Bioactive hydrogels for tissue engineering

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A thesis submitted in accordance with the requirements for the Doctor of Philosophy (PhD) degree

Materials Department, Imperial College London

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Declaration

The content of this thesis is entirely my own work except where explicitly stated.
Some of the material in Chapters 1-3 has previously been published in the peer reviewed papers:

Complexity in biomaterials for tissue engineering

Elsie S. Place, Nicholas D. Evans, Molly M. Stevens

Synthetic polymer scaffolds for tissue engineering

Elsie S. Place, Julian H. George, Charlotte K. Williams and Molly M. Stevens

Strontium- and zinc-alginate hydrogels for bone tissue engineering

Elsie S. Place, Luis Rojo Del Olmo, Eileen Gentleman, Jose Sardinha, Molly M. Stevens
Tissue Engineering (accepted, 2011)

Other papers and conference presentations

Manuscript in preparation:
Latent transforming growth factor-β (TGF-β) hydrogels for cartilage tissue engineering
Elsie S. Place, Rekha Nair, Helena Chia, Greg Szulgit, Molly M. Stevens

Conference presentation (poster):
Hydrogels for Orthopaedic Tissue Engineering, Gordon Research Conference: Signal Transduction by Engineered Extracellular Matrices, Biddeford, ME, 26.06.2010 – 02.07.2010
Abstract

Modern tissue engineering (TE) scaffolds are expected to actively promote tissue repair as well as meeting the traditional requirements of non-toxicity, degradability and structural integrity. This thesis presents two novel bioactive hydrogel systems for bone and cartilage TE. A series of alginate hydrogels were developed in which all or a fraction of the calcium normally used for crosslinking alginate was replaced by bioactive strontium and/or zinc ions. Strontium was chosen for its ability to stimulate bone formation, while zinc is essential for alkaline phosphatase activity. Due to an interaction between the crosslinking ion and alginate type, the hydrogel properties could be tailored independently of the crosslinking ion used – meaning that varying biological and materials requirements can be accommodated. Strontium release from alginate gels was of a physiologically relevant magnitude, and alkaline phosphatase protein activity in Saos-2 cells was highest in strontium gels. Secondly, a biomimetic strategy for transforming growth factor beta (TGF-β) presentation and release was evaluated. TGF-β in vivo is secreted as part of an inactive latent complex, which is sequestered in a stable form within extracellular matrix until released by cells. TGF-β was therefore incorporated into poly(ethylene glycol)-hyaluronic acid hydrogels in its latent form. When compared to free TGF-β, advantages were demonstrated in terms of lower protein adsorption to tissue culture plastic and relative biological inactivity. The latter implies that high doses may be loaded into TE scaffolds without exposing cells to excessive quantities of active growth factor, with TGF-β bioavailability then being controlled by gradual activation by cells. Increased metabolic activity and ECM deposition by bovine chondrocytes were seen after almost five weeks in culture with a single initial loading of LTGF-β. These innovations correspond to current TE trends, which seek to use biomimetic principles to evoke regenerative responses from transplanted or host cells, but to do so using technically and commercially feasible means.
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<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>AlgG</td>
<td>Alginate, 55-65% G residues (Protanal LF200DL, FMC Biopolymer)</td>
</tr>
<tr>
<td>AlgG-RGD</td>
<td>AlgG with covalently linked RGD peptide</td>
</tr>
<tr>
<td>AlgM</td>
<td>Alginate, 55-65% M residues (Protanal LF200M, FMC Biopolymer)</td>
</tr>
<tr>
<td>ALMC</td>
<td>Protease inhibitors: aprotinin, leupeptin hemisulphate, marimastat, CP471474</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ATS</td>
<td>Advanced Tissue Sciences, Inc.</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulphate</td>
</tr>
<tr>
<td>Cys-33</td>
<td>Cysteine-33 of LAP</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>DBM</td>
<td>Demineralised bone matrix</td>
</tr>
<tr>
<td>DS</td>
<td>Dermatan sulphate</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>G [residue]</td>
<td>α-L-guluronic acid</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GRGDS</td>
<td>Glycine-arginine-glycine-aspartic acid-serine</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia inducible factor 1α</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>ICP</td>
<td>Inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>KS</td>
<td>Keratan sulphate</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency associated peptide</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LLC</td>
<td>Large latent complex</td>
</tr>
<tr>
<td>LTBP</td>
<td>Latent TGF-β binding protein</td>
</tr>
<tr>
<td>LTGF-β</td>
<td>Recombinant human small latent complex (R&amp;D Systems Europe)</td>
</tr>
<tr>
<td>M [residue]</td>
<td>β-D- mannuronic acid</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>Mw</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>PAA</td>
<td>Poly(acrylamide)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(caprolactone)</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PEGDA</td>
<td>Poly(ethylene glycol) diacrylate</td>
</tr>
<tr>
<td>PEG-HA-LTGF-β</td>
<td>Poly(ethylene glycol) diacrylate-hyaluronic acid-latent TGF-β</td>
</tr>
<tr>
<td>PEG-LTGF-β</td>
<td>PEGylated latent TGF-β</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glycolic acid)</td>
</tr>
<tr>
<td>Phospho-Smad3</td>
<td>Phosphorylated Smad3</td>
</tr>
<tr>
<td>PHSRN</td>
<td>proline-histidine-serine-arginine-asparagine</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>PNIPAAm</td>
<td>Poly(N-iso-propylacrylamide)</td>
</tr>
<tr>
<td>pNpp</td>
<td>p-Nitrophenyl phosphate</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl alcohol)</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate poly(acrylamide) gel electrophoresis</td>
</tr>
<tr>
<td>SFF</td>
<td>Solid freeform fabrication</td>
</tr>
<tr>
<td>SLC</td>
<td>Small latent complex</td>
</tr>
<tr>
<td>SPB</td>
<td>Sodium phosphate buffer</td>
</tr>
<tr>
<td>SPEB</td>
<td>SPB, 5 mM EDTA</td>
</tr>
<tr>
<td>SPEB+</td>
<td>SPB, 100 mM EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>TBS, 0.1% v/v Tween</td>
</tr>
<tr>
<td>TCP</td>
<td>Tissue culture plastic</td>
</tr>
<tr>
<td>TE</td>
<td>Tissue engineering</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TβRI</td>
<td>Type I TGF-β receptor (Alk5)</td>
</tr>
<tr>
<td>TβRII</td>
<td>Type II TGF-β receptor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>PIMC</td>
<td>Protease inhibitors: Roche EDTA-free complete mini, marimastat, CP471474</td>
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<tr>
<td>TGFBRI</td>
<td>TGF-β receptor inhibitor (SB-431542, Ascent Scientific)</td>
</tr>
<tr>
<td>SH-LTGF-β</td>
<td>Thiolated LTGF-β</td>
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Chapter 1

