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Biomimetic Nano-Biomaterials for Bone Repair and Regeneration

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

Ayesha Azeem

Research Supervisor: Dr Dimitrios I. Zeugolis

February 2017

Regenerative, Modular & Developmental Engineering Laboratory
(REMODEL)

Science Foundation Ireland (SFI) Centre for Research in Medical Devices
(CÚRAM)

National University of Ireland Galway (NUI Galway)
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Plagiarism statement

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

Ayesha Azeem
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Lastly, but never last I would like to thank my dear husband Toqeer, you have always supported and encouraged me with the final stages of this thesis. Let’s look forward to finishing this and the arrival of our little one!
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<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
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<td>APS</td>
<td>Ammonium persulfate</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CaP</td>
<td>Calcium phosphate</td>
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<td>CS</td>
<td>Chondroitin sulphate</td>
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<td>CTRL</td>
<td>Control</td>
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<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
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<tr>
<td>DHT</td>
<td>Dehydrothermal</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<td>DPPA</td>
<td>Diphenylphosphoryl azide</td>
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<td>DS</td>
<td>Dermatan sulphate</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
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<td>EDTA</td>
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<td>FBS</td>
<td>Foetal bovine serum</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GTA</td>
<td>Glutaraldehyde</td>
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<td>Hydroxyapatite</td>
</tr>
<tr>
<td>HAS</td>
<td>Hyaluronan synthases</td>
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<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<td>IMS</td>
<td>Industrial methylated spirit</td>
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<td>Ingenuity pathway analysis</td>
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<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
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<td>Nano-imprint lithography</td>
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<td>OD</td>
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<td>Phosphate buffered saline</td>
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<td>Poly(glycolide-co-lactide)</td>
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<td>Poly(methyl methacrylate)</td>
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<td>RPM</td>
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<td>Scanning electron microscope</td>
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<td>TCaP</td>
<td>Tricalcium phosphate</td>
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<td>Full Form</td>
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<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
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<td>TLDA</td>
<td>TaqMan® Low Density Array</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Abstract

Currently, over 1.7 billion people suffer from a musculoskeletal disorder; over 200 million operations take place annually; and in excess of 1 million new clinical cases are reported per year. Further, the annual healthcare expenditure exceeds US$ 950 billion Orthopaedic pathologies, such as osteoarthritis, tendinopathies, fractures, osteoporosis, intervertebral disc degeneration, low back pain, tumours and congenital deformities are among the largest group of debilitating diseases today. Therefore, the need for innovative regenerative strategies that are free from dysfunctional tissue healing remains ever relevant. Despite significant advances in the field, current biomaterial-based products for musculoskeletal repair and regeneration do not mimic the complex hierarchical structure and osteoinductive signals of the host native bone microenvironment. Herein, it is hypothesised that nano-textured biomaterials functionalised with carbohydrates moieties, by mimicking the native bone hierarchy and composition, would control osteoblast response \textit{in vitro} and promote functional bone regeneration \textit{in vivo}. Starting with nano-imprinting, this work revealed that topographical features in the middle nano-range (~306 nm) and low micron-range (~2,046 nm) induce cellular and matrix alignment and upregulation of osteogenic markers \textit{in vitro}. However, these topographical features did not induce directional cell growth and neotissue formation \textit{in vivo}. These data suggest that topographical features in two-dimensional scaffold (surface) can be utilised to maintain cell phenotype during \textit{in vitro} cell expansion, enhancing that way clinical translation and commercialisation of cell-based therapies.
With respect to electro-spinning, as low as 0.01 % functionalisation of electro-spun scaffolds with non-sulphated polysaccharides not only did not compromise the mechanical properties of the produced scaffolds, but also didn’t suppress osteoblast adhesion, growth, proliferation and early *in vitro* osteogenesis. These data suggest that functionalisation of three-dimensional implantable devices with carbohydrates moieties (in this case non-sulphated polysaccharides) could promote functional bone repair and regeneration.
Chapter One

Literature Review

Parts of this chapter have been published in:


1.1. Introduction

Musculoskeletal tissue diseases and injuries are the major causes of chronic morbidity in modern society. Indeed, musculoskeletal injuries and pathologies, such as osteoarthritis, tendinopathies, fractures, osteoporosis, intervertebral disc degeneration, low back pain, tumours and congenital deformities are among the largest group of debilitating diseases today. In fact, currently, over 1.7 billion people suffer from a musculoskeletal disorder; over 200 million operations take place annually; and in excess of 1 million new clinical cases are reported per year. Further, the annual healthcare expenditure exceeds US$ 950 billion [1-5]. Therefore, the need for innovative regenerative strategies that are free from dysfunctional tissue healing remains ever relevant.

Regenerative therapies to recapitulate native tissue function are based on an appropriate scaffold material alone or in combination with therapeutic / bioactive molecules and / or a potent cell population. The scaffold material, not only provides mechanical integrity, but also facilitates cell growth and ultimately neotissue formation. The first generation of scaffolds aimed to provide structural support, by imitating the mechanical properties of the tissue to be replaced and to allow nutrient / waste transfer, by creating a porous architecture. Whereas this fundamental design tenet has been successful in numerous proof-of-concept studies, it is now clear from an accumulation of failure paradigms that this simplistic approach does not recapitulate the intricate hierarchical organisation of physical structures enshrined within the native extracellular matrix (ECM) environments. To this end, numerous bottom-up and top-down nano- to micro-fabrication biomimetic technologies have been developed, not only to control cellular functions \textit{in vitro}, but also to expand and augment the functionality of
biomaterials in engineered tissue constructs [6-13]. Specifically, such technologies aim to incorporate a hierarchical element, often in the form of controlled porosity or aligned structures, to imitate the native tissue spatial architecture.

As well as mimicking the presence of biophysical cues of the native ECM environment, the incorporation of biochemical cues in a scaffold has been shown as an imperative factor to regenerate musculoskeletal tissues [14, 15]. These cues can be presented in many different forms, including growth factors, genes and drugs [15]. These signals upregulate and/or downregulate as required functions of the host and/or transplanted cells to give them a specific role in the reparative process [16]. Despite significant advances in the field, current tissue engineering products do not mimic the complex hierarchical structure and osteoinductive signals of the host native environment [17]. Thus, tissue engineering scaffolds have to be designed that will guide regeneration, up-regulate local cellular function, degrade in a controlled manner and provide mechanical strength until the host tissue can regain its normal function.

Herein, we discuss hierarchical nano- and micro-fabrication technologies and their impact in musculoskeletal tissue engineering. Further, we are discussing functionalisation strategies specific to bone tissue engineering.
1.2. Self-assembly

Molecular self-assembly, an inherent and ubiquitous biological process, is identified across the breadth of biology with length scales ranging from DNA base pairing, to microtubule fabrication, and up to macro-scale tissue morphogenesis [18, 19]. Through self-assembly, the extracellular environment is synthesised and organised in hierarchical motifs, existing in a dynamic equilibrium, that conform the structural and chemical milieu needed to promote a range of physiological functions, including cell morphology, proliferation, attachment, migration, fate and tissue morphogenesis [20-22]. This exact sophistication and success of nature, self-assembled scaffold fabrication technologies aim to imitate to produce hierarchical three-dimensional tissue equivalents. Structural conformable molecules with chemical complementarity spontaneously self-assemble, under appropriate conditions of temperature, pH, anionic strength, into supramolecular architectures that are hold together by reversible, non-covalent bonds [23-25]. Although individually these bonds are inherently weak and readily reversible, their collective interaction yields strong and stable complexes [26]. Largely attributed to the mild processing conditions and conformability, self-assembled natural- [27-29], synthetic- [30-32] or peptide- [33-35] derived hydrogels and micro-gels have the ability to capture and deliver live cells, whilst controlling their fate. They also immobilise and control the release of highly potent biological / bioactive molecules, whilst preserving their molecular conformation and bioactivity. Further, their injectability / minimal invasive nature affords localisation of their at the site of injury, thus minimising dose requirements. However, customarily used hydrogel / micro-gel fabrication technologies give rise to randomly orientated sub-fibrillar
architectures, imposing the need for the development of more refined systems that would closely imitate the hierarchy of native tissues. Although synthetic- [36, 37] and peptide- [38-42] based aligned hydrogels have been reported, to-date, by far, most of the work is focused in developing aligned collagen hydrogels.

Extruded collagen nano-textured micro-fibres (Figure 1.1), produced through extrusion of a collagen solution into a series of neutral phosphate buffers and cross-linking / functionalisation solutions maintained at 37 °C, represents one of the most striking attempts at recapitulating the hierarchical architectural organisation of native tendon and ligaments [43-61]. The cross-linking solution allows production of fibres with mechanical integrity similar to native tissues, whilst the functionalisation solution adds another element of functionality. The extrusion of the collagen solution through fine laboratory tubing facilitates production of fibres with high level of crystallinity and structural alignment at sub-fibrillar level, as revealed by ultra-structural analysis, responsible for undulations and crevices running parallel to the longitudinal fibre axis. In vitro studies have revealed that such structural characteristics are responsible for bidirectional cell migration along the fibre axis. For example, when these fibres were used for tendon prosthesis in small animal models, they rapidly resorbed and allowed formation of aligned connective tissue, similar to the one resulted from the implantation of an autologous tendon graft and almost identical to normal tendon, avoiding problems associated with long-term foreign body implantation or granuloma that could interfere with the smooth gliding of tendon repair [62-64]. Similarly, when the scaffold was used for anterior cruciate ligament replacement in small animal preclinical models, it was completely
remodelled into host tissue by 12 to 20 weeks post implantation and was replaced by an organised, crimped neo-ligament tissue [65, 66]. Using a sheep patellar tendon model, carbodiimide cross-linked fibres exhibited significantly lower mechanical properties to native tendon, whilst histological analysis demonstrated integration with the native tendon, partially resorption and tissue ingrowth by month 6 post-operative [67].
**Figure 1.1:** Extruding collagen nano-textured micro-fibres are comprised of aligned fibrils exhibiting the characteristics D periodicity of collagen (a). This sub-fibrillar structure is responsible for undulations and crevices running parallel to the fibres axis (b). These surface characteristics facilitate bidirectional cell growth (c). Using transglutaminase such fibres can be functionalised to allow sustained and localised delivery of therapeutics (d).
Isoelectric focusing (Figure 1.2) constitutes an alternative strategy to create bidirectionally aligned collagen hydrogels with sufficient mechanical integrity and similar biomimicry to native tissues [68, 69]. In this case, a collagen solution is subjected to electric stimulation. The applied current induces the collagen monomers to migrate towards and focus at their iso-electric point, where the global charge is neutral. Subsequent neutralisation and cross-linking / functionalisation results in the formation of a stable micro-hydrogel that is composed of interconnected and aligned along the uniaxial plane sub-micron-fibrils. This technology has been used extensively to recapitulate the native milieu of tendon tissues [70-74], with in vitro data even demonstrating that such substrates can differentiate bone marrow stem cells towards tenogenic lineage, making the addition of biological means in the culture media (e.g. bone morphogenic protein 12, BMP-12) superfluous [75]. In the only available in vivo study to-date, braided iso-electric focused fibres were implanted in a rabbit patellar tendon model; four months post-operative they were associated with a low-grade granulomatous inflammation, whilst 8 months post-operative, they were mostly absorbed [76].
Figure 1.2: Isoelectric focused collagen hydrogels are characterised by anisotropically orientated collagen fibrils (a). This sub-fibrillar structure is responsible for the biaxial surface architecture of these hydrogels (b). This anisotropic surface topography facilitates bidirectional elongation of various cells, including embryonic rat dorsal root ganglia (c). Using micro-spheres with a fluorescent peptide, we provide evidence that such substrates can be functionalised to enhance further their molecular activity (d).
Magnetic fields have also been incorporated into the self-assembly process of collagen as means to produce bidirectionally-aligned matrices [77-79]. Briefly, a collagen solution is neutralised and subsequently exposed to a high strength magnetic field (>5 T) at controlled temperature (37 °C), followed by cross-linking. Such systems have been assessed in bone [80] and cartilage [81] tissue engineering, when combined with calcium phosphate and hyaluronic acid, respectively. Although in vivo studies have yet to be reported in musculoskeletal tissue engineering using magnetically aligned hydrogels, preliminary in vivo data in a mice sciatic nerve model using a nerve conduit loaded with magnetically aligned collagen hydrogels are promising [82]. The addition of magnetic beads into a collagen solution has also been investigated as means to produce aligned thin and thick hydrogels [83, 84]. Alternative, yet under-investigated, methods to produce aligned collagen hydrogels include: molecular crowding [85], dynamic shear [86, 87], mica surfaces [88-90], mechanical loading [91-93] and centrifugation, followed by self-assembly at 37 °C and cyclic stretching [94, 95].
1.3. Freeze-drying

Freeze-drying or lyophilisation or cryo-desiccation is an inexpensive and highly reproducible method to produce reticulated porous scaffolds out of natural, synthetic or combination of polymers thereof by freezing and subsequent subliming of the polymeric suspension. Of significant importance during the fabrication process is the pore size, pore shape and pore volume fraction to allow cell attachment, growth and migration; nutrient / waste transfer; matrix deposition; and tissue growth [96-98]. Further, a uniform architecture is critical for mechanical resilience. Although freeze-drying appears to be a rather simple technique, every step of the process should be closely monitored to ensure reproducibility. For example, the solute, the solute concentration, the solute molecular weight, the pH of the solution, the freezing temperature / time and the freezing rate are critical for pore structure and morphology control [99-104]. Controlling the thermal events within the solution during the solidification, nucleation and annealing processes have also been shown to offer control over the scaffold’s architecture [105]. The cross-linker concentration has also been shown to affect pore size and porosity [106]. The pre-freezing temperature has also been shown to influence pore size and distribution; interconnectivity; and mechanical properties [107]. The pressure and the depressurisation processes has also been shown to affect the pore size and the interconnectivity of membranes prepared by foaming in supercritical carbon dioxide [108]. Using moulds of various configurations, differences in heat transfer rates were achieved during the freeze-drying process that resulted in scaffolds with complex pore orientations, and anisotropic pore size and alignment [109]. Using moulds of higher order shapes, the production of macro-structures, such as multichannel
conduits [110-112] or open-end capsules, tubules and sheets [104] has been reported. Freeze casting has also been used as means to develop metal [113] and ceramic [114] scaffolds with aligned pores and controlled porosity. Further, utilisation of embossing ice particulates of variable size as a template and controlling the freezing temperature resulted in a funnel-like sponge with controlled pore structure [115-117]. Spinning of the solution in a cylindrical copper mould around its longitudinal axis at variable angular velocities and for different times, followed by immediate freezing in liquid nitrogen, for rapid solidification, and subsequent drying resulted in a tubular scaffold with radially aligned pore structures [118]. Particle / porogen leaching may also be used alone [119-121] or in combination with moulds for the development of hierarchical scaffolds of controlled porosity, pore size, orientation and interconnectivity [122]. Directional freezing can also be used to produce two- and three-dimensional structures with controlled architecture [123].

Given the hierarchical and porous structure of bone, freeze dried materials have been extensively used in bone tissue engineering. Freeze-dried collagen sponges with pore diameter between 85 $\mu$m and 325 $\mu$m have been demonstrated to enhance osteoblast migration and proliferation, whilst cell migration was reduced by smaller pore size [124-126]. When collagen sponges with 380 $\mu$m pore diameter and 20% porosity were combined with calcium phosphate (Figure 1.3), in vitro data demonstrated increased osteoblast proliferation and differentiation that resulted in increased alkaline phosphatase, osteocalcin and collagen type I gene expression [110], whilst further functionalisation with VEGF165 plasmid resulted in significantly increased repaired bone volume in vivo [127]. Scaffolds with smaller pore sizes (hydroxyapatite / chitosan-gelatin
with pore sizes ranging from 300 μm to 500 [128] and hydroxyapatite particles / collagen with average pore size of 120 μm [129]) have also been shown to stimulate natural bone tissue formation.
Figure 1.3: Porous collagen type I / calcium phosphate scaffolds (a) not only promote cell infiltration and spreading \textit{in vitro} (b), but also when implanted in a murine intra-femoral model are surrounded by compact bone and degraded scaffolds are replaced by new bone (c) [127].
For cartilage tissue engineering, it has been shown that large pore sizes (in the range of 300 μm) stimulate proliferation, chondrogenic gene expression and cartilage-like matrix deposition of mesenchymal stem cells differentiated towards chondrogenic lineage [130, 131]. However, scaffolds with or without bone marrow mesenchymal stem cells and with broad pore sizes (up to 433 μm in diameter) have also been shown to enhance repair of osteochondral defects in porcine models [132, 133]. Stratification has also been recently incorporated into the scaffold design as means to produce implantable devices with different degrees of pore size range and porosities and to simulate the complex zonal organisation of mammalian skeletal tissues (e.g. osteochondral junction and ligament / bone interface) [134, 135]. For example, a triphasic (Phase A for ligament formation, Phase B for the interface and Phase C for the bone region) scaffold has been shown to support cellular interactions, tissue infiltration and abundant matrix production over 8 weeks in an athymic rat model [136].

