pixels)</th>
<th>Area (pixels²)</th>
<th>Mean measured area (pixels²)</th>
<th>Standard deviation (pixels²)</th>
<th>Relative standard deviation (%)</th>
<th>Relative error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x2</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5x5</td>
<td>25</td>
<td>21.9</td>
<td>1.25</td>
<td>5.70</td>
<td>12.6</td>
</tr>
<tr>
<td>10x10</td>
<td>100</td>
<td>97.2</td>
<td>1.52</td>
<td>1.57</td>
<td>2.75</td>
</tr>
<tr>
<td>20x20</td>
<td>400</td>
<td>398.4</td>
<td>5.08</td>
<td>1.27</td>
<td>0.41</td>
</tr>
</tbody>
</table>

The images tested represent challenging objects to accurately segment, being nearly circular in the case of the ellipses, very small with sharp corners in the case of the squares. Testing these shapes showed that the algorithm used is sensitive to small relative changes in dimension, being able to determine a preferred direction for ellipses with a 1% difference in long and short axes. It is less accurate in measuring absolute dimensions leading to errors in determining the surface area and small changes in aspect ratio. For small objects, the relative error in these measurements is significant. Objects with a diameter of 10 pixels represent the minimum default size suggested in the object identification pipeline, which as can be seen from Table 9-4, represent objects that can be measured with an accuracy that is acceptable for the application of
The software pipeline used to test the size and shape of the objects is in Table 9-5 and the actual pipeline used to generate cell orientation and aspect ratio is to be found in Table 9-6.

**Table 9-5: Pipeline used to segment and measure test shapes.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Module Name</th>
<th>Key Parameters</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IdentifyPrimaryObjects</td>
<td>Discard objects touching border of image. Threshold strategy: Automatic</td>
<td>Takes a greyscale or binary image and identifies objects</td>
</tr>
<tr>
<td>2</td>
<td>MeasureObjectSizeShape</td>
<td>Measures pixels within an identified object to determine area, perimeter, lengths and orientation of long axis along maximum length of object.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>DisplayHistogram</td>
<td>For display purposes only, allows</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>ExportToSpreadsheet</td>
<td>Saves data</td>
<td></td>
</tr>
<tr>
<td>Step</td>
<td>Module Name</td>
<td>Key Parameters</td>
<td>Description</td>
</tr>
<tr>
<td>------</td>
<td>--------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>ColorToGray</td>
<td>Splits RGB channel into three and converts each to greyscale</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>IdentifyPrimaryObjects</td>
<td>Uses blue channel (nuclei) to identify cells. Discard objects touching border of image. Threshold strategy: Automatic</td>
<td>Each cell is assumed to have only one nucleus.</td>
</tr>
<tr>
<td>3</td>
<td>IdentifySecondaryObjects</td>
<td>Takes red channel (cell membrane). Uses identified nuclei as 'seeds' around which to construct cells. The Watershed-Gradient algorithm is used to assign pixels to the cells</td>
<td>The watershed algorithm looks at the intensity gradient of the object to separate it from background.</td>
</tr>
<tr>
<td>4</td>
<td>MeasureObjectSizeShape</td>
<td>Measures pixels within an identified object to determine area, perimeter, lengths and orientation of long axis along maximum length of object.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>OverlayOutlines</td>
<td>Overlays identified nuclei and cell outlines onto copies of the original image</td>
<td>Saving these images allows the quality of fit to be assessed</td>
</tr>
<tr>
<td>6</td>
<td>ExportToSpreadsheet</td>
<td>Saves data</td>
<td></td>
</tr>
</tbody>
</table>