Introduction and scope of thesis

1.1 Tissue engineering: historical context and scaffolds

Tissue engineering (TE) aims to generate replacement biological tissues and organs for a wide range of medical conditions involving tissue loss or dysfunction. The clinical scope of TE is enormous, with therapeutic potential in many of the ageing and lifestyle diseases prevalent in Western populations – such as heart disease, osteoarthritis and osteoporosis – as well as a host of other major afflictions including spinal cord injury, cirrhosis, type I diabetes, retinopathy and facial disfigurement (1). The general strategy is usually to seed cells within a scaffold, a structural device which defines the geometry of the replacement tissue and provides environmental cues that promote tissue regeneration (Figure 1.1). In many cases an in vitro maturation step may be included, to allow for newly seeded cells to attach to the scaffold, differentiate, and/or deposit functional ECM. These activities may be facilitated by growth factor treatment regimes, or mechanical conditioning for those devices expected to take on an immediate load-bearing role such as tendon, cartilage, or heart valve replacements (2)(3). Alternatively the scaffold may be placed directly into the recipient (4)(5)(6). In this instance a compartment of the host’s own body functions as a bioreactor, supporting in situ maturation and – potentially – the early establishment of a blood supply; and raising the possibility of cell harvest, scaffold seeding, and implantation occurring in a single surgical event. Taking this approach one step further, the scaffolding material is sometimes inserted without cells; regeneration then relies on the recruitment of native cells into the implant and the subsequent deposition of ECM. Whichever approach is taken, the scaffold itself is critical to the success of the implant, and in many cases actively directs the behaviour of the cells within.
Figure 1.1: TE approaches. In traditional TE, cellularised scaffolds are given time to mature \textit{in vitro} before being introduced into the patient, while for \textit{in vivo} TE, the scaffold is implanted directly into the patient, with or without cells. These approaches are currently being applied in therapy for a range of tissues and organs, including those indicated (not an exhaustive list).

The success of these principles can be seen from the large number of products in development or active use. A fast-growing arsenal of tissue replacement devices are in clinical trials or already approved as therapies for tissues including cartilage, bone, blood vessel and pancreas (1). These advances represent the culmination of more than thirty years of research. The roots of TE extend at least as far back as the 1970s, with independent attempts from the groups of Green, Yannas and Bell – all at Massachusetts Institute of Technology – to create skin substitutes using cultured cell sheets, or collagen and glycosaminoglycans (GAGs) (7). A shift in emphasis from that of using natural scaffolds towards the use of synthetic polymers signalled the genesis of the field as we know it today, leading to the first use of a tissue
engineered device in a human, in 1991 (8). In this procedure, a team led by Joseph Vacanti implanted a synthetic polymer scaffold, seeded with the patient’s own cartilage cells, into a patient with a congenital sternal deficiency (Poland’s syndrome). Also present was Vacanti’s brother Charles, who went on to develop the controversial ‘mouse with a human ear’ – ‘Auriculosaurus’. The modern understanding of the term ‘tissue engineering’ was consolidated with a highly cited 1993 paper by Langer and Vacanti (9). TE went from strength to strength through the 1990s, only to run into a period of gloom in the early millennium (Figure 1.2). Early TE efforts were plagued by product issues related to scale-up, shelf-life, quality control and distribution, and suffered from inappropriate business models and withdrawal of private finance in the early ‘00s (10)(11). Since then the field has matured, evidenced by the return of large scale investment and the first regenerative medicine companies becoming profitable (12).