In tendon tissue engineering, where the field recognises that alignment is of paramount importance, collagen-glycosaminoglycans (GAGs) sponges with aligned pores increased cell attachment, collagen synthesis and maintained tenogenic phenotype [137]. The addition of growth factors in the three-dimensional conformation of the scaffold, as opposed to media supplementation, further increased bioactivity, as evidenced by higher cell recruitment, cell proliferation, collagen synthesis and gene stability [138, 139].
1.4. Additive manufacturing

Additive manufacturing (AM), layered manufacturing, solid freeform fabrication, rapid prototyping or 3D printing are terms used interchangeably to describe a class of scaffold fabrication technology with growing appeal in musculoskeletal tissue engineering. The distinctive characteristic of this method is that it can create three-dimensional objects via layer-upon-layer direct deposition of material substrates based on data received from a computer aided design system. The key strength of AM is its ability to produce complex implantable devices of highly precise geometry, architecture and reproducibility to closely imitate the tissue to be replaced [140-142]. AM in the field of musculoskeletal engineering utilises thermoplastic polymers, ceramics, metal powders and composites of thereof to develop porous implantable devices (Figure 1.4), often printed via fused deposition modelling [143]. Poly ε caprolactone (PCL) and PCL-ceramics [e.g. calcium phosphate (CaP) and tricalcium phosphate (TCP)] scaffolds have been fabricated with pore geometric tinkering (e.g. square, hexagonal and honeycomb geometries), strut filament dimensions, porosities and pore size ranges to enhance in vitro and in vivo performance [144].
Figure 1.4: PCL-TCaP scaffolds, using fused deposition modelling, can be fabricated with a range of pore morphologies (a). These scaffolds promote bone marrow stem cell attachment and spreading along the axis of the struts (b). Implanted cell-scaffold constructs revealed mineralized tissue ingrowth as star shaped osteocytes with numerous canaliculated align along the long axis of the strut filament (c).
Indirect AM describes the fabrication of sacrificial templates or inverse moulds [145]. Such systems have become increasingly necessary to widen the scope of applicable substrates and to enable the production of scaffolds with intricate internal architectures, including 100% pore interconnectivity and predefined intricate internal architectures, using even proteins and polysaccharide substrates [146]. Such scaffolds, not only promoted chondrocyte proliferation and prolonged phenotype maintenance [147], but also formed a cartilage-like tissue in subcutaneous nude mice model [147].

A key recent advancement in AM technology is coupling the traditional scaffold fabrication method with photolithography (micro-stereo-lithography). This system combines projection lithography (photolithography) with traditional stereo-lithography to fabricate highly complex scaffolds or substrates with controlled biochemical, mechanical and internal micro-architectures. Using this platform, intricate 3D scaffolds have been designed and engineered to mimic the microarchitecture of tissues [148]. Variations in the mechanical properties of the scaffolds were obtained by tuning the structure and the polymer concentration [148]. 3D direct-write photolithography, 3D laser lithography and projection micro-stereo-lithography have also been recently employed to fabricate nano-scale, ultra-light, high-strength-to-weight and size-dependent strengthening scaffolds [149, 150]. Such materials were termed ‘mechanical meta-materials’ due to the fact that their specific mechanical properties are defined by their geometry, rather than their composition [150].

A substantial amount of research effort is currently focused on increasing the functionality of the produced 3D constructs. To this end, PCL-TCaP loaded with collagen or alginate gels, as carriers of bone marrow stem cells and BMP-2, have
shown tremendous potential to induce new bone formation [151-154]. An exciting alternative strategy to functionalise implantable devices using AM technologies is the use of water-based binders or bio-inks that are jet-extruded to print 2D and 3D nano- and micro- architectures [155]. Bioactive molecules and cells suspended in aqueous dextran, fibrin, collagen, gelatin, hyaluronan, and alginate have been successfully incorporated into these bio-inks [156-158]. This bio-printing technology offers opportunities for the generation of engineered 3D tissues with controlled cell behaviour and compositionally optimised scaffold components [159]. Furthermore, cells and multifunctional molecules can be incorporated and transferred into scaffolds with hierarchical organisation and with morphologies / geometries similar to native musculoskeletal tissues [157, 160]. Such systems have already been shown to control the fate of tendon/ligament [160-162], bone [160] and cartilage [163] cells and stem cells [164].
1.5. Electro-spinning

Electro-spinning is considered one of the most facile scaffold fabrication processes available today [165, 166]. Historically, electro-spinning was first used in textile and filtering industries [167]. The compositional diversity, coupled with the ability to precisely control mechanical properties and structural features, along with the functionalisation potential have been instrumental for the wide utilisation of electro-spinning in the biomedical field [168-176]. The successful alignment of electro-spun fibres represented a landmark development, as we were able for first time to create artificial hierarchical biomimicry (Figure 1.5).

Over the years, numerous studies have demonstrated that fibre alignment promotes formation of long lamellipodia extensions parallel to the direction of the fibres, resulting in directional cell orientation and migration, via the mechanism of contact guidance, reminiscent of native tissue environmental signalling [177-179].

Cellular orientation is particularly relevant to the biomechanics of musculoskeletal tissue, as it determines the pattern of ECM deposition [180], which is an essential factor in the functionality of bone, tendon, ligament and cartilage [181]. Specifically to bone tissue engineering, fibre alignment and consequent cellular alignment, with or without further functionalisation, have been shown to regulate cell adhesion and migration, to promote osteogenic phenotype, to differentiate stem cells towards osteogenic lineage and to enhance mineralisation and osteogenesis in vitro and in vivo [182-189].
Figure 1.5: Using a rotating collector, electro-spun fibres can be produced in bidirectional fashion (a). Continuous extrusion can produce 3D implantable devices (b). Using a collector with predefined shape, 3D implantable devices can be produced with specific architecture (c). Such materials can be further functionalised either with particles (d) or with chemical / biological (e) linking systems (transglutaminase in this case). Bidirectionally aligned electro-spun fibres facilitate parallel to the fibre axis cell attachment and elongation (f).
The fibre alignment associated with electro-spun fibres closely resembles the orientation and architecture of collagen fibrils present in native tendon tissue. Such anisotropic constructs promote elongated physiological cell morphology, phenotype maintenance of tendon-derived cells, differentiation of other cell types towards tenogenic lineage and parallel to the fibre axis neotissue formation in the presence or absence of functional molecules and/or cells [190-196]. The porous 3D nature of electro-spun scaffolds, with or without functional molecules and/or cells, also provides an ideal environment for chondrogenic phenotype maintenance, chondrogenic differentiation of stem cells and neotissue formation \textit{in vivo} in both cartilage and osteochondral defects [197-202].

Besides fibre orientation, fibre size has been demonstrated to be an important variable in maintaining cell phenotype and function. In cartilage tissue engineering, for example, nano-scale fibres, as opposed to micro-scale fibres, have been shown to maintain chondrogenic phenotype [203, 204]. Likewise, scaffold fibre diameter has been reported to be crucial in tenogenic phenotype maintenance and differentiation of bone marrow stem cells towards tenogenic lineage, even to greater extent than alignment, with fibre diameter greater than 2 \( \mu m \) to be more suitable for \textit{in vitro} development of tendon/ligament tissue [205].

To enhance the functionalisation potential of electro-spun scaffolds blending [206, 207], chemical (polydopamine) [208] or biological (transglutaminase) [61] immobilisation, plasma treatment [209], hydrogels [210-212] and nano- / micro-particles [213-215] have been incorporated within the 3D scaffold. An alternative strategy has been based on the use of emulsion or multi-axial (co-axial or tri-axial) nozzle to produce multicomponent core-sheath fibres with
multiple, often immiscible, components. Such systems have been used extensively for bone [216-218], cartilage [219] and tendon [220] repair with very positive results to-date, in both in vitro and in vivo setting.

Electro-spinning has also been successfully combined with other scaffold fabrication technologies with optimal outputs for various clinical targets. Electro-spinning, for example, combined with freeze-dried materials and bone marrow stem cells enhanced osteochondral regeneration with improved compressive moduli in a rabbit model [221]. Bimodal and multiphasic scaffolds can be fabricated using electro-spinning and additive manufacturing [222, 223]. The resultant multi-hierarchical scaffold contained large size pores, essential for cell and mass transportation, whilst the fibrous component provided suitable structures for cell attachment. The modular system allowed development of implantable devices suitable for interface (e.g. bone-ligament [224] and muscle-tendon [225]) tissue engineering. Electro-spinning has also been extensively combined with hydrogels to enable localisation and sustained delivery of cells and therapeutics [226-228].
1.6. Imprinting

Given that the ECM provides cells with nano- to micro-scale biophysical signals necessary for maintaining and regulating vital cellular functions, an increasing number of studies utilise imprinted patterns onto engineered scaffolds (Figure 1.6) in an attempt to understand how topographical cues influence cell behaviour and consequently rationalise the design of implantable devices. Molecular imprinting [229-231], photo-induced imprinting [232], nano-imprint lithography (NIL) [233, 234], electron beam lithography [235, 236] and soft lithography [237] are the most widely used techniques to imprint patterns on substrates. Molecular imprinting refers to the topographical patterning of substrate surfaces based on sacrificial templates or ‘stamps’ designed to introduce precise structural patterns that will act as complementary binding sites and thus impact the ability to recognise specific receptors on the substrate. The substrates are usually polymers and the templates are either molecules or synthetic compounds that can be readily removed by washing or extraction. Conversely, photo-induced imprinting involves the exposure of selected areas of the substrate surface to laser light reactions to convert the monomer units into dimers and the non-exposed monomeric portions are selectively removed to produce desirable topographical patterns [238]. Various nano- and micro-scale topographical outlines, including grooves, lines, holes and pillars have been imprinted at sizes ranging from a few nano-meters to several hundreds of micro-meters [238]. Anisotropic arrays of nano- / micro-grooves, ridges and other topographical patterns have been imprinted on scaffolds as potent tools to maintain phenotype of primary cells, such as tenocytes and osteocytes [236, 239, 240]. Similarly, the use of imprinting of distinct patterns has found potential application in the
differentiation of stem cells towards musculoskeletal and other connective tissue lineages, with or without chemically inductive media supplements [241-244]. Specifically to musculoskeletal tissue engineering, imprinted interfaces have been used extensively to either maintain native phenotype or to direct cell phenotype towards specific lineage. Indeed, numerous studies have already shown that topographical features in the nano- and micro- scale can maintain tissue-like phenotype and differentiate stem cells towards osteogenic [233, 236, 245] or tenogenic [246, 247] lineage. Studies have also shown that lost phenotype, during \textit{in vitro} expansion, of tendon-derived cells can be restored using imprinted substrates that imitate the anisotropy of tendon tissues [248]. However, due to practical reasons, only the effect of a few topographies can be assesses simultaneously on cell function. Thus, a significant progress in the field was the development of a chip that can assess numerous topographies concurrently [242]. Given though the literally infinite number of potential patterns that can be imprinted, a rather reverse engineering method was used recently to maintain tenogenic phenotype of tendon-derived cells. Specifically, tendon slices were used as stamps to imprint true tendon topography on polydimethylsiloxane (PDMS) scaffolds, thereby replicating the natural ECM topography of native tendon, resulting in tenogenic phenotype maintenance of tendon-derived cells and tenogenic differentiation of stem cells [240].
Figure 1.6: Thermal imprinting techniques are frequently employed for the generation of topographical features at the nano- to micro- scale and subsequently assess cellular behaviour at the bio-interface. Anisotropic grooves (a) and pits (b) are frequently utilised as a means to study cellular attachment (c) and to control cell phenotype \textit{in vitro} (d). Pits are frequently used for sequestering individual cells to study single-cell processes, modulate juxtacrine signalling or mediate differential function (e). Anisotropic grooves are often used to study dimensionality induced cellular morphology, polarity and adhesion.
1.7. Bone dimensional hierarchy

Bone is one of the most complex and highly hierarchical composite materials found in a biological system (Figure 1.7). It is composed primarily of collagenous proteins and minerals, mainly hydroxyapatite (HAp) [249, 250]. Due to its complex structure, bone is constantly remodelling and changing shape to adapt to the daily forces placed upon it. The mechanical properties, such as strength, toughness and stiffness, are influenced by bone’s matrix nano- and micro-structures [251-254]. Its adaptation to mechanical environment is due to alterations in its shape and internal structure comprising of changes in its mechanical properties [254]. The structure of bone is a cascaded arrangement of building blocks at defined length scales. These building blocks form the hierarchical structures that control the above-mentioned properties [251]. Bone tissue can either be cortical (compact) or trabecular (spongy). Cortical bone is the dense bone that is found at the outer surfaces of long bones. The levels of bone hierarchical structural organisation consist of: (1) the macro-structure of cortical and cancellous bone; (2) the micro-structure (7 to 500 μm) of haversian system, osteons and single trabeculae; (3) the sub-microstructure (1 to 10 μm) of lamellae, collagenous proteins and minerals; the (4) nano- and the (5) sub-nano structure of various proteins and minerals [255, 256].
Figure 1.7: The hierarchical structure of bone illustrating the seven levels of hierarchy [257].
Although, this hierarchical structure may be irregular, it is highly organised in orientation of the components making the bone composition heterogeneous and anisotropic. In doing so, the macro-scale organisation of the osteons, osteoids and haversian canals provide the long bones with their mechanical anisotropy. The micro-scale porosity of bone is necessary for cell migration and vascularisation, whereas the nano- and sub-nano scale structures act as cell and mineral binding motifs [258, 259].

The natural ECM of bone is a complex system, composed of an intricate interweaving of protein fibres, such as collagen type I, which forms fibrils with a banding width of 68 nm, with a 3-5 nm banding depth and 35 nm inter-fibrillar spacing [260-262]. The ECM provides the cells with continuous nano-topographical cues and nano-scale adhesive proteins, such as fibronectin and laminin. The presence of these nano-features have been shown to directly modulate the expression and turn-over of transmembrane receptors [263] and to regulate vital cellular functions, such as proliferation, morphology, migration and differentiation [239, 241, 264-266]. At the nano-scale level of bone hierarchy, each collagen fibre is composed of an array of mineralised collagen fibrils (100 nm diameter, ~5-10 μm length). Collagen molecules (300 nm long and 1.5 nm thick) are deposited by osteoblast into extracellular space and assemble into fibrils [267-269]. These fibrils bind to tiny HAp (~10-50 nm length) mineral crystals that assemble in the gap between the fibrils [249, 270]. Both the size and orientation of HAp crystals are controlled by the orientation of collagen fibres, this specific relationship between the mineralised crystals and collagen fibres (hence mineralised collagen fibres) is critical to the strength and resilience of bone. Studies have shown the nano-scale organisation of these
ECM signals is required to promote differentiation and bone formation \textit{in vivo} [259].
1.8. Advances of topographical features in bone repair

The intricate hierarchical structure of bone is responsible not only for its mechanical properties, but also for progenitor and primary bone cell survival and bone formation at the micro- and nano-scale. The topographical and adhesive features of the ECM proteins coordinate complex multicellular behaviours. Due to direct deposition of the ECM proteins and their binding to the cytoskeleton through cellular receptors, cells sense and respond to the matrix biochemical and biophysical cues by converting them into intracellular signalling, thus resulting in general phenotypic behaviour, gene expression and protein production over time [271-273]. The nano- and micro-scale architecture of collagen fibrils and fibres influence cell polarity, shape and promote migration along the collagen fibres by providing contact guidance cues [274-276]. These nano-scale features of the ECM structures influence cellular behaviour in native tissues, as the cells also contain nano-scale features, such as focal contacts, and fine processes, such as cilia and filopodia, that interact with these ECM structures.

Biomaterial-based approaches aim to mimic this natural topography of bone. It is hypothesised that bone forming cells will identify these bio-inspired artificial topographies as their own native extracellular microenvironment, promoting enhanced cellular integration and therefore improved tissue formation around the implant [261, 265, 277]. Recently, many studies have been performed that have shown that either stem cells or bone cells respond positively to topographical features that mimic the size scale of bone ECM (Table 1.1).
Table 1.1: Indicative examples of the influence of topographical features in bone repair.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Groove Depth (nm)</th>
<th>Line Width (nm)</th>
<th>Groove Width (nm)</th>
<th>Pitch (nm)</th>
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<td>Human mesenchymal stem cells</td>
<td>300</td>
<td>200</td>
<td>200</td>
<td>400, 1,400, 4,000</td>
<td>400 nm pitch improves osteogenic differentiation, up-regulates osteogenic markers and induces bone mineral formation</td>
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<td>Human mesenchymal stem cells</td>
<td>350</td>
<td>350</td>
<td>350</td>
<td>700</td>
<td>350 nm gratings induced lower expression of integrin subunits</td>
<td>[279]</td>
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<tr>
<td>Human mesenchymal stem cells</td>
<td>350</td>
<td>500</td>
<td>500</td>
<td>1,000</td>
<td>Topography induce changes in nuclei shape</td>
<td>[280]</td>
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<tr>
<td></td>
<td>Human osteoblasts</td>
<td>Rat bone marrow cells differentiated into osteoblasts</td>
<td>Rat osteoblasts</td>
<td>10 μm induced cellular alignment and reduce adhesion. 100 μm increased adhesion, cell spreading and upregulation of bone genes</td>
<td>Ridge to groove ratio controlled osteoblast motility</td>
<td>Osteoblasts were responsive to patterns down to 75 nm width and 33 nm depth. Mineral deposition was detected at 50 nm width and 17 nm depth</td>
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<td>1:1, 1:3, 3:1 (Ridge to groove)</td>
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<td>1:1, 1:3, 3:1 (Ridge to groove)</td>
<td>1:1, 1:3, 3:1 (Ridge to groove)</td>
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1.9. Biofunctionalisation of scaffolds for bone regeneration

In regeneration of bone, as well as the scaffold architecture, the presence of biomolecules is imperative for cells to synthesise new ECM. Biomolecules, such as chemotactic factors, growth factors and adhesive proteins, have been used extensively in bone regeneration as a means to promote osteogenesis [184, 284-286]. Glycans are a major class of macromolecules that regulate signals that control cell proliferation, cell differentiation, normal physiology and cell-cell and cell-matrix communications [287-290]. They are synthesised in the rough endoplasmic reticulum and in the Golgi apparatus, where most of the structural modifications occur. The multitude of variable glycan conformations is the result of two possible anomeric configurations of sugars (α / β), their various linkages and ring sizes and site-specific substitutions, such as acetylation, phosphorylation and sulphation [290, 291].