There are considerable scientific challenges involved in bringing TE products to the clinic in a cost effective manner, including generating large numbers of cells from small samples, and achieving adequate vascularisation to implanted material. But despite these challenges, significant progress has been made towards addressing the central aims of TE and indeed, sales of regenerative biomaterials now exceed USD 240 million per annum (12). TE constructs for skin replacement were one of the earlier breakthroughs: TransCyte®, a biosynthetic covering produced by Advanced Tissue Sciences Inc. (ATS), was approved by the U.S. Food and Drug Administration (FDA) in 1997, followed by several related materials. The first clinical trial of a TE organ was reported by Atala and his team in 2006, who carried out reconstructive surgery on seven patients with end-stage bladder disease using TE bladders (Figure 1.2) (13). Here, three-dimensional (3D) moulded polymers were seeded with the patients’ own cells expanded from biopsy. Thirty-one months later the engineered bladders displayed histologically normal trilaminar walls, and capacity and compliance were improved. Meanwhile growing numbers of patients are benefiting from TE techniques in clinical trials of vascular grafts and cartilage, among others (14)(2). Advances in scaffold design, as well as in cell culture and surgical techniques, promise to consolidate and extend these successes to a wider range of tissues and anatomical sites, and ultimately to large numbers of patients.
<table>
<thead>
<tr>
<th>Date</th>
<th>Event</th>
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<tbody>
<tr>
<td>2010</td>
<td>Continuing legal disputes over use of embryonic stem cells in the U.S.</td>
</tr>
<tr>
<td>2008</td>
<td>Successful transplantation of TE airway</td>
</tr>
</tbody>
</table>
| 2007  | • Creation of induced pluripotent stem cells from adult human skin cells  
       • Osiris named Biotech Company of the year                           
       • ~170 companies offering TE products or services, sales in excess of USD 1.3 billion;  
         >1,000,000 patients treated; aggregate economic activity five-fold higher than in 2002  
       • Organogenesis breaking even, reinvesting profits; Apligraf® sales of USD 60 million per year |
| 2006  | • TE bladder appears in the Lancet                                   
       • Launch of Proteus Venture Capital Fund, the first dedicated regenerative medicine fund |
| 2005  | Carticel® becomes profitable                                          |
| 2003  | Organogenesis emerges from bankruptcy                                 |
| 2002  | • 'Integra® Dermal Regeneration Template' by Intergra Life Sciences approved for treatment of severe burns  
       • Organogenesis and ATS, both previously valued at USD 1 billion, file for bankruptcy  
       • TE activity halved since 2000, loss of 800 full time employees, capital value of publicly traded TE corporations drops from USD 2.5 billion to USD 300 million  
       • Circe bioartificial liver completes Phase III clinical trial with statistically significant benefit for a subset of patients; FDA approval not granted as patient group was not identified in advance  
       • 42% increase in stem cell firms, coining of term “Regenerative Medicine”     |
| 2001  | President Bush restricts federal funding for embryonic stem cell research |
| 2000  | • Time magazine names Tissue Engineer as 'Hottest Job' for the future, 3000 people pursue TE careers  
       • USD 580 million spent annually on TE research and development, public TE companies valued at USD 2.5 billion |
| 1998  | • FDA approves Apligraf®, first allogeneic TE product                 
       • Human embryonic stem cells isolated                               |
| 1997  | • TransCyte® by ATS becomes first US FDA approved TE product         
       • Carticel® autologous cartilage implant approved by FDA             |
| 1996  | The Tissue Engineering Society founded (now Tissue Engineering Regenerative Medicine International Society - TERMIS) |
| 1990s | USD 3.5 billion invested worldwide in TE, 90% from private finance    |
| Late 1980s | Early TE work in Massachusetts, term TE appears in literature         |
| Late 1970s | Cells combined with biomaterials in artificial skin and biohybrid pancreas research |
| 1968  | First bone marrow transplant                                         |
| 1950s | Organ transplantation with identical twins                            |

**Figure 1.2: Tissue engineering timeline.** TE gained in profile through the nineties, hitting a peak around the turn of the millennium, but several early commercial ventures failed and large-scale private financing was withdrawn. Improved business planning and a sounder scientific base have since propelled TE towards a new era of success (7)(13)(15)(12)(16).
1.2 Scope of thesis

This thesis is concerned with how scaffolds can be made to actively support tissue repair. In recent years, TE scaffolds have come to be regarded not simply as passive cell carriers satisfying the minimum requirements of non-toxicity, degradability and structural integrity, but as instructive devices with the potential to be loaded with information that can be interpreted by transplanted or host cells (17). Materials can now be made to engage with cells in biologically-meaningful ways – for example by remodelling an artificial environment in a manner analogous to a natural ECM, or proliferating or differentiating in response to cues imposed by the chemist or biologist (5)(18)(19). The thesis consists of a literature review (Chapter 2) followed by two experimental chapters (Chapters 3, 4) describing novel hydrogel systems for bone and cartilage TE, respectively.

The literature review is concerned with the principles of ‘bottom up’ scaffold design, according to which scaffolds are assembled in the lab from one or more constituent materials, with a major focus on engineering bioactivity. The opposing strategy – the ‘top-down’ derivation of decellularised extracellular matrix (ECM) from natural tissue – has generated some spectacular recent results and will be considered briefly in the Chapter 2 section 2.2.1 Extracellular matrix and ECM scaffolds. Particular attention throughout will be given to hydrogel scaffolds, although many of the strategies described are valid for different scaffold types. The review is divided into four main sections. The first section, 2.1 Engineered scaffolds: form and bulk properties gives a brief introduction to TE scaffolds, hydrogel crosslinking, and control of two important bulk properties of scaffolds – degradation and mechanics – with an emphasis on hydrogels. Following this, three sections are devoted to examining the types of cues to which cells respond, and how we might incorporate related signals into ‘bioactive’ scaffolds. 2.2 Bioactive scaffolds I: Physicochemical environment describes how cell adhesion sites, enzymatically degradable polymers, and physical properties of the matrix can be used to fabricate ‘artificial extracellular matrices’ that cells can interact with physically (a convenient oversimplification). 2.3 Bioactive scaffolds II: Growth factors discusses the use of growth factors within TE scaffolds, and in particular how advanced delivery strategies can improve functional outcome. Finally, 2.4 Bioactive scaffolds III: Complexity considers how commercial considerations are helping to drive the development of scaffold biomaterials with increasing functional complexity, while keeping other forms of complexity (structural, synthetic, procedural etc.) to a minimum.
Chapters 3 and 4 present novel bioactive hydrogel systems for bone and cartilage TE, respectively. Materials innovations that promote the regeneration of these tissues could help to satisfy major global demands, which exist due to the high incidence of complications following bone fracture, the high and increasing prevalence of osteoarthritis, and the drawbacks of current treatment options for these conditions (20)(21)(22)(23). In Chapter 3, a series of alginate hydrogels is described in which all or a fraction of the calcium normally used for crosslinking is replaced by bioactive strontium and/or zinc ions. Strontium was chosen for its ability to stimulate bone formation, while zinc is essential for alkaline phosphatase (ALP) activity. Calcium- and strontium-alginate hydrogels were assessed in terms of stiffness, degradation, and ion release, in order to determine how these properties were affected by the crosslinking ion and alginate type; to demonstrate control over these properties; and to evaluate the suitability of alginate as a slow release system for physiologically active concentrations of strontium.

Saos-2 (human osteosarcoma cells) were encapsulated within hydrogels crosslinked by calcium-, strontium-, and/or zinc, with covalently coupled glycine-arginine-glycine-aspartic acid-serine (GRGDS) peptide to provide integrin anchorage sites. Total metabolic activity (alamarBlue® reduction) and lactate dehydrogenase (LDH) activity were assayed as measures of proliferation, to determine how the crosslinking ion(s) and material properties of the hydrogels affected cell viability. Osteogenic activity was evaluated by examining ALP activity and expression of the osteoblast phenotypic marker genes RUNX2, COL1A1 and BSP. This strategy relates to one of the main themes of the literature review as it is biologically inspired, yet technically simple, inexpensive, and flexible. It has the potential to be combined with other alginate-based systems for bone tissue engineering, or adapted to other tissue engineering applications, for example by choosing other bioactive ions such as cobalt (24).

A second major theme of the literature review is how growth factors can be deployed to maximum effect via advanced schemes for their presentation and release. Chapter 4 explores the possibility of supplying transforming growth factor beta (TGF-β) within TE scaffolds in its inactive latent form, rather than as a free, active dimer. TGF-β in vivo is secreted as an inactive complex, non-covalently bound to a latency associated peptide (LAP), and via LAP to latent TGF-β binding proteins (LTBPs). The approach described here was to load TGF-β within TE hydrogel scaffolds while still complexed with LAP (‘latent TGF-β’, LTGF-β). To explore some potential advantages of this approach, the concentrations of free and latent TGF-β
were measured over 48 hours on tissue culture plastic (TCP) as an indicator of their tendencies to adsorb to surfaces. The Smad3 phosphorylation responses of HFF-1 (human foreskin fibroblasts) to free and latent TGF-β were compared at different concentrations and in the presence of various pharmacological inhibitors of LTGF-β activation pathways, to infer how the dynamics of exposure to TGF-β might be altered by using the latent form. The utility of LTGF-β for cartilage TE was assessed by tethering the complex inside poly(ethylene glycol) diacrylate (PEGDA) and hyaluronic acid (HA) hydrogels, using Michael addition chemistry to conjugate the protein and crosslink the hydrogels. The biological activity of the PEGylated protein was investigated to ascertain whether the modification procedure had diminished its potency. Finally, bovine articular chondrocytes were cultured within the PEG-HA-LTGF-β hydrogels, and their viability and ECM deposition were examined as indices of TE success. The work presented in Chapters 3 and 4 provide proof-of-concept for these novel TE strategies, and in each case several routes for future optimisation or development are readily apparent. To begin, then, general consideration will be given to TE scaffolds: their form, bulk properties, and the many ways in which bioactivity can be engineered into them.
Chapter 2