GAGs are a class of linear anionic polysaccharides consisting of repetitive disaccharide units that are either sulphated or non-sulphated. GAGs ineract with multiple ECM constituents, including proteins, growth factors, cytokines and enzymes [292, 293]. Thus far, four different families of GAGs present in the bone ECM: chondroitin sulphate (CS), heparin sulphate (HS), dermatan sulphate (DS) and hyaluronic acid (HA). HA is the only non-sulphated GAG in the body. HS and CS are attached to a protein core forming proteoglycans (PGs) [294]. Numerous studies have demonstrated their capacity to induce osteoblast proliferation and differentiation in vitro and bone formation in vivo, clearly stressing their essential role in bone regeneration [295, 296].
1.10. Hyaluronic acid (HA)

HA is the only non-sulphated GAG, interacting with numerous receptors and ECM proteins [297, 298]. HA synthesis occurs at the surface of the cytoplasmic membrane via the polymeric activity of hyaluronan synthases (HAS). There are three different forms of these transmembrane proteins present: HAS1, HAS2 and HAS3. Although, the specific roles of these enzymes are unclear, they are activated by various growth factors [e.g. transforming growth factor beta (TGF-β) and BMP] and produce HA with several levels of polymerisation and stability. The three isomers of these enzymes secrete high molecular weight (2 x 10^6 to 4 x 10^6 Da - HAS1 and HAS2) and low molecular weight (HAS3) chains of HA. The size and the molecular weight of HA molecules play essential roles in its structural and biological events [299, 300].

HA functions in cell communication, motility and morphogenesis by its interaction with hyaladherins [297, 301-303]. At biophysical level, HA performs a structural role when it is at its high molecular weight form. It is highly charged, osmotically active and its volume at a hydrated state fulfils the role space filling, lubricant and shock absorber [297, 304, 305]. From a biological perspective, high molecular weight HA functions as anti-inflammatory, immunosuppressive and anti-angiogenic agent. It performs these functions by inhibiting the entrance of pro-inflammatory molecules and cells to the tissue and by creating a voluminous pericellular matrix that prevents the action inflammatory molecules. High molecular weight HA binds to the cell surface receptors CD168 and CD44 and inactivates these receptors [306]. By inhibiting the activation of these receptors, HA impairs the production of inflammatory cytokines, macrophage activation and phagocytosis [307]. In pathophysiology, injuries and during
remodelling, high molecular weight HA is cleaved to form low molecular weight HA. Low molecular weight HA fragments are associated with the release of inflammatory cytokines, stimulation of angiogenesis and tissue remodelling [300, 308].

1.11. Ficoll
In comparison to HA, ficoll, a large, neutral polydisperse macromolecule has been associated with increased collagenous ECM deposition in cell culture. Similarly to HA it provides biochemical signals to the cells as present to them in their host tissue environment. It has been used previously as a macromolecular crowding polysucrose to crowd the extracellular environment to enhance the deposition of collagen and crosslinking in 2D cell culture system. These studies have been performed with adding a range of molecular weights (70-400 KD) of Ficoll in the growth media solution for the particular cell type. As it is a polar-hydrophilic molecule it creates large hydration shells around the mass of the molecule creating a large EVE. Previous studies using increasing concentrations of Ficoll 400 (0-15 mg/ml) have demonstrated collagen nucleation and fibre growth. Thus macromolecules are preferred compact conformations in the presence of a high concentration of macromolecules and suggest the significance of a crowded environment for the folding and stabilization of globular proteins.

When used as a functionalisation molecule, it can also enhance cellular attachment and proliferation.

Thus far there have no through studies performed to evaluate the influence Ficoll on bone regeneration. Therefore, in this research project by imitating the
biochemical features of the bone ECM with electrospun isotropic fibres functionalized with high molecular weight (> 1,000,000 Da) HA and FC will further enhance the *in vitro* osteogenesis potential of electro-spun poly(glycolide-co-lactide) (PLGA) fibres alone we aim to functionalise electrospun and nanoimprinted scaffolds with HA and Ficoll to evaluate osteoblast behaviour *in vitro* and bone formation *in vivo*.
1.12. Limitations of state-of-the-art

Currently various fabrication techniques and biofunctionalisation strategies are available for bone tissue engineering. Yet again, the optimal substrate dimensionality has yet to be identified, whilst the influence of non-sulphated polysaccharides on bone repair and regeneration is unclear.

1.13. Aim, hypotheses and objectives

The ultimate goal of this study is to create a bio-inspired scaffold that with its ideal dimensionality and composition will maintain osteoblast phenotype in vitro and direct neotissue formation in vivo.

Phase I hypothesis

There is an optimal anisotropic topography that will maintain osteoblast phenotype in vitro and direct neotissue formation in vivo.

Phase I objectives

- To fabricate anisotropically ordered substrates from nano- to micro- scale using imprinting technologies.
- To assess osteoblast response as a function of topography.
- To assess neotissue formation as a function of topography.
Phase II hypothesis

Multifactorial approaches based on topographical features and carbohydrate functionalisation will maintain osteoblast phenotype *in vitro*.

Phase II objectives

- To fabricate functionalised scaffolds with carbohydrate moieties.
- To assess their structural, physical and biological properties.

Note:

The initial aim of this project was to develop functionalised imprinted substrates. However, phase I clearly demonstrates that such substrates are not clinically relevant. For this reason, in phase II, electro-spun scaffolds were utilised.
1.14. References


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Chapter Two

The influence of anisotropic nano- to micro- topography on in vitro and in vivo osteogenesis

Parts of this chapter have been published in:


Chapter 2 - Imprinting

2.1. Introduction

The extracellular matrix (ECM) is a complex system of highly organised nano- to micron-scale components [1-3] responsible for the mechanical integrity of tissues [4-6]. The ECM also provides cells with continuous topographical cues that directly modulate the expression and turnover of trans-membrane receptors and regulate vital cellular functions, such as proliferation, morphology, migration and differentiation [7-10]. Thus, biomaterial-based strategies focus on developing tissue-specific implantable devices and *in vitro* tools with topographical features similar to tissue to be replaced [11-16]. It is hypothesised that host cells will recognise these features as inherent and therefore promote integration.

The field of cell-based therapies also requires substantial number of functional stem cells and permanently differentiated cells. However, *in vitro* expansion is often associated with phenotypic drift, cellular senescence and loss of cells’ therapeutic potential. Thus, significant research is on-going to develop functional *in vitro* microenvironments, using primarily topographical features [17-22]. It is hypothesised that by emulating the topographical characteristics of the tissue from which the cells were extracted from, cell phenotype and function will be maintained during their *in vitro* expansion, enhancing that way the clinical translation and commercialisation of cell-based therapies.

To-date, numerous bottom-up and top-down approaches are under intense investigation to develop materials with precisely controlled topographical features. Among the bottom up approaches, electro-spinning [23-35], self-assembly [36-41] and isoelectric focusing [42-44] can produce fibrous scaffolds with controllable mechanical properties and the ability to control / direct cell fate.
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_in vitro_ and guide neotissue formation _in vivo_. However, none of these methods can produce materials with controlled spatial architecture. To this end, top-down approaches based on lithography [45-55] are under research and development, in both academia and industry setting. Commercially available natural biodegradable polymers (e.g. collagen, gelatin, chitosan, silk), synthetic biodegradable and bioresorbable polymers (e.g. poly-glycolide, poly-lactide, poly-dioxanone, poly-caprolactone, polyethylene glycol) and synthetic non-absorbable polymers (e.g. poly-tetra-fluoro-ethylene, poly-propylene, silicone) drive the design and fabrication of medical devices for tissue engineering applications [56-65]. Bottom-up approaches, such as self-assembly, favour the use of biodegradable natural or synthetic polymers and peptides due to mild processing conditions [66-75], whilst top-down approaches, such as lithography, primarily utilise non-absorbable synthetic polymers [76-86], due to the more robust nature of the polymers. However, advances in engineering have now make available capillary force lithography [87, 88] and cast-mould techniques [89, 90] that allow incorporation of nano-features on biodegradable polymers. Specifically to bone tissue engineering, studies focused on anisotropic arrays of grooves and ridges have implicated topographical modification as a potent tool to either maintain osteogenic phenotype [91, 92] or to differentiate stem cells towards osteogenic lineage, with or without osteoinductive growth media [93-96]. Although the influence of various groove widths and/or distances between grooves has been assessed [97-105], the influence of groove depth is understudied [106-108], especially across length scales. Further, although these studies have rationalised a number of physiological processes occurring at the _in vitro_ bio-interface, an optimal groove dimension that translates _in vitro_
functional behaviour of osteoblasts to *in vivo* functional neotissue formation, as a function of substrate topography, has yet to be determined.
2.2. Materials and methods

2.2.1. Fabrication of silicon masters and topographical patterning of poly-lactic-co-glycolic acid (PLGA) substrates

Anisotropically ordered (constant groove width of ~1,860 nm and line width of ~2,220 nm and variable groove depth of ~35 nm, ~306 nm and ~2,046 nm) PLGA substrates were fabricated using standard photolithography followed by imprinting lithography, as described in detail below. These groove depth mimics the size scale of collagen fibres of bone extracellular matrix. As control group, isotropic / planar PLGA substrates were used with an inherent surface roughness (Ra) of ~115 nm over 10 \( \mu \text{m}^2 \), as revealed by AFM (Figure 2.1).

Silicon master moulds with anisotropic topographies were fabricated using a photolithography process followed by reactive ion etching. The masters consisted of a 1.5 x 1.5 cm\(^2\) pattern area containing line gratings with consistent pitch and variable groove depths. The groove width was ~1,860 nm, whilst the line width was ~2,220 nm. Silicon samples (3.0 x 3.0 cm\(^2\)) were first spin-coated with a positive photoresist film (S1813 PR, Shipley) and then exposed to these masks using OAI Mask Aligner (Model MBA800). Following photoresist development (MF 319 developer) at 20 °C chamber temperature for 9 sec (~35 nm depth), 80 sec (~306 nm depth) and 8 min (~2,046 nm depth), the master mould was etched by reactive ion etching (Oxford ICP etcher) using CHF\(_3 \) + SF\(_6\) ionised gas. Remaining photoresist was removed using acetone.

Silicon masters were then analysed by scanning electron microscopy (SEM, Hitachi Scanning Microscope S-4700, Hitachi-Hisco Europe Gmbh, UK) and atomic force microscopy (AFM, Veeco Dimension 3100 AFM, USA). The moulds were finally silanised with octadecyltrichlorosilane (OTS, 5 mM, Sigma
Aldrich, Ireland) solution to obtain a low energy surface and to facilitate imprint release. Silanisation entailed placing of the Si substrates in a closed drier for 6-8 h, followed by rinsing with OTS and drying with N\textsubscript{2}.

PLGA (lactide to glycolide ration of 85:15, M\textsubscript{w} 90 KDa; Purac, The Netherlands) substrates were compression moulded and laser micro-machined to size.
**Figure 2.1:** Three-dimensional (3D) AFM topography analysis of isotropic substrates (A) and substrates with groove depth of ~35 nm (B), ~306 nm (C) and ~2,046 nm (D). Quantification of isotropic control roughness, groove width, ridge width and groove depth (E).
2.2.2. Topographical patterning

Thermal imprinting process was used to pattern the PLGA substrates. Specifically, PLGA substrates (2.0 x 2.0 cm$^2$) were imprinted using Specac Hydraulic Press (15 T & 25 T) at 120 °C and at pressure of 5 MPa, which was applied for 5 min before cooling down to 30 °C (below glass transition temperature of PLGA = 45 – 55 °C [109, 110]) facilitating substrate release. The imprinted gratings on the PLGA substrates were subsequently analysed by SEM and AFM.

2.2.3. Measuring and cutting of samples

The PLGA samples (2 cm x 2 cm), as shown in Figure 2.2, were divided in smaller pieces to ensure all appropriate assays can be carried out. Due to the different size requirements of the different assays, the samples were cut into different sizes (Figure 2.3).
**Figure 2.2:** Illustration of the 2 cm x 2 cm grooved sample with a 1.5 cm x 1.5 cm patterned area in the centre.
**Figure 2.3:** Illustration of how the 2 cm x 2 cm samples were cut.

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<th>Cut</th>
<th>Result</th>
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<td><img src="image14" alt="Cut 5 Image" /></td>
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<tr>
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<td><img src="image20" alt="Cut 8 Image" /></td>
<td><img src="image21" alt="Result Image" /></td>
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<tr>
<td><img src="image22" alt="Start Image" /></td>
<td><img src="image23" alt="Cut 9 - 12 Image" /></td>
<td><img src="image24" alt="Result Image" /></td>
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2.2.4. Structural characterisation

For SEM analysis (Hitachi Scanning Microscope S-4700, Hitachi-Hisco Europe GmbH, UK), the samples were mounted using carbon tape on SEM sample holders and gold-sputter coated before imaging. For AFM analysis (Veeco Dimension 3100 AFM, USA), initially Nano World TESP tips (Force Constant: 42 N/m; Resonance Frequency: 320 kHz; Nano and More Gmbh, Germany) were used, however they were suboptimal for the analysis of micron-scale gratings. To circumvent this, high aspect ratio AR5-NCLR (Force Constant: 48 N/m; Resonance Frequency: 190 kHz; Nano Sensors™, Switzerland) tips were used. Imaging was performed with a scan rate of 0.5 Hz over an area of 10 μm².

2.2.5. Cell culture

Primary human osteoblasts were derived from femoral head biopsies of male subjects (purchased from Lonza, USA and PromoCell, Germany). The cells were cultured in Dulbecco’s modified Eagle’s medium (1,000 mg glucose, Sigma Aldrich, Ireland) supplemented with 10 % foetal bovine serum and 1 % penicillin/streptomycin (Sigma Aldrich, Ireland). The osteoblast culture was maintained at 37 °C in a humidified 5 % CO₂ incubator, until the cells were approximately 80 % confluent. The culture media was changed every 4 days. The cells (passages 4–5) were seeded for 1, 11 and 21 days on imprinted substrates in 8-well (Lab-Tek®, UK) and 12-well (Ibidi®, Ireland) chamber slides at a cell density of 2 x 10⁴ / 0.8 cm² and 7 x 10³ / 0.35 cm², respectively. For mineralisation studies, the osteoblast growth medium was supplemented with 50 μg/ml ascorbic acid, 10 nM dexamethasone and 5mM beta-glycerophosphate.
After samples were cut to size, each sample was securely positioned in to the appropriate chamber slide. The samples were washed once with 70 % ethanol or industrial methylated spirit (IMS). During the second wash with 70 % ethanol or IMS the samples were place under UV for 1 h. The samples were then washed twice with Hank's Balanced Salt Solution. The samples were then stored overnight in media. Prior to seeding, the samples were again washed with media.

2.2.6. Cellular and nuclear morphological analysis

Cell attachment, spreading, alignment and elongation were analysed using fluorescent microscopy. A cell density of $7 \times 10^3 / 0.35 \text{ cm}^2$ was seeded for these experiments. At the end of the culture time, the cells were fixed with 4 % paraformaldehyde for 30 min at room temperature (RT). Once fixed, the cells were washed three times with phosphate buffered saline (PBS, Sigma Aldrich, Ireland) and permeabilised with 0.2 % TritonX (Sigma Aldrich, Ireland) for 5 min at RT, followed by three 5 min washes with PBS. The actin cytoskeleton of the cells was stained with rhodamine-conjugated phalloidin (Molecular Probes, Ireland) for 1 h and the nuclei were then stained with 4,0,6-diamidino-2-phenylindole (DAPI, Molecular Probes, Ireland) for 5 min. Three 5 min washes with PBS were carried out between and after these stainings. Images were captured at 10X magnification using an inverted BX51 Olympus fluorescence microscope.

The spreading, alignment and elongation of the cytoskeleton and the nuclei of osteoblasts were quantified using NIH ImageJ software following previously described protocols [94, 111]. Briefly, inclusion criteria for the cell cytoskeleton measurements were based on cells that were adherent with no distinct cleavage
furrows and had no cell-cell contact. A threshold according to the area of cells /
nuclei was applied and the total area, aspect ratio (the ratio of the major axis
divided by the minor axis of each cell/nuclei based on a fitted ellipse), circularity
(4 \times \pi \times \text{area} / \text{perimeter}^2, \text{a value of 1 represents a circle}) and the specific angle
of orientation of cells/nuclei to the grooves and ridges was measured. Cells and
nuclei were considered aligned to the underlying topography when this angle
was within 10 degrees of being parallel to the ridges and grooves. Assessment
technique illustrated in figure

2.2.7. SEM analysis

The samples were prepared for SEM analysis using well-established protocols
[44, 102]. A cell density of 7 \times 10^3 / 0.35 \text{ cm}^2 was seeded for this experiment.
Briefly, osteoblasts were washed for 2 min in 0.1 M 1,4 piperazine bis-2-ethano
sulphonic acid (PIPES), pH 7.4 (Sigma Aldrich, Ireland), followed by
stabilisation in 4 % paraformaldehyde and then in 2.5 % glutaraldehyde (Sigma
Aldrich, Ireland) for 5 min in PIPES buffer. For contrast, 1 % osmium tetroxide
(Agar Scientific, UK) was used for 90 min. The cells were then dehydrated in a
graded ddH_2O/ethanol series: 50 %, 60 %, 70 %, 80 %, 90 %, 95 %, and 100 %
for 5 min. Following the ethanol washes, the cells were further dehydrated in a
graded ethanol / hexamethyldisilazane (HMDS, Sigma Aldrich, Ireland) series:
33 %, 50 %, 60 %, and 100 % for 15 min each. The dehydrated samples were
mounted using carbon tape on SEM sample holders and gold-sputter coated
before imaging with SEM (Hitachi Scanning Microscope S-4700, Hitachi-Hisco
Europe Gmbh, UK).
Figure 2.4. Illustration of ImageJ analysis
2.2.8. Cell metabolic activity, cell viability and cell proliferation analysis

Cell metabolic activity was determined on days 1, 11, and 21 using alamarBlue® cell metabolic activity assay (Thermo Scientific, UK). A cell density of $7 \times 10^3 / 0.35 \text{ cm}^2$ was seeded for these experiments. Briefly, alamarBlue® dye was diluted with Hank’s balance salt solution (HBSS, Sigma Aldrich, Ireland) to make a 10 % (v/v) alamarBlue® solution. Media was removed from each well and 0.2 ml alamarBlue® solution was added to each well. The cells were incubated for 4 h at 37 °C and then absorbance was measured at wavelengths of 570 nm and 600 nm using a microplate reader (Varioskan Flash, Thermo Scientific, UK). The level of metabolic activity was calculated using the simplified method of calculating % reduction, according to the supplier’s protocol.

Live/Dead® assay (Molecular Probes, Invitrogen, Ireland) was used to measure the influence of topographical features on osteoblast viability, as per manufacturer’s protocol. Briefly, at each time point, osteoblasts were washed with HBSS and the culture was incubated with Live/Dead® staining solution (2 $\mu$mol/L calcein-AM-green and 0.5 $\mu$mol/L ethidium homodimer-1 in a pH adjusted buffer) for 30 minutes at 37 °C and 5% CO$_2$ in a humidified incubator. The culture was washed with HBSS after discarding the Live/Dead® staining solution and the fluorescence images were captured using an Olympus IX-81 inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan) using FITC and Texas Red filter for live (green) and dead (red) cells respectively.

To assess proliferation, the total viable cell number was calculated by counting the DAPI stained nuclei from six randomly selected images from each patterned and control substrates.
2.2.9. Gene expression analysis

The expression of 36 genes (Table 2.1), grouped as osteogenic, adhesion, collagenous and non-collagenous, was assessed using TaqMan® Low Density Array (TLDA; Applied Biosystems, USA), as described below. 18S rRNA, TOP1 and EIF4A2 were used as housekeepers / internal controls.

2.2.10. RNA extraction

A cell density of $2 \times 10^4 / 0.8 \text{ cm}^2$ was seeded on the imprinted and control substrates located in a 8-well (Lab-Tek®, UK) chamber slide and total RNA was extracted at 0, 1, 11 and 21 days. The osteoblasts were lysed in Trizol reagent ($400 \mu\text{g/well}$, Sigma Aldrich, UK) using a cell scraper. RNA was isolated using a tri-spin protocol. In brief, $125 \mu\text{g/ml}$ glycogen (Sigma Aldrich, UK) was added and incubated at RT for 3 min. 2/5 sample volume of chloroform (Invitrogen, UK) was added to the samples, which were shaken vigorously for 15 sec and then incubated at RT for 5 min. After centrifugation ($12,000 \times \text{g}$ for 15 min) the upper phase was transferred into a fresh 1.5 ml tube, an equal volume of isopropanol (Sigma Aldrich, UK) was added and samples were incubated at RT for 10 min. Samples were then centrifuged for 10 min at $12,000 \times \text{g}$ and the supernatant was discarded. Pellets were washed with 1 ml ethanol (Sigma Aldrich, UK), then vortexed and centrifuged for 5 min at $7,500 \times \text{g}$ before the ethanol was removed. The samples were then air-dried and re-suspended in $50 \mu\text{l}$ of analytical grade water. The RNA concentration of samples was estimated using a nanodrop spectrophotometer. RNA samples with $A_{260/280}$ ratio of $\sim 2$ were considered acceptable.
2.2.11. Reverse transcription

RNA was primed with random hexamers and reverse transcribed with the superscript II kit (Life Technologies, UK) according to manufacturer’s protocol. In brief, RNA was incubated at 70 °C for 10 min with 200 ng random hexamers. 4 µl 5x sample buffer, 10 mM DTT, 0.125 mM dNTP’s (2.5 mM), 200 units Superscript II and 40 units RNase inhibitor were added and incubated for 1h at 42 °C and at 70 °C for 10 min (all reagents apart from the random hexamers were from the superscript II kit).

2.2.12. TLDA analysis

cDNA (100 ng) and universal PCR master-mix (50 µl) were loaded into the fill reservoirs (100 µl/reservoir) and the plate was run according to manufacturer’s protocol, using the Applied Biosystems 7900HT real-time PCR system (UK) and Applied Biosystems sequence detection system (SDS 2.3 and RQ manager 1.2) software (UK). The thermal cycles were as follows: 50 °C for 2 min 94.5 °C for 10 min followed by 40 cycles of 97 °C for 30 sec and 59.7 °C for 1 min. To calculate the relative expression of each target gene, the $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ methods were used as described previously [112]. Mean Ct values of each target gene were normalised to the housekeeping gene values. To analyse the changes in gene expression between the isotropic control and the structured substrates at all time points, the $2^{-\Delta\Delta Ct}$ method was used. Using QIAGEN's Ingenuity Pathway Analysis (IPA, USA) software of complex ‘omics data, Ingenuity® systems, the fold changes in gene expression was computed into canonical and functional pathway analysis between the control (isotropic substrate) and each condition.
structured substrates) at various time points. The $p$-values were determined using a right-tailed Fisher’s exact test.
Table 2.1: Genes and their transcripts assessed using TLDA.

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<th>Gene Symbol</th>
<th>NCBI Ref. Seq.</th>
<th>TaqMan® Transcript</th>
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<td>Hs00959010_m1</td>
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<td>Bone Sialoprotein</td>
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2.2.13. Matrix mineralisation analysis

A cell density of $2 \times 10^4 / 0.8 \text{ cm}^2$ was seeded on the imprinted and control substrates located in a 8-well (Lab-Tek®, UK) chamber slide. On days 7, 14 and 21 osteoblasts were assessed for calcium deposition via alizarin red staining. The cells on each substrate were fixed in 10 % formalin (Sigma Aldrich, Ireland) for 30 min at RT. Cells were then stained with 40 mM (pH 4.1) alizarin red (ARS, Sigma Aldrich, Ireland) for 30 min, before washed with dH$_2$O. Stained monolayer were visualised by bright-field microscopy, using an inverted microscope (Leica S 40/0.45, 10X, Ireland). Alizarin red was extracted and quantified by adding 10 % v/v acetic acid to each sample for 30 min. The slurry was overlaid with mineral oil (Sigma Aldrich, Ireland) and heated to 85 °C for 10 min, then transferred to ice for 5 min, followed by centrifugation at 20,000g for 15 min. 10 % v/v ammonium hydroxide was added to the supernatant from each sample to neutralise the acid, the pH was confirmed to be between 4.1 and 4.5. Aliquots of the supernatant were read at 405 nm in 96-well plate on a microplate reader (Thermo Scientific, Varioskan Flash). The results were normalised to total cellular protein values, measured in cell lysates by the Bradford method (Bio-Rad Laboratories, Inc.).

2.2.14. Alkaline phosphatase (ALP) activity analysis

A cell density of $2 \times 10^4 / 0.8 \text{ cm}^2$ was seeded on the imprinted and control substrates located in a 8-well (Lab-Tek®, UK) chamber slide. ALP activity of osteoblasts was measured on days 7, 14 and 21. On these days, the cells were washed twice with HBSS to remove medium serum proteins. The cell layer was lysed with lysis buffer (0.1 % Triton-X in 10mM Trizma-Base, pH 7.4; Sigma
Aldrich, Ireland) at 4-8 °C under agitation for minimum 1 h. In each 1.5 ml centrifuge tube, 250 μl of alkaline buffer (1.5 M, at pH 10.3, Sigma Aldrich, Ireland), 50 μl of substrate solution (4-nitrophenyl phosphate, Sigma Aldrich, Ireland) dissolved in 1 M diethanolamine buffer (in 0.5 mM MgCl₂; Sigma Aldrich, Ireland), 100 μl of cell lysate and 100 μl of H₂O was added. Following incubation at 37 °C for 30 min, the reaction was stopped by adding 500 μl of 0.1 M NaOH. Using p-nitrophenol (10 mM, Sigma Aldrich, Ireland) as standard, the optical density (OD) was measured at 405 nm using a spectrophotometer. The activity of ALP was expressed as the OD divided by the incubation time and the total protein content was assayed by the Bio-Rad (UK) protein assay. All materials for this assay were bought from Sigma Aldrich (Ireland).

2.2.15. Gelatin zymography analysis and densitometric analysis

A cell density of 7 x 10³ / 0.35 cm² was seeded for this experiment. All reagents were purchased from Sigma Aldrich, Ireland and a well-established gelatin zymography protocol was followed [113]. Briefly, a 10 % acrylamide gelatin gel, using gelatin, 1.5 M Tris (pH 8.8), 30 % acrylamide-bis-acrylamide, 50 % glycerol, 10 % SDS, TEMED and 10 % APS, was prepared for polyacrylamide migration. Stacking gel contained 4 % polyacrylamide in 0.125 mM Tris (pH 6.8) and 0.1 % SDS. These gels were polymerised by adding 50 μl of 10 % ammonium persulfate and 10 μl of TEMED. At each culture time point (days 1, 11, and 21), media was collected and diluted in 0.125 mM Tris (pH 6.8), 50 % glycerol and 0.4 % bromophenol blue. 10 μg / lane of each sample was added. The gels were run for 1 h at 20 mA / gel (0.75 mm thick mini-gel in SDS-buffer without a reducing agent). Following electrophoresis, the zymogram was
developed by washing (incubate 1 h at RT on a rotating shaker to remove the SDS and to renature proteinases) with 2.5 % TritonX 100 in 50 mM Tris (pH 7.4), 5 mM CaCl$_2$ and 1 mM ZnCl$_2$ followed by rinsing briefly with deionised water. The gels were then incubated overnight at 37 °C in 50 mM Tris (pH 7.4), 5 mM CaCl$_2$ and 1 mM ZnCl$_2$. They were then stained with Coomassie G250 in 30 % ethanol, 10 % acetic acid for 30 min and destained in 30 % ethanol / 10 % acetic acid until clear bands were visible, followed by stopping the staining and rehydration overnight in 2 % acetic acid. The gels were then scanned and proteinase activity was evidenced as cleared area. Densitometric analysis of gels was performed using GeneTools analysis software (Syngene, UK).

2.2.16. *In vivo* analysis

Six male Merino sheep (approximately 6 years old and 50 kg body weight) were used in this study, which was approved by the Queensland University of Technology, Brisbane, Australia, Animal Ethics Committee (Ethics Approval Number 1200000189). Merino sheep were supplied by a local veterinarian research farm and were inducted at the farm (standard protocol involving tagging, blood analysis, drenching and vaccinations) after assessment by the facility veterinarian.

On the day of surgery, the sheep were anaesthetised with an intravenous induction of propofol (3 mg/kg), and maintained with 50 % oxygen in air, and isofluorane (2 to 2.5 %) using a mechanical ventilator. Prior to surgery, the surgical site was infiltrated with local anaesthetic (2.2 mL 2 % Lidocaine and 1:80,000 Adrenaline, Lignospan Special, Septodon, UK). Buprenorphine (0.005 mg/kg) and ketorolac (0.5 mg/kg) were given for pre-emptive and post-operative
bi-modal pain management. All sheep received prophylactic parenteral antibiotic regimen of ciprofloxacin (200 mg/100mL), kefzol (20 mg/kg) and gentamicin (5 mg/kg). A sagittal skin incision was made extending from in between the horns to the occiput. The subcutaneous soft tissue was carefully split and retracted to expose the calvaria. The periosteum was incised longitudinally and carefully elevated stripping it from the underlying bone. Rectangular (1x1 cm in dimension) isotropic and anisotropic PLGA substrates were placed on the denuded calvaria (Figure 2.4 A). Particular attention was paid to ensure good contact between the PLGA substrates and the underlying bone, ensuring that the anisotropic features were in direct contact with the bony tissue. Each substrate was then fixed to the skull using titanium bone tacks (2.5 mm each, due to the thickness of sheep calvarial bone) placed in each corner of the substrate to ensure appropriate stability (Figure 2.4 B). After securing the substrates in place, the periosteum was replaced over them and was sutured closed. Afterwards, subcutaneous soft tissue and skin were closed with layered sutures. Antibiotic spray was applied and the surgical site then covered with sterile dressings. Due to limitations in the sample availability, three animals were sacrificed by intravenous injection of 60 mg/kg sodium pentobarbital (Lethabarb©, Virbac, Australia) at each 4 weeks and 8 weeks post-implantation. Calvarial bones and adjacent soft tissue were explanted and immediately fixed with 4 % paraformaldehyde in PBS at pH 7.4 for 48 h prior to further analysis.
Figure 2.5: Schematic illustration of the position of the PLGA substrates on sheep calvaria (A). Calvaria appearance, following substrate implantation (B).
2.2.17. Histological analysis

Histological analysis for non-demineralised bone was performed in all explanted tissue specimens. The specimens were rinsed with water and sectioned using the Exakt 300 diamond band saw (Exact Apatbau GmbH, Germany). Each sample was sectioned to a thickness of 4 mm and subsequently dehydrated in sequential graded series of ethanol concentrations prior to resin infiltration. Once fully infiltrated, the specimens were embedded in Tecknovit 7200 resin. Light polymerisation system was used prior to mounting them on a plastic slide. Following this, 250 µm thick sections were obtained using the Exakt 300 band saw and the slides were polished down to 10 µm, using the Exakt 400 CS Micro-Grinding System. The slides were stained with haematoxylin and eosin according to protocols established for resin embedded samples. The number of specimens demonstrating direct bone to substrate contact in each group was calculated and compared to the total number of samples within the same group. Histomorphometry analysis was also performed by measuring the percentage of direct bone-to-substrate contact, defined as the length of direct bone contact, as seen from histology, divided by the total length of the substrate). Osteomeasure software (OsteoMetrics Inc, USA) was utilised for this analysis and the regions of interest were manually drawn on images taken at x8 magnification.

2.2.18. Statistical analysis

All data were analysed using GraphPad Prism® 5 (Graphpad Software, USA) and/or PASW Statistics 17.0 (SPSS Inc, IL). Analysis of variance (ANOVA) and Tukey’s multiple comparison post-hoc tests were performed. Statistical significance was accepted at $p < 0.05$. 
Note:

Prof Dietmar Hutmacher and his team conducted the *in vivo* study and the associated analysis.
2.3. Results

2.3.1. Cell morphometric analysis
SEM analysis revealed that as early as 24 h after seeding, osteoblasts aligned and elongated parallel to the substrate orientation on imprinted substrates with groove depth of ~306 nm and ~2,046 nm; a spread morphology was induced on isotropic controls and on imprinted substrates with groove depth of ~35 nm (Figure 2.5). Rhodamine-conjugated phallolidin and DAPI staining indicated that at all time points (day 1, 11 and 21), osteoblasts exhibited a well-spread morphology on isotropic and imprinted substrates with ~35 nm groove depth and an aligned orientation and an elongated morphology, parallel to the substrate topography, on imprinted substrates with groove depth of ~306 nm and ~2,046 nm (Figure 2.6). Complementary image analysis further demonstrated that osteoblasts aligned parallel to substrates with groove depths of ~306 nm and ~2,046 nm, as they displayed an angle of deviation relative to the groove orientation of 0-10 ° (Figure 2.7). The cytoskeleton was significantly more elongated on substrates with groove depths of ~306 nm and ~2,046 nm, as compared to control substrates and substrates with groove depth of ~35 nm (Figure 2.8). No significant difference was observed among the groups with respect to cell area (Figure 2.9). On day 1 and 11, substrates with groove depth of ~306 nm and ~2,046 nm induced statistically significant higher nuclei elongation than isotropic substrates and substrates with groove depth of 35 nm; on day 21 only substrates with ~2,046 nm induced the highest nuclei elongation (Figure 2.10).
Figure 2.6: SEM micrographs demonstrate fusiform morphology for osteoblasts seeded on isotropic substrates and substrates with groove depth of ~35 nm (A). Osteoblasts aligned parallel to the underlying topography on groove depths of ~306 nm (B) and ~2,046 nm (C).
Figure 2.7: At 24 hrs, DAPI (blue) and rhodamine-conjugated phalloidin (red) staining indicates that osteoblasts aligned parallel to the substrate topography of groove depths of ~306 nm and ~2,046 nm, whilst a random morphology was observed on isotropic substrates and substrates with groove depth of ~35 nm.
Figure 2.8: At 24 hrs, orientation of osteoblasts on the nano-imprinted substrates. This was further confirmed, when the angle of cells parallel to the underlying topography was between 0 – 10 ° on substrates with groove depth of ~306 nm and ~2,046 nm. Data presented as mean ± SD. One-way and two-way ANOVA was performed. *p < 0.05, **p < 0.01.
Figure 2.9: At 24 hrs, elongation of osteoblast cytoskeleton. Substrates with groove depth of \(~306\) nm and \(~2,046\) nm induced the highest cellular elongation. Data presented as mean ± SD. One-way and two-way ANOVA was performed. **\(p < 0.01\).
Figure 2.10: At 24 hrs, area of osteoblast cytoskeleton. No significant difference was observed in cellular area as a function of the surface topography. Data presented as mean ± SD. One-way and two-way ANOVA was performed.
Figure 2.11: Elongation of osteoblast nuclei. By day 21, substrates with ~2,046 nm groove depth exhibited the highest nuclei elongation among the groups (E). Data presented as mean ± SD. One-way and two-way ANOVA was performed.