Literature review

2.1 Engineered scaffolds: Form and bulk properties

2.1.1 Scaffolds and hydrogels

TE scaffolds can take a variety of forms, although most can be classified as either solid, fibrous or hydrogel (Figure 2.1). Various engineering approaches can produce solid implants from polymers, ceramics or metals, which are relatively resistant to mechanical forces and incorporate an interconnected network of pores for cell movement and mass transport of nutrients. Porous solid scaffold fabrication techniques include particulate leaching, foaming by chemical means or by CO\textsubscript{2} expansion, microsphere sintering, and phase separation, while electrospinning and phase separation are the principle means by which to create fibrous scaffolds (25)(26)(27)(28)(29). Technologically advanced options for solid or hydrogel scaffolds include a range of solid freeform fabrication (SFF) techniques. Some of these are computer assisted systems whereby the layer-by-layer deposition of materials including, potentially, cells, into a pre-specified 3D shape is achieved either through printing or by the extrusion of a polymer melt. Other groups have used laser beams to solidify polymers into complex shapes by photopolymerisation or sintering as they sweep over polymer liquids or layers of powder, respectively (30)(31). These technologies afford precise control over scaffold architectures, and when coupled to established medical imaging techniques they can produce solid TE devices fitted to the shape and dimensions of individual defect sites.

However, damaged tissue should ideally be treated so as to minimise further injury to the surrounding tissue – and one minimally invasive way to achieve this is to inject a polymer matrix into the site of damage in the form of a hydrogel (with or without cells). Hydrogels are highly hydrated networks of
hydrophilic, crosslinked polymer chains. They can be formed from both synthetic or natural polymers, and crosslinked in a variety of ways. Commonly exploited synthetic polymers include poly(ethylene glycol) (PEG) and poly(methyl methacrylate), although a host of newer polymers are also under evaluation. Examples of natural polymers in common use include the proteins collagen, gelatin and fibrin; and the polysaccharides alginate, chitosan and agar. Where a gel is covalently crosslinked, it is known as a chemical hydrogel. These are often produced by reacting functionalised polymers with small molecule crosslinking functionalities, or alternatively by radical production through ultraviolet (UV) exposure or the use of redox initiators. One chemistry that has been widely adopted is the Michael addition between thiols and acrylates or vinylsulphones, which proceeds at physiological pH over a period of a few minutes, and is well tolerated by cells. The PEG-HA gels used in Chapter 4 (Latent TGF-β hydrogels for cartilage tissue engineering) are crosslinked in this way.

Conversely, the crosslinks of physical hydrogels consist of non-covalent interactions, such as hydrogen bonds, crystallised segments, or electrostatic or hydrophobic interactions. Alginate and pectin are examples of ionic gels: they crosslink in the presence of divalent or multivalent cations due to affinity between the positively-charged ions and the negative carboxylic acid groups of the polymers. Alginate crosslinking is discussed further in Chapter 3 (Strontium- and zinc- alginate hydrogels for bone tissue engineering). Furthermore, specific interactions between biological molecules – which are typically enforced by non-covalent interactions – can also induce gelation. All the major classes of biopolymers can be grafted to polymer chains leading to gel formation by a variety of different interactions. Examples include complementary oligonucleotide sequences, oppositely charged peptides that dimerise into coiled-coil superhelices, and self-assembling peptide nanofibres. Heparin, an ECM polysaccharide that binds a multitude of proteins including growth factors, has been conjugated to PEG by Yamaguchi and co-workers, and the solution gelled by the addition of growth factors.
**Figure 2.1: TE scaffolds, and hydrogel crosslinking.** (Top) Hydrogel, fibrous and porous scaffolds are used in TE. Custom morphologies can be created using SFF techniques. (Bottom) Permanent, covalent links between polymer chains can be created by UV irradiation or through the use of reactive groups, with or without initiators, creating 'chemical' gels (bottom left). Physical crosslinks are reversible bonds based on a variety of non-covalent interactions, and this approach can be extended through the use of biological agents such as complementary oligonucleotides and oppositely charged peptides. Custom and porous scaffold images are from (31) and (48), respectively. Image of fibrous scaffold courtesy of Dr. Seth McCullen, Imperial College London.
Physical hydrogels have less aggressive reactivity than some chemical gels, and they are formed in equilibrium reactions which can be controlled by the solvent, salt concentration, pH or temperature. This raises the option of designing crosslinking groups so as to obtain materials that gel when introduced to a physiological environment, after moulding precisely to fill irregularly shaped defects. Thermally reversible polymers which undergo sol-gel transitions with increasing temperature are one such example. One way to engineer thermogelling systems is to synthesise block copolymers where the blocks have different solubilities in aqueous media. In this approach, PEG is usually coupled to hydrophobic blocks such as poly(caprolactone) (PCL) or poly(propylene fumarate). By varying the block length and molecular weight (Mw) of the copolymers, the sol-to-gel transition temperature can be coaxied into a physiologically useful range and mechanical performance optimised (45). An alternative is to make use of the temperature-sensitive polymer poly(N-iso-propylacrylamide) (PNIPAAm), which forms a solution below the lower critical solution temperature, and a gel at higher temperatures due to dehydration and aggregation (46). One novel application of this unusual property is in ‘cell sheet engineering’, whereby cells are grown to confluence on a sheet of PNIPAAm, and harvested as an intact monolayer simply by lowering the temperature to 20°C, causing the substrate to dissolve. This strategy has been deployed to excellent clinical benefit in corneal repair (47).
2.1.2 Controlling degradation