*p < 0.05, **p < 0.01.
2.3.2. Cell metabolic activity, viability and proliferation analysis

Cell metabolic activity (Figure 2.11), proliferation (Figure 2.12) and viability (Figure 2.13) and significantly increased from day 1 to day 11 for all groups and plateaued thereafter. However, no difference was observed within the groups at any given time point.
**Figure 2.12:** Evaluation of osteoblast metabolic activity. Metabolic activity was significantly increased from day 1 to day 11 for all groups and plateaued from then onwards. Data presented as mean ± SD. One-way and two-way ANOVA was performed. ***p < 0.001.
Figure 2.13: Cell proliferation was significantly increased from day 1 to 11 for all groups and plateaued from then onwards. On day 21, significantly higher cell proliferation was observed for all textured substrates. Data presented as mean ± SD. One-way and two-way ANOVA was performed. ***p < 0.001.
Figure 2.14: Osteoblast viability. Cell viability appeared to significantly increase from day 1 to 11 and to remain constant from day 11 to day 21 for all groups. Overall, no difference was observed within the groups at any given time point (we consider the observed difference at day 11 for the 300 nm asymptomatic).
2.3.3. Gene analysis

Hierarchical clustering diagrams of gene analysis demonstrated an overall upregulation of the assessed genes as a function of increased groove depth at a given time point. Further, the highest upregulation (0.5 to 1.5 fold) of osteoblast phenotype (bone sialoprotein, osteonectin and RNX2); adhesion (ITGA2, ITGA4, ITGA5, ITGA6, ITGA10, ITGB1, ITGB2, ITGB3, ITGB5 and CD44); and ECM (collagens III, XI, XII, XIV, decorin, cartilage oligomeric protein, matrix gla protein, fibromodulin, aggrecan, biglycan) genes was observed on substrates with groove depth of ~2,046 nm (Figure 2.14). Similarly, IPA indicated significant increases in the expression of genes involved in functional and canonical pathways of cellular assembly and cytoskeleton organisation, cellular adhesion and movement and phenotype maintenance on grooved substrates, with more profound effect induced by the ~2,046 nm groove depth (Figure 2.15, Figure 2.16).
**Figure 2.15:** Hierarchical clustering of gene analysis. It indicates that substrates with groove depth of ~2046 nm induced the highest upregulation of genes associated with phenotype, adhesion and ECM.
Figure 2.16: IPA indicated substrates with groove depth of ~2,046 nm induced the highest cytoskeleton organisation, cellular movement and skeletal development (A) and stimulated the highest integrin and paxillin signalling (B).
Figure 2.17: Network analysis demonstrates the interconnectivity of functional and canonical pathways on substrates with groove depth of ~35 nm (A), ~306 nm (B) and ~2,046 nm (C) at day 1 (C); ~35 nm (D), ~306 nm (E) and ~2,046 nm (F) at day 11; and ~35 nm (G), ~306 nm (H) and ~2,046 nm (I) at day 21. The biochemical pathways are interconnected by grey lines. The networks show gene expression for adhesion, cytoskeleton organisation and skeletal development. FX indicates functional pathways and CP indicates canonical pathways. (Green = downregulated genes; Red = upregulated genes). See Appendix … for detailed images.
2.3.4. Matrix mineralisation and ALP activity analysis

Aligned mineral deposition was observed only on substrates with groove depth of ~2046 nm by day 21 (Figure 2.17). Calcium deposition was not significantly affected as a function of the substrate used at any time-point (Figure 2.18). At day 7, ALP activity was significantly increased on ~35 nm and ~2046 nm in groove depth substrates, as compared to ~306 nm in depth groove substrate and control, isotropic, substrate (Figure 2.19). At day 14 and 21, no significant difference was observed in ALP activity between all structured substrates and the control, isotropic, substrate (Figure 2.19).
Figure 2.18: Osteoblast calcium deposition. Only substrates with groove depth of ~2046 nm induced aligned mineralised matrix by day 21.

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Figure 2.19: Quantification of osteoblast calcium deposition. No significant difference between the groups was observed in calcium deposition. Data presented as mean ± SD. One-way and two-way ANOVA was performed.
**Figure 2.20:** Alkaline phosphatase activity of Osteoblasts. At day 7, ALP activity was significantly increased on ~35 nm and ~2,046 nm in groove depth substrates, as compared to ~306 nm in depth groove substrate and control, isotropic, substrate. At day 14 and 21, no significant difference was observed in ALP activity between all structured substrates and the control, isotropic, substrate. Data presented as mean ± SD. One-way and two-way ANOVA was performed. *$p < 0.05$
2.3.5. Proteolytic activity analysis

Gelatin zymography and supplementary densitometric analysis (Figure 2.20) demonstrated no difference in matrix metalloproteinase (MMP2) expression among the groups. However, MMP2 expression was increased as a function of time in culture.
**Figure 2.21:** Zymography analysis (A) and complementary densitometric analysis (B) demonstrated no difference in MMP2 expression among the groups. MMP2 expression was increased as a function of time in culture.
2.3.6. *In vivo* analysis

Non-decalcified tissue sectioning and histology revealed that none of the substrates presented any sign of obvious degradation, at any of the time points assessed, as they were still clearly visible upon close examination (*Figure 2.21*). Post-operative healing was normal, with gross morphological analysis of the explanted specimens demonstrating a peri-implant fibrous tissue formation over them (*Figure 2.22*). Haematoxylin and eosin staining (*Figure 2.22*) of the various implants, at both time points, demonstrated that in all specimens a fibrous tissue was intercalated between the resident bone and the substrate. The frequency of bone-to-substrate contact was calculated at both time points and it was observed that bone contact occurred more frequently at the earlier time point (*Table 2.2*). However, the percentage coverage of bone-to-substrate contact remained in all cases extremely low (< 2 %) at both time points (*Figure 2.22 F*).
Figure 2.22: Morphology of the calvaria 4 weeks post-implantation.
Figure 2.23: Representative haematoxylin and eosin staining demonstrates the intercalation of fibrous tissue (FT) between the resident bone and the PLGA substrates. (A) Substrate with ~35 nm groove depth; (B) Substrate with ~306 nm groove depth; (C) Substrate with ~2,046 nm groove depth; (D) Control, isotropic substrate (no topographical features); (E) Example of direct bone-to-substrate contact; (F) Percentage coverage of bone-to-substrate contact remained under 2% for all substrates and both time points and no statistical difference was found between the groups and time points.
Table 2.2: Frequency of bone-to-substrate contact, as evaluated by histological analysis.

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2.4. Discussion

Bottom-up (e.g. self-assembly) and top-down (e.g. imprinting) fabrication technologies have attracted great scientific and technological interest the recent years as means to fabricate biomaterials with instructive signals to accurately control cellular functions at the bio-interface and influence permanently differentiated and stem cell function. Despite the wealth of information available to-date from \textit{in vitro} experimentation, only a few studies have assessed the influence of nano- and micro-textured substrates on host response [114]. Herein, the influence of physiological relevant size-scale topographical features on osteoblast response \textit{in vitro} and whether such substrates can direct functional neotissue formation \textit{in vivo} were assessed.

Starting with morphological analysis, it is evidenced that substrates with deeper grooves (~306 nm and ~2,046 nm) induced morphological changes in both osteoblasts’ cytoskeleton and nuclei. The theory of direct mechano-transduction proposes that intracellular tension in elongated and aligned cytoskeleton actin filaments is transferred to the nucleus through cytoskeletal elements to induce the repositioning of the chromosomal interphase centromere [115-120]. Substrates with groove depth of ~35 nm were considered as too shallow to induce morphological changes, whilst substrates with deeper grooves induced directional cell growth, as have been observed previously with imprinting / lithography technologies [99, 102, 121, 122], fibrous constructs [34, 123, 124] and machined / etched titanium surfaces [125-128].

Subsequent cell metabolic activity, viability and proliferation assays revealed no significant differences among the isotropic and the anisotropic substrates assessed. This observation indicates that although surface topography induces
cellular elongation parallel to the substrate orientation, as mechanical loading does in the direction of the applied load [129, 130], surface-induced cellular elongation does not activate apoptotic pathways, as mechanical tension may bring about [131-134].

Further functional and canonical pathway analysis indicated upregulation of cytoskeleton and nuclei organisation genes on groove depths of ~306 nm and ~2,046 nm. This observation was validated using DAPI and rhodamine-conjugated phalloidin staining analysis, where only substrates with groove depth of ~306 nm and ~2,046 nm induced parallel to the substrate cellular elongation.

Gene analysis also indicated upregulation of integrin and paxillin signalling on ~2,046 nm, which denotes rapid turnover of focal adhesion proteins and possibly increased motility through activation of FAK, Rho and Rac signalling [135-138]. This observation can be validated through the evidenced increase in MMP2 activity, which induced proteolysis that facilitated cell migration, through remodelling of the ECM [139, 140]. This observation is in agreement with previous work, where it was reported that temporal MMP expression increases in osteoblasts cultured on micron-scale grooves, indicating that surface topography, through preferential MMP activation, modulates remodelling [141].

Gene analysis further indicated upregulation of integrins and their recruitment by paxillin on the textured substrates, which corroborates their affinity to ECM molecules, such as collagen, laminin, fibronectin, osteopontin and thrombospondin, which are responsible for the production of mineralised bone matrix [142-144]. Moreover, at 21 days of culture, osteogenic markers (e.g. bone sialoprotein, osteonectin and RUNX2) were also upregulated on substrates with groove depth of ~2,046 nm. Indeed, upregulation of bone sialoprotein, a gene
that encodes non-collagenous components of bone ECM, and RUNX2, a gene that promotes osteogenic differentiation, indicate that these substrates induced osteogenesis [145-147]. However, the gene expression of osteopontin remained unaffected and the gene expression of alkaline phosphatase and osteocalcin were downregulated at day 21 at ~2,046 nm groove depth substrate. In addition, alkaline phosphatase activity and calcium deposition remained unaffected as a function of the substrate used. It is believed that this indifference in mineralisation is due to the identical substrate stiffness between the substrates used, which has been demonstrated (substrate stiffness) to strongly regulate protein expression, cell phenotype maintenance and stem cell differentiation [148-151], suggesting that substrate stiffness overwhelms surface topography. In fact, the upregulation of cartilage oligomeric protein and aggrecan, genes associated with chondrogenesis and a tissue of lower stiffness than bone and closer to PLGA substrate stiffness (Young’s modulus; 2.0 GPa), reinforce this notion. This is in accordance to previous observation, where in the absence of mechanical loading, bovine tenocytes aligned perpendicularly to substrate orientation [34], suggesting that mechanical loading overpowers surface topography.

As in vitro bone-specific protein assays fail to recognise differences between isotropic and anisotropic substrates, similarly, no differences were observed among them in the in vivo model. Further, at both time points and for all substrates, the percentage coverage of bone-to-substrate contact was found to be less than 2 %, exemplifying the fact that when bone-to-substrate contact occurred, it was nearly negligible. This indicates that the frequency and the extent of bone-to-substrate contact were unrelated to the topographical features
and that osteogenesis onto the various substrates occurred in a random manner. It is worth pointing out that this observation contradicts previous results, where similar size-scale features induced directional *in vitro* cell growth and neotissue formation for various clinical targets, including neural [152-155] tendon, [156-158] bone [30, 159, 160] and skin [161-163]. However, these studies used three-dimensional nano- and micro-fibrous constructs that allowed cell penetration within the three-dimensional fibrous matrix and subsequent cell-guidance and neotissue growth within this contained / restricted architecture / environment.
2.5. Conclusions

The work presented herein indicates that topographical features in the middle nano-range (~306 nm) and low micron-range (~2,046 nm), rather than in the low nano-scale (~35 nm) groove depth, induce cellular and matrix alignment and upregulation of osteogenic markers \textit{in vitro}. However, these topographical features did not translate \textit{in vitro} contact guidance, directional cell growth and matrix alignment into enhanced interfacial integration and directional neotissue formation \textit{in vivo}. This study suggests that topographical features in two-dimensional content (surface) can be effectively utilised to maintain cell phenotype and function \textit{in vitro}, where dilute culture media and low cell numbers are customarily used. Cell-based therapies require \textit{ex vivo} cell propagation to obtain a suitable cell number. However, during \textit{ex vivo} growth, on far from physiological smooth tissue culture plastics, cells lose their phenotype and function. Such structured substrates can protect cell phenotype and function \textit{ex vivo}, and ultimately enable clinical translation and commercialisation of cell-based therapies.
2.6. References


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Chapter 2 - Imprinting


Chapter Three

Evaluation of osteoblast behaviour *in vitro* on carbohydrate
functionalised electro-spun scaffolds

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Kumar, P, Keeney, M, Rooney, N, Pandit, A, Zeugolis, DI. Journal of

The influence of non-sulphated polysaccharides on the properties of
electro-spun poly(lactic-co-glycolic acid) fibres. *Azeem, A*, Marani, L,
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Science & Engineering. ACS Biomaterials Science and Engineering. 2016,
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3.1. Introduction

Advancements in engineering, materials science, polymer chemistry and biology have made available numerous nano- to micro- fabrication processes and functionalised osteoinductive biomaterials to meet the increasing worldwide demand for functional implantable devices for bone repair and regeneration [1-4]. Among the various fabrication technologies, electro-spinning has been favoured for biomedical applications, as it produces two- and three- dimensional scaffolds that closely imitate the structural and physical properties of native bone tissue and offers the opportunity to deliver therapeutics, biologics and viable cell populations at the side of interest [5-12]. To further enhance biomimicry, hydroxyapatite has been primarily incorporated into these scaffolds to improve mechanical properties, mineralisation, osteogenesis and cell recruitment, adhesion and proliferation [13-18]. Growth factors have also been incorporated into electro-spun meshes and the resultant scaffolds have been shown to significantly improve osteogenesis in vitro and in vivo. Specifically, electro-spun meshes loaded with FGF2-FGF18 have been shown to significantly stimulate in vitro rat mesenchymal stem cell proliferation, induction of alkaline phosphate activity and mineralisation and to significantly enhance bone-forming ability, in terms of bone volume and density, in vivo [19]. PDGF-BB loaded scaffolds have been shown to induce increased expression of osteogenic markers of human mesenchymal stem cells [20]. VEGF loaded scaffolds promoted attachment, proliferation and alkaline phosphatase production of human osteoblasts, whilst histological analysis revealed new bone matrix formation after 10 weeks of implantation [21]. BMP-2 and BMP-2 / dexamethasone loaded scaffolds exhibited enhanced stem cell attachment, proliferation and strong ability to
differentiate the stem cells towards osteogenic lineage in vitro and enhanced osteogenesis in vivo [22-24]. Stromal cell derived factor-1α induced stimulated chemotactic migration of stem cells in vitro and increased the amount of bone formed in vivo [25]. The short half-life of growth factors [26, 27], along with the literally infinite number of permutations (e.g. concentration, combination and timing) has triggered investigation into alternative functionalisation molecules.

Glycosaminoglycans (GAGs) are crucial in numerous physiological processes, whilst their absence or down-regulation is associated with pathophysiologicals [28, 29]. Specifically to bone tissue, heparin sulphate is key regulator of endochondral ossification and osteochondroma development [30]. Of significant importance is in vivo data demonstrating the superiority of heparin sulphate over BMP-2 in promoting critical size bone defect healing [31], possibly due to the recruitment and enhancement of host growth factors at the side of injury [32]. Chondroitin sulphate has shown to influence osteoblast behaviour and stem cell differentiation towards osteogenic lineage in vitro and to enhance new bone formation in vivo [33, 34]. Further, chondroitin sulphate and glucosamine have been used extensively in clinic for osteoarthritis management [35-38]. Heparin has been shown to increase mRNA expression of alkaline phosphatase and BMP-4, to promote mineralisation and overall to support osteogenic differentiation of stem cells in vitro [39]. A heparin / bFGF / poly-ε-caprolactone scaffold [40] and a heparin / methacrylamide / BMP-2 scaffold [41] enhanced cell attachment and proliferation and induced high alkaline phosphate activity. A heparin / BMP-2 / titanium scaffold improved osteoblast function in vitro and new bone formation in vivo [42]. A heparin / collagen / hydroxyapatite / VEGF scaffold improve mineralisation and stimulated vascularisation [43]. Although
hyaluronic acid (HA), the sole non-sulphated GAG, has extensively been studied in tendon repair and regeneration [44-46], its potential in bone repair and regeneration has only recently started taking off. Specifically, chitosan / HA containing calcium phosphate cements have been shown to support growth and enhance alkaline phosphatase activity in osteoblast culture [47]. HA inhibited expression of MMP (matrix metalloproteinases) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) in murine osteoblast culture [48]. A HA / gelatin / biphasic calcium phosphate scaffold resulted in a significant increase in stem cell proliferation and high alkaline phosphatase activity in vitro, whilst in vivo, it induced rapid new bone formation and a high rate of collagen mineralisation [49]. Although low molecular weight HA has been shown to effectively maintain murine embryonic stem cells in a viable and undifferentiated state [50], high molecular weight HA has been shown to decrease cell adhesion, as a function of increasing the concentration of HA, to increase mRNA expression of alkaline phosphatase, osteocalcin and RUNX-2 and to promote bone formation [51]. Of significant importance is recent data demonstrating the superiority of HA, over chondroitin-4-sulphate, chondroitin-6-sulphate, dermatan sulphate and heparin, in osteogenic differentiation of human bone marrow mesenchymal stem cells and bone matrix synthesis [52]. Further, although increasing data demonstrate the suitability of GAG-derivatives in the design of functional biomaterials [53], only the potential of sulphated polysaccharides has been assessed for bone repair [54]. Thus, herein we ventured to assess the influence of HA and Ficoll™ (FC) on the structural, thermal, physical and biological properties of electro-spun scaffolds. FC is a neutral non-sulphated polysaccharides that has been shown to enhance ECM deposition,
when it is used as macromolecular crowder [55, 56] and to enhance cell attachment and growth, when it is used as functionalisation molecule [57].
3.2. Materials and methods

3.2.1. Fabrication of isotropic electro-spun fibres

To create isotropic carbohydrate functionlised electrospun mats typical protocols for electro-spinning were utilised, as has been described before [58-61]. Briefly, 8 % w/v 85/15 poly(lactic-co-glycolic acid) (PLGA, Purac, The Netherlands) was dissolved in 2,2,2-trifluoroethanol (TFE, Sigma-Aldrich, Ireland) and was extruded at 10 μl/min through an 18 G stainless steel needle (BD, Ireland). Upon application of high voltage (~12 KV) between the needle and the collector (~18 cm distance), the solvent was evaporated and the fibres were collected on the aluminium collector. 0.01 %, 0.1 % and 1.0 % solutions of HA (851,000 – 1,190,000 Da; Lifecore Biomedical, Chaska, MN, USA) and FC (1,000,000 Da; TdB Consultancy, Sweden) in TFE were prepared at room temperature using sonication amplitude of 40 % for 30 min. An 8 % w/v PLGA was added to these solutions, which were subsequently electro-spun, as described above.

3.2.2. Electro-spun fibre morphology assessment

Isotropic electro-spun mats were mounted onto a carbon tape, gold-sputter coated and imaged using a Hitachi Scanning Electron Microscope S-4700 (Hitachi High-Technologies Europe GmbH, Germany). Fibre diameter analysis was conducted using NIH ImageJ software. The SEM image was loaded onto the image tool. Using the scale bar on the image, a scale was set identifying pixels/μm. A line was drawn across the diameter of the fiber and measured.
3.2.3. Differential scanning calorimetry (DSC) assessment

Thermal analysis was carried out using the DSC-60 (Shimadzu Corp, Japan) equipment, based on well-established protocols [62]. Briefly, all scaffolds were rehydrated in phosphate buffer saline (PBS) overnight at room temperature. Using tissue paper excess PBS was removed and subsequently heating was applied at a constant temperature ramp of 10 °C / min in the temperature range of 25 °C and 100 °C. The endothermic transition was recorded as a peak. Onset (temperature at which the tangent to the initial power versus temperature line crosses the baseline) and peak (temperature of maximum power absorption) temperatures were recorded.

3.2.4. Mechanical assessment

Mechanical properties were assessed under uniaxial tension, using a Zwick/Roell (Leominster, Herefordshire, UK) Z005 testing machine, loaded with a 10 N load cell, as has been described previously [63, 64]. The samples were pre-cut into a dog-bone shape, as per ASTM D882-2010 guidelines. Prior to testing, all samples were incubated overnight at room temperature in PBS and tissue paper was used to remove excess PBS. The samples thickness was measured using digital callipers (Scienceware®, Digi-Max™, Sigma-Aldrich, Ireland). The samples were hand-tightened between the vertical grips, which were set at 5 cm gauge length. Scaffolds that broke at contact points with the grips were rejected from the analysis. The extension rate was set at 10 mm/min. The following definitions were used to calculate mechanical data: force at break defined as the force required to cause failure, stress at break was defined as the load at failure divided by the original cross-sectional area (engineering stress) and strain at
break was defined as the increase in scaffold length required to cause failure divided by the original length.
3.2.5. Osteoblast culture

Primary human osteoblasts were derived from femoral head biopsies of male subjects (Lonza, USA and PromoCell, Germany). The cells were cultured in Dulbecco’s modified Eagle’s medium (1000 mg glucose, Sigma Aldrich, Ireland) supplemented with 10 % foetal bovine serum and 1 % penicillin/streptomycin (Sigma Aldrich, Ireland). The osteoblast culture was maintained at 37 °C in a humidified 5 % CO₂ incubator, until the cells were approximately 80 % confluent. The culture media was changed every 4 days. The cells (passage 4 to 5) were seeded for 1, 11 and 21 or 7, 14 and 21 days on the functionalised substrates in 24-well plates (Lab-Tek®, UK) at a cell density of $4 \times 10^4 / 1.9 \text{ cm}^2$. The sample size of the fibres was the same as the well plate, 1.9 cm². For mineralisation studies, the osteoblast growth medium was supplemented with $50 \mu\text{g/ml ascorbic acid, 10 nM dexamethasone}$ and $5 \text{ mM } \beta$-glycerophosphate.

3.2.6. Osteoblast metabolic activity, viability and proliferation assessment

Cell metabolic activity was determined on days 1, 11 and 21 using alamarBlue® assay (Thermo Scientific, UK). Briefly, alamarBlue® dye was diluted with Hank’s balance salt solution (HBSS, Sigma Aldrich, Ireland) to make a 10 % (v/v) alamarBlue® solution. Media was removed from each well and 0.2 ml alamarBlue® solution was added to each well. The cells were incubated for 4 h at 37 °C and then absorbance was measured at wavelengths of 570 nm and 600 nm using a microplate reader (Varioskan Flash, Thermo Scientific, UK). The level of metabolic activity was calculated using the simplified method of calculating % reduction, according to the supplier’s protocol. Cell viability was assessed at
Chapter 3 - Electrospinning

days 1, 11 and 21, using the Pierce lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (CytoTex 96®, Promega), as per manufacturer’s protocol. Briefly, the LDH released into the medium was transferred to a new plate and mixed with Reaction Mixture. After 30 min at 37 °C, reactions were stopped using the Stop Solution. Absorbance at 490 nm and 680 nm was measured using a microplate reader (Varioskan Flash, Thermo Scientific, UK) to determine LDH activity. To assess osteoblast proliferation, the total viable cell number was calculated by counting the DAPI stained nuclei (see section 2.9) from six randomly selected images from each group at a given time point.

3.2.7. Osteoblast alkaline phosphatase (ALP) activity assessment

ALP activity of osteoblasts was measured on days 7, 14 and 21. At each time point, cells were washed x2 with HBSS to remove media serum proteins. The cell layers were then lysed (0.1 % Triton-X in 10mM Trizma-Base, pH 7.4; Sigma Aldrich, Ireland) at 4-8 °C under agitation for 1 h. 250 μl alkaline buffer (1.5 M, at pH 10.3, Sigma Aldrich, Ireland) and 50 μl substrate solution (4-nitrophenyl phosphate, Sigma Aldrich, Ireland) were dissolved in 1 M diethanolamine buffer (in 0.5 mM MgCl₂; Sigma Aldrich, Ireland) and 100 μl of cell lysate and 100 μl of H₂O were added. Following incubation at 37 °C for 30 min, the reaction was stopped using 500 μl of 0.1 M NaOH. Using p-nitrophenol (10 mM, Sigma Aldrich, Ireland) as standard, the optical density (OD) was measured at 405 nm (Varioskan Flash, Thermo Scientific, UK). The activity of ALP was expressed as the OD divided by the incubation time and the total protein content was assayed by the Bio-Rad Laboratories (UK) protein assay. All materials for this assay were bought from Sigma Aldrich (Ireland).
3.2.8. Osteoblast mineralisation assessment

Osteoblasts mineralisation was assessed using alizarin red staining at days 7, 14 and 21. Cells were fixed in 10 % formalin (Sigma Aldrich, Ireland) for 30 min at room temperature and then stained with 40 mM (pH 4.1) alizarin red (Sigma Aldrich, Ireland) for 30 min, before washed with distilled H$_2$O. Stained monolayers were visualised by bright-field microscopy, using an inverted microscope (Leica S 40/0.45, 10X). Alizarin red was extracted and quantified by adding 10 % v/v acetic acid to each sample for 30 min. The slurry was overlaid with mineral oil (Sigma Aldrich, Ireland) and heated to 85 °C for 10 min, then transferred to ice for 5 min, followed by centrifugation at 20,000 g for 15 min. 10 % v/v ammonium hydroxide was added to the supernatant to neutralise the acid, the pH was confirmed to be between 4.1 and 4.5. Aliquots of the supernatant were read at 405 nm in 96-well plate using a microplate reader (Thermo Scientific, Varioskan Flash). The results were normalised to total cellular protein values, measured in cell lysates by the Bradford method (Bio-Rad Laboratories, UK).

3.2.9. Immunofluorescence assessment of osteocalcin (OCN) and osteopontin (OPN)

At the end of the culture time, the cells were fixed with 4 % paraformaldehyde for 30 min at room temperature. The cells were then washed x3 with phosphate buffered saline (PBS, Sigma Aldrich, Ireland) and permeabilised with 0.2 % TritonX-100 (Sigma Aldrich, Ireland) for 10 min at room temperature, followed by x3 5 min washes with PBS. The cytoskeleton of the cells was stained with rhodamine-conjugated phalloidin (Molecular Probes, Ireland) for 1 h and the
nuclei were then stained with 4,0,6-diamidino-2-phenylindole (DAPI, Molecular Probes, Ireland) for 5 min. Three 5 min washes with PBS were carried out between and after these stains. Images were captured at 10X magnification using an inverted BX51 Olympus (Japan) fluorescence microscope. Following permeabilisation with Triton X (see above), all samples were incubated for 1 hour at room temperature in 1 % BSA / PBS followed by addition of anti-OCN antibody (1:100, v-19, sc-18319, Santa Cruz Biotechnology, USA) / anti-OPN antibody (1:100, k-20, sc-10591, Santa Cruz Biotechnology, USA) and incubated overnight at 4 °C. The cells were then washed x3 for 5 min in PBS. Images were captured at 10X magnification using an inverted BX51 Olympus (Japan) fluorescence microscope.

3.2.10. Statistical analysis

Data were analysed using SPSS 21.0 software for Mac (SPSS Inc., Chicago, IL, USA). Normal distribution was assessed using Kolmogorov-Smirnov test, whilst equal variance was assessed using Levine test. For parametric statistics, one-way analysis of variance (ANOVA) was employed for multiple comparisons, whilst for non-parametric statistics Kruskal-Wallis was used for multiple comparisons. Statistical significant was accepted at $p < 0.05$. Data are expressed as mean ± standard deviation.
3.3. Results

3.3.1. Fibre morphology assessment

SEM micrographs (Figure 3.1) revealed that all scaffolds exhibited a bead-free morphology. Fibre diameter distribution analysis (Figure 3.2) showed that all treatments had an average fibre diameter in the range of 350 to 480 nm. The addition of HA and FC appear to decrease fibre diameter; this trend became significant ($p < 0.001$) for the 0.1 % and 1 % HA and FC concentrations (Table 3.1).
**Figure 3.1:** SEM analysis revealed that all fibres exhibited a bead-free morphology.
Figure 3.2: Fibre diameter distribution analysis revealed that all groups had an average fibre diameter in the range of 350 to 480 nm. A: PLGA; B: PLGA + 0.01 % HA; C: PLGA + 0.1 % HA; D: PLGA + 1 % HA; E: PLGA + 0.01 % FC; F: PLGA + 0.1 % FC; G: PLGA + 1 % FC.
3.3.2. Thermal and mechanical assessment

Table 3.1 summarises the thermal and mechanical properties of the scaffolds produced in this study. Neither the addition of HA nor the addition of FC significantly affected the thermal properties of the electro-spun fibres ($p > 0.05$). From all the HA and FC concentrations (0.01 %, 0.1 % and 1 %), only the 1 % HA significantly reduced force at break ($p < 0.001$), in comparison to PLGA alone. The addition of 1 % HA and FC resulted in significant decrease of stress at break and strain at break values ($p < 0.001$), in comparison to PLGA alone.

At 0.01 % functional molecule concentration, FC fibres had significantly higher force at break ($p < 0.001$), but no difference was observed in stress and strain at break values ($p > 0.05$). At 0.1 % functional molecule concentration, no significant differences were observed between HA and FC fibres ($p > 0.05$). At 1 % functional molecule concentration, FC fibres exhibited significantly higher force at break values ($p < 0.001$), but no difference was observed in stress or strain at break values ($p > 0.05$).
Table 3.1: Thermal, structural and mechanical properties of the scaffolds produced in this study. The addition of HA and FC did not significantly affect the thermal properties of the electro-spun fibres ($p > 0.05$). Only the addition of 0.1 % and 1 % HA and FC concentrations significantly decreased fibre diameter ($p < 0.001$). The 0.01 % FC fibres had significantly higher force at break ($p < 0.001$) than the 0.01 % HA fibres, but no difference was observed in stress and strain at break values ($p > 0.05$). No significant differences were observed between HA and FC fibres at 0.01 % concentration ($p > 0.05$). The 1 % FC fibres exhibited significantly higher force and stress at break values ($p < 0.001$) than the 1 % HA fibres, but no difference was observed in strain at break values ($p > 0.05$).

<table>
<thead>
<tr>
<th></th>
<th>Onset (°C)</th>
<th>Peak (°C)</th>
<th>Thickness (mm)</th>
<th>Fibre Diameter (μm)</th>
<th>Force at Break (N)</th>
<th>Stress at Break (MPa)</th>
<th>Strain at Break (%)</th>
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<tbody>
<tr>
<td><strong>PLGA</strong></td>
<td>44.32 ± 3.05</td>
<td>50.45 ± 1.76</td>
<td>0.10 ± 0.02</td>
<td>0.48 ± 0.12</td>
<td>3.18 ± 1.36</td>
<td>2.69 ± 1.88</td>
<td>21.33 ± 5.84</td>
</tr>
<tr>
<td><strong>0.01 % HA</strong></td>
<td>43.31 ± 7.04</td>
<td>48.15 ± 3.29</td>
<td>0.15 ± 0.02</td>
<td>0.44 ± 0.10</td>
<td>2.62 ± 0.63</td>
<td>1.84 ± 0.64</td>
<td>20.34 ± 4.21</td>
</tr>
<tr>
<td><strong>0.1 % HA</strong></td>
<td>46.09 ± 1.11</td>
<td>50.01 ± 0.39</td>
<td>0.22 ± 0.05</td>
<td>0.35 ± 0.11</td>
<td>2.92 ± 1.94</td>
<td>1.33 ± 0.82</td>
<td>17.82 ± 7.54</td>
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<tr>
<td><strong>1 % HA</strong></td>
<td>40.40 ± 8.79</td>
<td>51.78 ± 0.76</td>
<td>0.24 ± 0.06</td>
<td>0.35 ± 0.07</td>
<td>0.61 ± 0.25</td>
<td>0.27 ± 0.13</td>
<td>8.61 ± 5.67</td>
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<tr>
<td>FC</td>
<td>Value 1 ± Standard Deviation</td>
<td>Value 2 ± Standard Deviation</td>
<td>Value 3 ± Standard Deviation</td>
<td>Value 4 ± Standard Deviation</td>
<td>Value 5 ± Standard Deviation</td>
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<tr>
<td>0.01%</td>
<td>48.07 ± 0.77</td>
<td>50.03 ± 4.45</td>
<td>0.10 ± 0.09</td>
<td>0.41 ± 0.15</td>
<td>3.23 ± 0.77</td>
<td>2.04 ± 1.28</td>
<td>19.63 ± 3.78</td>
</tr>
<tr>
<td>0.1%</td>
<td>45.58 ± 1.53</td>
<td>49.92 ± 0.68</td>
<td>0.15 ± 0.03</td>
<td>0.44 ± 0.07</td>
<td>2.67 ± 0.92</td>
<td>1.85 ± 0.75</td>
<td>17.89 ± 0.75</td>
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<tr>
<td>1%</td>
<td>45.84 ± 0.9</td>
<td>50.7 ± 0.12</td>
<td>0.21 ± 0.04</td>
<td>0.37 ± 0.09</td>
<td>2.85 ± 1.35</td>
<td>1.31 ± 1.48</td>
<td>12.35 ± 2.92</td>
</tr>
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</table>
3.3.3. Osteoblast morphology, metabolic activity, viability and proliferation assessment

Immunofluorescence staining demonstrated that all preparations supported osteoblast growth and electro-spun scaffolds, in particular HA and FC loaded scaffolds (qualitative analysis), restricted osteoblast spreading, as opposed to tissue culture plastic (Figure 3.3). Osteoblast viability (Figure 3.4), metabolic activity (Figure 3.5) and proliferation (Figure 3.6) were increased ($p < 0.05$) at day 11 and day 21, as compared to day 1, but no significant difference ($p > 0.05$) was detected at a given time point between the groups.
Figure 3.3: All scaffolds supported osteoblast growth and the electro-spun scaffolds, especially the HA and FC functionalised (qualitative observation), restricted cytoskeleton spreading. Red = Actin cytoskeleton, Blue = DAPI stained nuclei.

**Red:** Actin fibres (Rhodamine Phalloidin), **Blue:** Nuclei (DAPI)
Figure 3.4: Osteoblast viability was significantly higher \((p < 0.05)\) at day 11 and day 21 than at day 1, but no significant difference \((p > 0.05)\) was observed between the groups at a given time point.
Figure 3.5: Osteoblast metabolic activity was significantly higher ($p < 0.05$) at day 11 and day 21 than at day 1, but no significant difference ($p > 0.05$) was observed between the groups at a given time point.
Figure 3.6: Osteoblast proliferation was significantly higher ($p < 0.05$) at day 11 and day 21 than at day 1, but no significant difference ($p > 0.05$) was observed between the groups at a given time point.
3.3.4. Osteoblasts’ ALP activity and mineralisation assessment

At day 7, no significant difference ($p > 0.05$) was observed in alkaline phosphatase activity between the groups (Figure 3.7 a). At day 14 and 21, 1 % HA and 1 % FC exhibited significantly higher ($p < 0.05$) alkaline phosphatase activity than the PLGA alone scaffolds and tissue culture plastic (Figure 3.7 a). In general, an increase in alkaline phosphatase activity was observed at a given time point as the concentration of HA or FC were increased (Figure 3.7 a).

At day 7 and day 14, the 1 % HA electro-spun scaffolds induced the highest ($p < 0.05$) calcium deposition (Figure 3.7 b). At day 21, the 0.1 % and 1 % HA and the 1 % FC exhibited significantly higher ($p < 0.05$) calcium deposition than PLGA alone scaffolds and tissue culture plastic (Figure 3.7 b). In general, an increase in calcium deposition was observed at a given time point as the concentration of HA or FC were increased (Figure 3.7 b). Visual assessment of alizarin red stained samples (Figure 3.8) did not reveal any significant differences in matrix mineralisation between the groups (tissue culture plastic, PLGA alone and PLGA with various concentrations of HA and FC).
Figure 3.7: At day 21, 1% HA and 1% FC exhibited the highest alkaline phosphatase activity (a). At day 21, 0.1% HA, 1% HA and 1% FC exhibited significantly higher calcium deposition than PLGA alone scaffolds and tissue culture plastic (b). Data presented as mean ± SD. One-way and two-way ANOVA was performed. *p < 0.05
**Figure 3.8:** Visual assessment of alizarin red stained samples did not reveal any significant differences in matrix mineralisation between the groups.