Central to the TE approach is the use of non-permanent scaffold materials that, over time, become completely replaced by natural extracellular matrix. The objective is therefore to implant a scaffold that can persist in a robust state for long enough to allow for the formation of native tissue, but which will ultimately degrade and become replaced by newly synthesised matrix. In living tissues, cells migrate through natural ECM either by adapting their morphology to manoeuvre through a path of least resistance, or by clearing a trail through the matrix using secreted, locally activated or membrane bound proteases such as matrix metalloproteases (MMPs) and plasmin (49). Biomedical materials based on matrix constituents, like collagen, fibrin or hyaluronic acid, naturally harbour enzymatically-cleavable sequences – thus in many cases, natural scaffolds will eventually be cleared completely from the implant site.

Where mammals lack the enzymes to break down the material – alginate for example, which is derived from brown algae – chemical modification and/or manipulating molecular weight may go some way towards achieving the desired effect. Strategies applied to alginate have included gamma-irradiation to reduce molecular weight, encapsulation with microspheres containing the degradative enzyme alginate lyase, and periodate oxidation to increase the polymer’s susceptibility to hydrolysis (50)(51). Boontheekul and colleagues in the Mooney lab combined the latter approach with the use of a bimodal Mw distribution of alginate. Incorporating low Mw polymer accelerates degradation to the required speed, while the presence of high Mw chains prevents the loss of mechanical integrity that usually accompanies significant decreases in Mw.

Likewise, several groups have introduced degradable groups into non-degradable synthetic polymers such as PEG. In one example, Kutty and co-workers synthesised a modified PEG containing ester bonds, and used this as the basis for semi-interpenetrating networks alongside either gelatin, collagen or hyaluronic acid (52). Where hyaluronic acid was used, encapsulated fibroblasts spread throughout the hydrogel in a continuous, three-dimensional network. More biomimetic modes of hydrogel degradation are discussed later in the section 2.2 Bioactive scaffolds I: Physicochemical environment.
2.1.3 Recreating tissue mechanics

Many of the target tissues for tissue engineering exist to perform an essentially physical task, whether that be as part of the musculoskeletal, cardiovascular, or integumentary system. Artificial constructs which recreate the mechanical properties of the native tissue will tolerate the forces acting on them, minimise shear by deforming with their surroundings, and can also support the immediate mechanical needs at the implant site – and one need only to consider the example of an engineered heart valve to appreciate how essential this can be. However, the mechanics of living tissues are the product of a whole array of matrix components – their structures, abundance, organisation and interplay – and working to the complex mechanical specifications laid down by nature with our current toolkit is challenging. In some cases the best way to recreate these properties may be to perform *in vitro* conditioning, in which some aspect of the *in vivo* mechanical environment is simulated, prompting cells to deposit an ordered matrix (3). Often, however, the material itself may be optimised to possess mechanical properties approximating those of the target tissue. Each tissue presents its own set of demands: cortical bone, for example, is notable for its high strength and toughness while constructs for soft tissues such as muscle or skin need to be flexible and elastic. In many cases physical demands on scaffolding materials are complicated by the anisotropy inherent in most living tissues: the parallel arrangement of collagen fibres within tendons and the concentrically layered sheets of the intervertebral disc, for example.

With their high water content and rubbery consistency, hydrogels are potentially excellent mimetics of many biological tissues. Research is ongoing into producing hydrogels with mechanical properties suitable for soft tissue replacement. Poly(vinyl alcohol) (PVA) hydrogels crosslinked by thermal cycling are unusual in that their stress-strain relationships display an exponential shape similar to that of many natural tissues (53). Their physical crosslinks consist of crystallites which form and grow during each thermal cycle, resulting, in effect, in a nanocomposite with the crystalline regions reinforcing an amorphous matrix. By stretching the sample after a single cycle the primary crystallites, which have dimensions of just a few nanometres, can be made to align along the direction of stress, and further cycling leads to their subsequent growth. Increasing the initial strain applied to the gel leads to a higher degree of crystallite alignment and therefore anisotropy, whereas the stiffness in both directions increases in proportion to the number of thermal cycles as the volume fraction of crystallites continues to grow. Millon *et al.* applied strain during thermal cycling to produce an anisotropic PVA hydrogel whose
stress-strain relationship matched very closely that of porcine aorta in both the longitudinal and perpendicular directions (53).