<table>
<thead>
<tr>
<th></th>
<th>TCP</th>
<th>PLGA</th>
<th>PLGA + HA 0.01%</th>
<th>PLGA + HA 0.1%</th>
<th>PLGA + HA 1%</th>
<th>PLGA + FC 0.01%</th>
<th>PLGA + FC 0.1%</th>
<th>PLGA + FC 1%</th>
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3.3.5. Expression of osteonectin and osteopontin

At day 21, osteocalcin (Figure 3.9) was evidenced on tissue culture plastic and PLGA substrates, whilst osteopontin (Figure 3.10) was detected on all samples (tissue culture plastic, PLGA alone and PLGA with various concentrations of HA and FC).
Figure 3.9: Immunofluorescent staining for osteocalcin was positive on osteoblasts grown on tissue culture plastic and PLGA alone electro-spun fibres. Note: Staining at day 21. Red: Actin fibres (Rhodamine Phalloidin); Blue: Nuclei (DAPI); Green: Osteocalcin staining.
Figure 3.10: Immunofluorescent staining for osteopontin was positive on all samples assessed. Note: Staining at day 21. Red: Actin fibres (Rhodamine Phalloidin); Blue: Nuclei (DAPI); Green: Osteocalcin staining.
3.4. Discussion

Given that imprinting has failed to direct functional regeneration in vivo [65, 66], electro-spinning has remained the sole nano-fabrication technology that can produce functionalised with bioactive / therapeutic cargos three-dimensional substrates for regenerative medicine purposes [67-70]. Specifically to bone tissue engineering, electro-spun scaffolds, loaded with a growth factor [71-73] or a viable cell population [74, 75] or with both [76, 77], have been shown to promote functional repair and regeneration in various preclinical models. Despite these promising preliminary results, none of these technologies has been clinically translated. We attribute this limited clinical translation to the complexity of the proposed devices. Although scaffolds loaded with sulphated GAGs and carbohydrates have been shown to direct stem cell lineage commitment [78, 79] and to constitute a suitable biomimetic device for various clinical indications, including skin [80-82], cartilage [83-85], bone [33, 86, 87], scaffolds loaded with non-sulphated polysaccharides (e.g. HA) have primarily been studied as adhesion prevention devices [88-90]. Herein, as first, the influence of non-sulphated polysaccharides (HA and FC) on the biophysical and biomechanical properties of electro-spun scaffolds and their ability to promote in vitro osteogenesis was assessed.

Starting with structural characterisation, SEM micrographs revealed bead-free scaffold morphology, with mean fibre diameter in the range of 350 to 480 nm. This fibre diameter distribution is in accordance to previously produced electro-spun meshes out of, for example, gelatin and collagen [91], PLGA-gelatin-elastin [92], low concentration PLLA [93], PLLA-collagen-nano-hydroxyapatite [94] and 80 % HA – 20 % collagen [95]. It is worth pointing out other studies with
similar diameter of silk fibroin electro-spun constructs supported significantly higher cell proliferation and expression of extracellular matrix genes than electro-spun fibres with average fibre diameter of \( \sim 1 \mu \text{m} \) [96].

The addition of 0.01 % HA and FC did not appear to affect significantly fibre diameter, which is in accordance to previous studies, where PCL fibres were functionalised with various peptides [97]. The addition of higher concentrations (0.1 % and 1 %) of HA and FC significantly reduced fibre diameter. Although this observation is in contradiction with previous studies, where collagen and PCL fibre diameter was increased as the concentration of nano-hydroxyapatite was increased [98-100], it is in agreement with other studies, where the diameter of PLA was decreased as the concentration of multi-walled carbon nano-tubes was increased [101] and the diameter of PCL/PLA was decreased as the concentration of hydroxyapatite was increased [102]. In both cases, the authors speculated that this reduction in diameter was due to the reduction in the viscosity of the dispersion, which is known to affect fibre diameter. It is interesting to note that the addition of HA in collagen also resulted in increased fibre diameter up to ratio 8 g/ml collagen / 1.5 g/ml HA; however at ratio 8 g/ml collagen / 2 g/ml HA, the fibre diameter was decreased [103]. Unfortunately, higher ratios of HA to collagen were not assessed to see whether this trend would have continued. Intermediate PEG and NaCl concentrations (20% as opposed to 5 % and 40 %) have also been shown to reduce the diameter of extruded collagen fibres due to salting-in / salting-out effect [63, 104]. Further, physiologically speaking, it is of interest to note that excessive HA inhibits lateral growth of collagen fibrils [105], which again may explain this reduction in fibre diameter as a function of increased HA and FC concentration.
Neither the addition of HA nor the addition of FC affected the denaturation temperature of the scaffold. This is in accordance to previous publication, where the addition of FC did not affect the thermal properties of collagen films [57]. With respect to HA, it is worth pointing out that HA functionalised scaffolds have been reported twice to exhibit higher denaturation temperature than the non-functionalised scaffolds. However, the first study provided only the absolute mean values of two replicates and, as such, it is inconclusive [106] and, in the second study, only the 15 % HA loaded scaffold exhibited higher denaturation temperature than the non-functionalised scaffold and the 7.5 % HA functionalised scaffold [107]. In any case, all produced scaffolds herein exhibited denaturation temperature well-above body temperature, which clearly indicates their suitability for biomedical applications.

With respect to the biomechanical properties, all treatments but the 0.01 % FC, resulted in electro-spun fibres with reduced force, stress and strain at break values, as compared to the non-functionalised PLGA electro-spun fibres. Previous studies have also shown that increasing concentrations of PEG, NaCl and dermatan sulphate are associated with reduction in the biomechanical properties of reconstituted collagen [63, 104, 108]. Similar results have been observed with incorporation of hydroxyapatite in PLACL/gelatin [109] and PCL [100] electro-spun scaffolds; a reduction in tensile strength, tensile strain and elastic modulus was recorded. We speculate that at 0.01 % FC the optimal free water content / balance is maintained, whilst at higher concentration of FC and at all HA concentrations, all free water is removed. The influence of intra- and extra- cellular water on the mechanical properties of articular cartilage has been explained using a chemo-mechanical model [110]. It is worth pointing out that
Chapter 3 - Electrospinning

the fibres produced in this study had similar or superior mechanical properties similar to previously produced PLGA [58], collagen type I [111], collagen type II [112], gelatin [113], PCL [114, 115], hydroxyapatite-PCL [100, 116], peptide-PCL [97], gelatin-PCL [117], pullulan / dextran [118] electro-spun scaffolds, to mention only a few.

With respect to biological analysis, all scaffolds, independently of the presence or absence of HA and FC, supported osteoblast growth with no apparent differences in viability, metabolic activity and proliferation. This is in accordance to previous publications, where: increasing concentrations of HA in collagen electro-spun collagen fibres did not affect viability of human foreskin fibroblasts [103]; increasing concentrations of HA in collagen scaffolds did not affect pre-adipocyte proliferation [107]; increasing concentrations of multi-walled carbon nano-tubes in PLA electro-spun fibres did not affect osteoblast proliferation [101]; increasing concentrations of hydroxyapatite in PCL/PLA electro-spun fibres did not affect MC3T3-E1 osteoblast-like cell proliferation [102]; and incorporation of hydroxyapatite in PLACL/gelatin electro-spun scaffolds did not affect proliferation of human foetal osteoblast cells [109]. An increased osteoblast viability and proliferation has been reported previously when high concentrations of peptides were used to functionalise electro-spun PCL fibres [97]. Although FC has been shown to increase the metabolic activity and proliferation of lung fibroblasts [57], it has no effect in viability, proliferation and metabolic activity of skin, lung and corneal fibroblasts, when it is used as a macromolecular crowding agent [55, 56].

The addition of HA and FC in PLGA electro-spun fibres appeared to reduce osteoblast spreading. This is in accordance to previous work, where GAGs were
shown to inhibit both spreading and adherence of osteoclasts and their precursors, illustrating that way the therapeutic potential of GAGs in osteolytic diseases [119]. Further, the addition of HA and FC in PLGA electro-spun fibres appeared to enhance osteoblast mineralisation. This is a very interesting outcome, given that previous studies have shown that the addition of hydroxyapatite in: PLACL/gelatin electro-spun scaffolds did not affect osteoblast mineral deposition, not even after 30 days in culture [109]; PCL/PLA electro-spun scaffolds did not affect MC3T3-E1 osteoblast-like cell mineralisation for up to 15 days in culture [102]; PCL electro-spun scaffolds did not affect osteoblast mineralisation for up to 5 days in culture [100]. This observation further enhances previous studies, where sulphated glycosaminoglycans increased up to 28-fold alkaline phosphatase, osteoprotegerin and osteocalcin gene expression and up to 4-fold calcium deposition in osteoblast culture [34] and promoted osteoclastogenesis through interference with the NF-κB ligand / osteoprotegerin complex formation [120]. In addition, GAGs have been shown to enhance osteoblastic differentiation of mesenchymal stem cells [121], with HA to be the most potent GAG [52]. It is also worth noting that electro-spun GAG-mimetic peptide nano-fibres have been shown to promote osteogenic activity and mineralisation of osteoblastic cells through interaction with BMP-2 [122]. Osteopontin, middle to late stage bone formation marker, was evidenced in all scaffolds, as was observed previously on collagen-GAG scaffolds [123]. The rather limited osteocalcin (late bone formation marker) expression observed herein is not surprising, given that the cells were not extensively mineralised [124]. Further, similar to our data,
osteocalcin expression was not altered on artificial matrices comprised of collagen and different GAG derivatives [54].
3.5. Conclusion

Herein we demonstrated that as low as 0.01 % HA and FC functionalisation of PLGA electrospun fibres not only does not compromise the mechanical properties of the scaffold, but also enhances osteoblast adhesion, growth, proliferation and early osteogenesis. This study further supports current trends towards functionalisation of implantable devices with carbohydrates moieties for functional tissue engineering applications.
3.6. References


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Chapter Four

Summary and Future Studies
4.1. Summary

The aim of this study was to develop anisotropic substrates with optimal dimensionality and functionalised with sugar moieties that would control osteoblast response \textit{in vitro} and promote functional bone regeneration \textit{in vivo}. Imprinted work revealed that topographical features in the middle nano-range (~306 nm) and low micron-range (~2,046 nm) induce cellular and matrix alignment and upregulation of osteogenic markers \textit{in vitro}. However, these topographical features did not induce directional cell growth and neotissue formation \textit{in vivo}. These data suggest that topographical features in two-dimensional content (surface) can be utilised to maintain cell phenotype during \textit{in vitro} cell expansion, enhancing that way clinical translation and commercialisation of cell-based therapies. Although it was not shown specifically here, three-dimensional scaffold fabrication technologies (e.g. electro-spinning, freeze drying, additive manufacturing) are more likely to result in functional repair and regeneration \textit{in vivo}, as they allow cell infiltration and parallel to the porosity of the scaffold cell and matrix alignment. Although functionalisation strategies are traditionally utilising proteins, growth factors, genes and drugs, recent data advocate the use of carbohydrates, as sugars are essential components of the human body and are involved in numerous physiological processes. Preliminary data from electrospun isotropic substrates functionalised with sugars presented herein advocate the use of non-sulphated polysaccharides in bone repair and regeneration.
4.2. Future studies

4.2.1. Imprinting

**Project No 1:** Data presenting herein advocate the use of nano- to low micro-imprinting technologies to maintain cell phenotype *in vitro*. However, herein only three topographies were assessed with the biggest one being in the low micron range. It would be valuable to assess the influence of larger topographical features (e.g. 50, 100, 250, 500 μm) in neotissue formation.

**Project No 2:** Recent data have made apparent the importance of multifactorial approaches to maintain permanently differentiated cell phenotype and / or to direct stem cells towards specific lineage. In scaffold fabrication space, the significance of substrate rigidity in controlling cellular functions *in vitro* has been well established. However, the simultaneous effect of surface topography and substrate rigidity has yet to be elucidated. Thus, a potential future project could be to fabricate substrates with variable surface topography and substrate rigidity to assess permanently differentiated cell phenotype maintenance and / or stem cell lineage commitment.

**Project No 3:** Cell sheet utilises flat substrates that are unsuitable for hierarchical tissues, such as tendon or corneal stromal. It would be of significant importance to bring imprinting technologies in the field of cell sheet tissue engineering for the development of hierarchical tissues *in vitro.*
4.2.2. Electro-spinning

**Project No 4:** This study carried out a preliminary assessment of non-sulphated polysaccharides in osteoblast response *in vitro*. The obvious follow up study is to assess the optimal scaffold in a preclinical model.

**Project No 5:** Herein, the influence of non-sulphated polysaccharides was assessed. Of significant importance would be to assess the influence sulphated glycosaminoglycans and mimetics of thereof in cell phenotype maintenance *in vitro* and functional repair and regeneration *in vivo*.

**Project No 6:** Given that glycosaminoglycans in the body are linked to core proteins forming proteoglycans, it is of significant importance to assess the influence of proteoglycans on cell phenotype maintenance *in vitro* and on neotissue formation *in vivo*. 
Appendix
A. Electro-spinning

Materials and equipment

1. Personal protective equipment.
2. Laboratory coat, powder free gloves, face mask and eye goggles must be used when preparing the solution.
3. All solutions must be prepared inside the fume hood.
4. 50ml centrifuge tubes.
5. Parafilm.
6. Laboratory tube rotator (suitable for 50ml tube).
7. Leur-lock syringes.
8. Leur-lock blunt tip needles.

Procedure

1. Preparing the solution.
2. Weigh the polymer.
3. Place it in a 50 ml tube.
4. Add required amount of solvent to the polymer using a Pasteur pipette (to be done in fume hood).
5. Secure cap on tube.
6. Cover with tube cap with Parafilm™.
7. Place in rotating mixer until completely dissolved.
8. Using electro-spinning rig.

9. Turn on the fume hood.

10. Select the desired mandrel geometry.

11. Cover the mandrel with aluminium foil.

12. Connect the mandrel securely to the rotating motor by completely closing the mandrel chamber.

13. Under a fume hood fill the syringe with the desired amount of polymer solution.

14. Place the syringe in the syringe pump and lock in place.

15. Set the displacement of the syringe pump by adjusting the distance between the two limit switches on the linear table. Set the distance between the tip of the needle and the mandrel by moving the syringe pump platform (the platform should be placed in such a way that the linear table is parallel to the mandrel's longitudinal).

16. Turn the syringe pump on.

17. Set the pump’s parameters (syringe diameter, flow rate).

18. Start the syringe pump.

19. Close the fume hood (Verify that the inter-lock on the left of the fume hood is completely closed).

20. Over on the control panel, start the linear table, high voltage supply and rotating motor (never touch any cables, the alligator clip or the needle when the electrospinning rig is on).

21. In case of clot formation, stop the process, clean the tip and optimise the process (e.g. solution concentration, distance between needle and collector, voltage, extrusion rate).
Collecting the fibres and switching off

1. When processing is complete, stop all elements (linear table, high voltage supply, rotating motor and syringe pump).
2. Open the mandrel chamber and remove the mandrel.
3. With a blade, cut the processed construct and aluminium foil along the longitudinal axis of the mandrel.
4. Place the mandrel back in its chamber and close it completely.
5. Remove the syringe from the pump.
6. Dispose of the blunt tipped needle in the sharps bin and place the spent syringe in the sharps disposal bin – do not cover the used needle.
7. Make sure that the electrical supply is turned off at the mains, that the Electrospinning Rig is cleaned and close the fume-hood before leaving.
8. Substrate prep and sterilisation
9. Cell culture aseptic technique
10. To ensure that the interior of the hood was sprayed with Virkon for 5 minutes and then with 70 % IMS.
11. Everything entering the hood was sprayed with 70 IMS
12. Any containers must only be opened within the hood. Any containers opened outside the hood should be considered contaminated.
B. Changing media

1. Media was changed every three days.

2. Media and HBSS were placed in the water bath to warm to 37 °C.

3. Under the hood, pour out media and HBSS in to 50 ml falcon tubes and place these into the water bath.

4. Aseptic technique was used, the flask were removed from the incubator and sprayed with IMS.

5. The cells were viewed with the microscope to check how confluent the cells were and to ensure no contamination.

6. The media was removed from the flask and placed in the waste container, which had Virkon in it.

7. The cells were washed with HBSS and the fresh media was added.

8. The flasks were then returned to the incubator.
Chapter 5 - Appendix

C. Passaging cells

1. When cells reached approximately 80 % confluency, they were split into two or more flasks or frozen for later use.

2. Media, HBSS and were placed in the water bath to warm to 37 °C.

3. Aseptic technique was used, the flask were removed from the incubator and sprayed with IMS.

4. The media was removed from the flask and placed in the waste container, which had Virkon in it.

5. The cells were washed with HBSS.

6. Trypsin-EDTA (T/E) was added to the flask, ensuring complete coverage of the flask.

7. The flask was returned to the incubator for 5 minutes (enzyme is active at 37 °C).

8. The flask was examined under the microscope to see if cells had detached from the surface.

9. Cells should be rounded and floating in the media, if not lightly tapping the flask may remove the remaining cells or replace in the incubator for another 5 minutes. It is not recommended to have the cells in the incubator for longer than 10 minutes.

10. Once cells were detached from the surface the flask is placed back into the incubator.

11. Equal volume of media supplemented with 10 % serum was added to the flask. This deactivates the trypsin.
12. All of the liquid was then removed and put into a sterile centrifuge tube and centrifuged for 5 minutes at 1200 rpm (placing a counter weight in the centrifuge to ensure it is balanced.

13. The tubes were removed. The cells formed a pellet at the bottom of the tube.

14. The supernatant was removed and the pellet was re-suspended in fresh media.

15. 1 ml pipettes used to re-suspend the pellet and to add more media if required.

16. Air bubbles should be avoided, when re-suspending cells.

17. The cells were then counted and seeded into new flasks.
D. Freezing cells

1. To freeze cells, carry out the passaging protocol.

2. However, instead of resuspending the pellet in media, cells are resuspended in freezing media. Freezing media consists of media supplemented with 10% FBS and 10% filtered DMSO. (DMSO is a cryo-protective agent).

3. Generally, there is 1 ml per vial containing 500,000 to 2 million cells.

4. Cells were placed in Mr Frosty container and placed in the – 80 °C freezer.

5. Wear protective gloves and face shield when adding cryo-vials from liquid nitrogen cylinder.

6. Cells were then put into liquid nitrogen after 24 hours.
E. Thawing cells

1. Wear protective gloves and face shield when removing cryo-vials from liquid nitrogen cylinder.
2. Remove the required number of vials from the liquid nitrogen container.
3. Place the vial in the water bath, just long enough to thaw the solution. Do not cover the vial as this can lead to contamination.
4. Spray the vial with IMS before placing into the hood.
5. DMSO needs to be removed, as it is toxic.
6. Cells are immediately placed in to pre-warmed media in a sterile centrifuge tube.
7. The cells were centrifuged for 5 minutes at 1200 rpm.
8. The supernatant media was aspirated leaving the cell pellet at the bottom of the tube.
9. The cells were re-suspended in media.
10. The cells were counted and plated out at 4000 cells/cm².
F. Cell counting

1. 50 µl of cell suspension was mixed with 50 µl trypan blue

2. 10 µl of the cell / trypan blue solution was added to each side of the haemocytometer

3. Trypan blue is excluded by the live cells and penetrates the dead cells due to their damaged membranes – blue cells are dead, clear cells are alive.
G. Staining for rhodamine phalloidin and DAPI

Materials

1. HBSS
2. 1x PBS
3. DAPI (0.1 % ddH$_2$O)
4. Rhodamine Phalloidin (1:100)
5. 4 % Paraformaldehyde (PFA)
6. Triton X-100 (0.2 % PBS)
7. Blocking solution (1 % BSA in 1x PBS)

Methods

1. Remove media from the cells.
2. Wash the cells twice with HBSS.
3. Fix the cells with 4 % PFA for 15mins at room temperature.
4. Wash the cells 3 times with 1x PBS.
5. Permeabilise the cells with Triton X for 5 min at room temperature.
6. Block with the blocking solution for 30 min at room temperature (optional).
7. Wash the cells 3 times with 1x PBS.
8. Incubate with the rhodamine-phalloidin solution for 30 minutes to one hour, at room temperature, protected from light.
9. Wash the cells 3 times with 1x PBS.
10. Stain the nuclei with DAPI for 5mins at room temperature.
11. Wash the cells four times with 1x PBS and observe using an inverted fluorescence microscope.
H. Cell metabolic activity assay

Materials

1. Hank’s balanced salt solution
2. AlamarBlue®

Methods

1. Add 1000 μl of Hank’s balanced salt solution into the required number of wells in the sterile 24 well plate i.e. same number as samples to be tested.
2. Make up the solution of alamarBlue® in Hank's balanced salt solution (ratio 1:9 respectively). 500 μl is required per well.
3. Transfer the seeded scaffolds/tissue culture inserts from their original well plate to the Hanks solution well plate using the sterile tweezers.
4. Remove the Hanks solution from each well.
5. Cover the scaffolds and positive control (empty wells washed with Hanks) with 500 μl of the alamarBlue® in Hank's balanced salt solution (ratio 1:9).
6. Incubate for 2 hours at 37 °C.
7. After incubating for 2 hours, transfer 200 μl of the dye into the clear 96 well plate.
8. Measure the absorbance at 550 nm and 595 nm (0.5 seconds per well).
9. Calculate a viability value according to ‘simplified method of calculating % reduction’ available in the alamarBlue® handbook.
10. Subtract the absorbance values of Hank’s balanced salt solution only from the absorbance values of the alamarBlue® in Hank's balanced salt solution
(ratio 1:9). $AO_{LW} =$ absorbance of oxidized form at lower wavelength, and $AO_{HW} =$ absorbance of oxidized form at higher wavelength.


12. $R_O = AO_{LW}/AO_{HW}$

13. To calculate the % of reduced alamarBlue™:

14. $AR_{LW} = ALW - (AHW \times R_O) \times 100$
I. Live/Dead assay

1. Take kit out of freezer, defrost tubes.

2. Place in the centrifuge for a few seconds to ensure contents are at the bottom of the tube.

3. Media was removed from chamber/wells and cells were was washed with HBSS.

4. For a dead control immerse cells in 70 % Methanol.

5. Prepare staining solution.
   a. Calcein (live) is at 4 mM concentration in the tube. Use 4 µl.
   b. Dilute 1:1000 – 5 µl in 5 ml.

6. Ethidium homoiomer-1 (dead) is at 2 mM. use at 2 µM.
   a. Dilute 1:1000 – 5 µl in 5 ml.

7. Protect from light.

8. Add enough stain to cover the substrates.

9. Incubate for 30 minutes.

10. The live cells were imaged using FITC filter and the dead cells were imaged using the Texas red filter.
J. RNA extraction protocol

1. At the predefined time to analyse cells for RNA content, wash scaffold / cells with Hanks balanced Salt solution.
2. Microbiology Hood Ground Floor Lab.
3. Add 250 μl (12-well chamber slide, double for 8well chamber slide) of TRI Reagent® to wells containing scaffolds/cells.
4. Homogenize samples using the tip of a pipette (using a scrapping motion on entire surface, then aspirate 4-6 times) being careful not to contaminate adjacent wells. Make sure scaffolds have been completely homogenized.
5. Using a 1 ml pipette tip, aspirate the solution repetitively.
6. Store homogenate for 5 minutes at room temperature to dissociate nucleoprotein complexes.
7. Remove the TRI Reagent® solution to a sterile 1.5 ml eppendorf.
8. Note: Tri/Lysate solution can be frozen at this point at -80 °C for approximately a month.
9. Add 50 μl of chloroform per 0.25 ml of TRI Reagent®.
10. Sake vigorously for 15 seconds by inversion.
11. Incubate for 15 minutes at room temperature.
12. Centrifuge at 12,000g for 15 minutes at 4 °C. Following the centrifugation, 3 phases will appear; -a lower red phenol-chloroform phase, an inter-phase, and an aqueous phase (translucent). mRNA is located within the aqueous phase.
13. Remove the clear upper aqueous phase (~ 100 μl) to a sterile eppendorf. Be careful not to touch the interface. Leave a little of the upper phase to avoid contact with the interface.
14. Slowly add 1 volume of 70% ethanol and mix by inversion.
15. Add sample from step 12 to RNeasy column.
16. Centrifuge at 8,000g for 15 seconds and discard the collected solution.
17. Repeat step 13 and 14 for remaining sample.
18. Add 350 µl of RW1 buffer to centre of column, centrifuge at 8,000g for 15 seconds. Discard the collected solution.
19. Transfer column to new collection tube. Add 500 µl RPE to centre of column, centrifuge at 8,000g for 15 seconds. Discard the collected solution.
20. Add 500 µl RPE to centre of column, centrifuge at 8,000g for 2 seconds. Discard the collected solution.
21. Transfer column to a new 1.5 ml eppendorf. Add 20 µl RNase-free water onto the column, incubate at room temperature for 1 minute and centrifuge for 1 minute at 8,000g.
22. Place the flow through solution onto the column again, incubate at room temperature for 1 minute and centrifuge at 8,000g for 1 minute.
23. MDRG Lab NanoDrop
24. Determine the concentration using the NanoDrop and freeze at -80 °C.
25. Calibrate the spectrometer with water.
26. Place 1-1.2 µl on the NanoDrop. The purity is determined from the ratio between A260 and A280. The ratio A260/A280 should be above 1.8 to indicate a pure form of RNA.
27. Print of report and place in lab book comment on any irregularities.
K. Alkaline phosphatase assay

Materials

- Lysis Buffer: 0.1% Triton-X in 10mM Trizma-Base (or Trizma-HCl) (Sigma T6066), adjusted to pH 7.4 with HCl.
- Phosphatase substrate: 4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma, #N-2765, 20mg tablets)
- Standard Solution: p-Nitrophenol (Sigma, #N7660 10 mM) (not in solution)
- Alkaline Buffer Solution: 2-Amino-2-methylpropanol 1.5 M (Sigma, #A-9226) OR 2-Amino-2-methylpropanol 10.48M (Sigma, #A-9199 Syrup)
- Diethanolamine (Sigma, #D-0681, Syrup)
- MgCl₂ (Fluka, #63064)
- 96-well plate, flat bottomed and transparent
- 0.1M NaOH

Procedure

2D or 3D cultures of cells are washed with PBS (similar volume to media) and the cell layer is lysed with lysis buffer for at least 60 minutes at 4-8 °C on a gyratory shaker in the cold room.

ALP quantification

Solutions can be prepared depending on the number of samples to be evaluated and the volume of the cell lysate.

Preparation of samples
Samples are incubated with lysis buffer 0.1 % Triton-X in 10mM Tris-HCl, pH 7.4 and stored at 4 °C under agitation for min. 60 minutes. Samples are stored frozen at -20 °C until analysis.

**Preparation of solutions for ALP quantification**

- Prepare 0.5mM MgCl$_2$ (112.5 mg/l)
- Diethanolamine Buffer: 1M Diethanolamine in 0.5 mM MgCl$_2$:
  Add 424 µl of Diethanolamine to 4 ml of 0.5 mM MgCl$_2$, pH to 9.8 at room temperature.
- Alkaline Buffer: Adjust the 1.5M solution (#A9226) to a pH 10.3 at 25 °C OR Dilute the syrup (#A9199) to 1.5M by the addition of MilliQ water, with dilution of 1:7. Adjust the pH to 10.3 at 25 °C. Store at 4-8 °C.
- Substrate solution: Dissolve 5 tablets of Phosphatase substrate (20 mg) in 3.8 ml of the Diethanolamine Buffer to a final concentration of 25 mg/ml. The substrate solution can be stored at –20 °C for 6 weeks in a light-shielded tube.

**Preparation of standards**

- p-Nitrophenol Standard: Dilute 10mM P-Nitrophenol with lysis buffer (0.1 % Triton-X in 10mM Trizma-Base) 1:10 to a final concentration of 1 µM.
- Prepare 8 eppendorf with 0, 10, 60 and 70 µl of diluted p-nitrophenol, make up to 100 µl with lysis buffer.
- Cool to 4 °C for 30 minutes.
Place the tubes with standards on ice and pipette into the tubes and add 250 µl of alkaline buffer, 100 µl of H₂O, 50 µl of substrate solution.

Keep at 37°C in a heating block/water bath for exactly 15 minutes.

Add 500 µl of 0.1 M NaOH.

Store at room temperature for later measurement. Final concentration will be 0 to 70nM in the standard curve.

**Assaying samples**

- Thaw each sample and keep at 2-8°C for at least 30 min.
- Add the following reagents to a eppendorf tube (in this order): 250 µl Alkaline buffer, 50 µl substrate solution, 100 µl cell lysate, 100 µl H₂O

Note: For samples with expected high levels of ALP this can be diluted with lysate buffer or for samples where low levels of ALP are expected 200 µl of cell lysate can be added and the 100 µl H₂O omitted.

- Vortex.
- Immediately incubate at 37 °C in a heating block/water bath.
- After 15min check samples, if necessary incubate samples longer.
- Remove from 37 °C and stop the reaction by adding 500 µl of 0.1 M NaOH.
- Measure aliquot of each sample / standard in duplicate wells (250 µl each) on plate reader in a transparent flat-bottom well-plate at 405 nm.
• Add 50 μl of concentrated HCl to each well, and measure again.
  Subtract second measurement form first, to remove interference.

The measured values are divided by the incubation time in minutes. So the result represents the enzyme activity per minute, and should be represented as a function of total protein OR total DNA OR cell density.
L. Alizarin red staining

Materials

- Alizarin red
- Ammonium Hydroxide (10 %)
- Acetic acid (10 %)
- PBS and ddH2O
- Absorbance plate reader at 405 nm

Alizarin red dye preparation

1. ARS (40 mM) in dH2O: formula - C\textsubscript{14}H\textsubscript{7}O\textsubscript{7}Sna MW: 342.3 g/mole
2. Add 2 g into 100 ml ddH2O.
3. pH was adjusted to 4.1 using 0.5 % (v/v) ammonium hydroxide. pH is critical.
4. Solution can be kept in the dark for up to 6 months.
5. Filter using filter paper before use. Avoid contact and inhalation.

Staining

1. Monolayers in 6-well plates (10 cm\textsuperscript{2}/well) were washed with PBS.
2. Fixed in formalin for 30 min.
3. The monolayers were then washed 3x with PBS.
4. Add 1 mL of 40 mM ARS (pH 4.1) per well, incubate at RT for 5 min.
5. After aspiration of the unincorporated dye, the wells were washed four times with 4mL dH2O while shaking for 5 min and then incubated in PBS for 15 min to eliminate nonspecific staining.
6. Stained monolayers were visualised by bright field microscopy using an inverted microscope (Nikon): Calcium deposits are stained orange-red.

Quantifying extracted dye

1. 800 µL 10 % (v/v) acetic acid was added to each well
2. Incubated at room temperature for 30 min with shaking.
3. Scrape the monolayer and transferred with 10 % (v/v) acetic acid to a 1.5 mL micro-centrifuge tube with a wide-mouth pipette.
4. Vortex for 30 sec.
5. The slurry was overlaid with 500 µL mineral oil (Sigma–Aldrich),
6. Heated to exactly 85 °C for 10 min then transferred to ice for 5 min. Care was taken at this point to avoid opening of the tubes until fully cooled.
7. Centrifuged at 20,000g for 15 min.
8. Transfer 500 µL of the supernatant to a new 1.5 mL micro-centrifuge tube.
9. Add 200 µL 10 % (v/v) ammonium hydroxide to neutralize the acid. Check that pH is between 4.1 and 4.5.
10. 150 µl aliquots of the supernatant were read in triplicate at 405 nm in 96-well format using opaque-walled transparent-bottomed plates (Fisher Life Sciences).
11. Results were then normalised to total cellular protein values, as measured in cell lysate by the Bradford method (Bio-Rad Laboratories, Inc.).
M. Gelatin zymography

10 % acrylamide gelatin gel recipe:

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

8.3 ml of gelatin solution

5.25 ml 1.5 M Tris pH 8.8

7 ml 30% acrylamide-bisacrylamide

0.165 ml 50 % Glycerol

0.165 ml 10 % SDS

0.010 ml TEMED

0.100 ml 10 % APS

Sample preparation and electrophoresis

Add SDS-loading buffer WITHOUT a reducing agent

Add 10 µg or LESS total protein per lane (detection limit for MMP2/9 is on the order of a couple hundred pico gram). Adding more total protein just obscures the result, as abundant proteins will stain with the Coomassie.

Run the gel (20 mA/gel: 0.75 mm thick minigel)

Developing the zymogram

Wash (incubate 1 h at room temp on a rotating shaker to remove the SDS and renature your proteinases) with 2.5 % TritonX100 in 50 mM Tris pH 7.4, 5 mM CaCl₂, 1 mM ZnCl₂.

Rinse briefly with deionized water.

Incubate overnight at 37 °C with 50 mM Tris pH 7.4, 5 mM CaCl₂, 1 mM ZnCl₂, (optional 0.01 % sodium Azide).
Stain with 0.5 % Coomassie G250 in 30 % Ethanol, 10 % acetic acid for 30 minutes (Entire gel should be dark blue)

De-stain in 30 % Ethanol / 10 % acetic acid until you see clear bands -- This can happen fast (a couple of minutes).

Change to 2 % acetic acid to stop staining.

Rehydrate in 2 % acetic acid overnight

Polyacrylamide migration gel was performed at 10 % (ratio Acrylamide/Bis acrylamide 37:1) in 0.375 mM Tris-HCl pH 8.8 contained 1 mg/ml gelatin and 0.1 % sodium dodecyl sulphate (SDS).

Stacking gel contained 4 % polyacrylamide in 0.125 mM Tris, pH 6.8 and 0.1 % SDS. Gels were polymerised by adding 50 μl of 10 % ammonium persulfate and 10 μl of 0.1 % TEMED.

At each culture time point, media from hydrogels NCH or CCH3 were half-diluted in 0.125 mM Tris, pH 6.8, 50 % glycerol, 0.4 % bromophenol blue and deposited within gel wells ($n = 6$).

Then, gels were run under Laemmli conditions (24 mM Tris, 192 mM glycine, 3.47 mM SDS; 40 mA, 1 h).

Following electrophoresis, gels were washed twice (30 min each) in 200 ml of 2.5 % Triton X-100 under constant mechanical stirring and then incubated in 100 mM Tris-HCl pH 7.4, 30 mM CaCl$_2$ for 19 h at 37 °C.

Gels were stained with Coomassie blue R-250 for 2 h (50 % methanol, 10 % acetic acid) and de-stained appropriately (40 % ethanol, 10 % acetic acid). Proteinase activity was evidenced as cleared area.
Finally, the gels were rinsed for 1 h in 5 % ethanol, 7.5 % acetic acid and kept in sealed bags containing distilled water.
N. Detailed Image of Figure 2.17
O. Outputs

Manuscripts


Abstract publications


Conference papers


2. 08/2015: English A, Azeem A, Spanoudes K, Biggs M, Pandit A, Zeugolis DI. Surface topography: Is it clinically relevant? Podium Presentation at European Society for Biomaterials 2015 meeting, 30th of August to 3rd of September 2015, Krakow, Poland


5. 06/2012: Azeem A, English A, Tripathi B, Jones E, Rooney N, Legerlotz K, Riley G, Cross G, Hutmacher D, Pandit A, Zeugolis D. Guiding osteoblast behaviour at the nano-bio-interface by anisotropically ordered surfaces. Poster Presentation at Royal Academy of Medicine in Ireland (RAMI), Section of Biomedical Sciences, Bailey Allen Hall, National University of Ireland Galway, 14th of June 2012, Galway, Ireland

6. 06/2012: English A, Azeem A, Jones E, Legerlotz K, Rooney N, Riley G, Pandit A, Zeugolis D. Tenocytes align perpendicular to the substrate topography in the absence of mechanical loading. Podium Presentation at 9th World Biomaterials Congress, 1st to 5th of June 2012, Chengdu, China


at the 24th European Society for Biomaterials, 4th to 8th of September 2011, Dublin, Ireland


**Patents**


**Courses**

Completed LAST course, March 2011