In general, too much crosslinking leads to a brittle gel, but with too few crosslinks the material may be too weak to provide the necessary support. At the optimum range, a hydrogel will be both strong and elastic. Physical gels with reversible crosslinks, however, may tolerate higher strains than chemical gels due in part to the ability of the crosslinks to re-form once broken (54). Covalently crosslinked alginate gels under tension undergo very little deformation until a critical stress is reached, at which point the crosslinks suddenly ‘unzip’ along a propagating crack, resulting in catastrophic failure. Conversely, as stress builds within ionically crosslinked alginate, the polymer network rearranges via cycles of de-crosslinking, chain slippage, and new bond formation. The load bearing area thus expands, and greater toughness is achieved (54).

Of course, not all tissues are stiff, and some TE applications may have a specific requirement for very soft materials. The extreme softness of the vocal fold lamina propria (elastic modulus 100-1000 Pa) is essential for proper phonation and its function is easily impaired by scarring. This has motivated the development of soft \( (E \sim 500 \text{Pa}) \) highly elastic gels of double cross-linked hyaluronic acid microparticles for vocal fold TE (55). The particles are synthesised by crosslinking with divinyl sulphone, then surface oxidised and lightly cross-linked together using hyaluronic acid modified with adipic dihydrazide. The gels exhibit controllable viscoelasticity, and a reduction in dynamic viscosity with frequency occurs at a similar rate as in human vocal fold mucosa. This and the previous examples demonstrate how TE scaffolds can be engineered to have bulk properties appropriate to their application; but in addition to these structural roles, materials can now be used to actively manipulate cells into behaving in particular ways. The next three sections on ‘Bioactive scaffolds’ examine different ways in which this can be achieved. Somewhat artificially, the first part of this discussion is organised around the ‘physicochemical’ environment, referring to those factors that (among other things) enable cells to interact with matrices on a physical level, for example by attaching to a synthetic material and degrading it in a protease-dependent manner. As to how we identify and implement factors which influence cell behaviour, our best clues come from nature’s own solutions. The next section therefore begins by considering natural extracellular matrix scaffolds.
2.2 Bioactive scaffolds I: Physicochemical environment

2.2.1 Extracellular matrix and ECM scaffolds

In the early 1960s, the orthopaedic surgeon Urist noted that demineralised bone matrix (DBM) – bone tissue from which all cells and mineral have been removed, leaving only ECM macromolecules – would induce bone formation in the surrounding tissues when implanted at an ectopic site (56). This observation led to the discovery of bone morphogenetic proteins (BMPs), growth factors with numerous physiological roles including in bone and cartilage development (57). ECM is usually a fibre-reinforced gel containing a tissue-specific array of proteins, glycoproteins and glycosaminoglycans including sequestered growth factors, hyaluronic acid, collagens, laminins, and fibronectin (58). It is primarily this molecular information which confers bioactivity: ECM supplies a vast amount of information that helps to regulate cells during development, tissue healing, during normal homeostasis, and in a huge number of pathological conditions including cancer (58). Several companies currently produce DBM commercially from cadavers for implantation in bone defects, and many other cadaver- or animal-derived decellularised ECM products have an inherent bioactivity sufficient to assist healing and have found clinical use. Products derived from the small intestinal submucosa of pigs are used routinely in reconstructive surgery (e.g. Oasis Wound Matrix®), and ECM derived from the pericardium of horses can be used as a reconstructive material in the dura mater layer of the brain meninges following a craniotomy (1).

This approach has recently been propelled to the forefront of whole organ TE. In 2008 a 30-year old woman became the first person to receive a TE tracheal segment, a procedure that saved her left lung (15). The scaffold in this case was a decellularised human donor trachea that was repopulated with cells expanded from the patient's bronchial mucosa and bone marrow. In contrast with traditional transplant surgery, the decellularisation protocol solved the problem of tissue rejection by removing virtually all traces of human leukocyte antigens – proteins which to a large extent determine tissue compatibility – with the consequence that the patient required no immunosuppressive drugs. As well as immediately restoring airway patency, the device facilitated the rapid development of an internal cellular lining and blood vessel network. This followed earlier work describing the reseeding of decellularised whole rat hearts with cardiomyocytes with restoration of some (minimal) level of function (59). More recently still,
similar work has been described in liver TE, and elsewhere whole rat lungs have been repopulated with neonatal rat lung epithelial and lung microvascular endothelial cells (60)(61). Upon transplantation, the lungs became perfused and ventilated – although to a lesser degree than the native contralateral lung – and blood samples demonstrated efficient gas exchange.

Inspired by the intrinsic bioactivity of ECM, many groups have designed scaffolds based on direct mimicry of the ECM. One way of achieving this is to build materials from purified matrix constituents such as fibrin, collagen or gelatin (a low cost denaturation product of collagen), which contain various recognition elements that can be bound and interpreted by cells. HA is widely incorporated into scaffolds, and is used therapeutically as a putative lubricating factor in arthritic joints – although here and in TE it may also interact directly with cells via CD44 and other cell surface receptors (62). The ubiquity of ECM molecules such as collagens and HA in tissues has prompted the development of Extracel™, a modular ECM system based on derivatives of gelatin and HA (63). The components are combined in varying proportions to suit many different cell culture and in vivo applications, crosslinked with PEGDA and supplemented with additives such as heparin-bound growth factors as needed. For example, whereas equal quantities (w/w) of gelatin and HA are suitable for most tissue applications, a specialised composition enriched in HA (95:5 w/w HA:gelatin) is required for vocal fold repair. In terms of more tissue-specific influences, a subset of BMPs are now used in TE devices including the tremendously successful INFUSE® (12). The use of growth factors in TE scaffolds will be discussed in detail in the section 2.3 Bioactive scaffolds II: Growth factors.

Matrix constituents have disadvantages associated with purification and processing, and coupled with the desire for greater control over material properties, this has led to the investigation of fully synthetic bioactive systems. The ability to functionalise bioinert substances could improve the suitability for TE of a host of materials with remarkable properties, such as high strength synthetic polymers and (nano)composites for bone TE (64)(65). Of particular interest are systems amenable to minimally invasive delivery, including injectable or shape-memory materials that gel or regain their original form in response to stimuli such as UV illumination or physiological conditions (temperature, pH or solvent) (66)(45)(38)(67). Materials that do not adsorb protein, such as PEG gels, can effectively be used as a
blank canvas on which to confer bioactivity with the minimum amount of modification. This approach allows for a high level of control over the molecular influences presented to cells.

2.2.2 Engineering cell adhesion

Synthetic materials can be designed to interact with cells by emulating key molecular features of the ECM. One way to provide sites for integrin attachment in scaffolds is to include purified ECM proteins. In some cases specific functions of biopolymers can be attributed to small functional domains which may be included in place of the full protein (18)(43). The best known of these is the integrin-binding arginine-glycine-aspartic acid (RGD) sequence found in many ECM proteins, including fibronectin, laminin, collagen type IV, fibrinogen, vitronectin, tenascin and thrombospondin (68)(69). Integrins are heterodimeric cell surface receptors which form part of the docking complex between ECM and the actin cytoskeleton. One third (8 of 24) bind RGD, and even in isolation this sequence can engage with integrins, allowing force generation for movement, providing behavioural cues, and for many cell types acting as survival signals – for without matrix attachment signals a cell may undergo a specialised form of apoptosis known as anoikis (70)(71). Therefore, the incorporation of adhesion proteins into a scaffold can support the migration, proliferation, differentiation and even the survival of its cell population. RGD modification alone is sufficient to transform alginate from a relatively inert polysaccharide into a substance that supports the formation of growth-plate like structures when mouse osteoblasts and chondrocytes are co-cultured within it (Figure 2.2) (72).

Figure 2.2: Cell adhesive hydrogels. Formation of growth-plate-like structures in RGD-modified alginate hydrogels with co-transplanted osteoblasts and chondrocytes, showing cartilage (left), transition (middle), and bone and marrow space (right) regions. Magnification x200. From ref (72).
Since the late 1980s, RGD has been incorporated into a wide range of surfaces, scaffolds and hydrogels. Various structural variations have been investigated as means by which to increase binding affinity, including the use of a cyclic peptide conformation, optimal flanking amino acids (GRGDS is commonly used), and the inclusion of a PEG spacer arm in order to suppress protein adsorption and present the motif to cells in a sterically favourable fashion (73)(74). The concentration and spatial presentation of the peptide can profoundly affect cell response. For example Massia et al. determined that an average distance of 440 nm between RGD peptides was sufficient to enable fibroblast attachment and spreading, but a significantly higher density – an interpeptide distance of 140 nm – was required for focal contact formation and cytoskeletal reorganisation into stress fibres (75). The sequence proline-histidine-serine-arginine-asparagine (PHSRN) from fibronectin binds synergistically with RGD, and has been shown to improve adhesion and focal contact formation when combined with RGD (69). In the intact protein, the synergy site (which includes, but is not restricted to, PHSRN) strengthens integrin-ECM bonds by engaging the alpha integrin subunit. Prior binding of RGD to the beta subunit and tensioning of this bond, usually through cytoskeletal contractility, are prerequisites for this secondary binding (76). When presented within biomaterials, the spacing between these two motifs may be an important factor to consider if this synergistic effect is to be achieved (69).

It has been suggested that patterning of the RGD peptide into clusters may facilitate the binding of ECM adhesion complexes – large assemblies of integrins and various docking proteins which connect the cytoskeleton (indirectly) to the matrix. In agreement with this hypothesis, YRGD peptides support cell migration at considerably lower densities when presented in clusters than when evenly spread (77). There is also some evidence that different behavioural responses – namely adhesion, migration, proliferation and differentiation – are affected differently by various pattern parameters (number of ligands per cluster, distance between clusters, overall ligand density), suggesting a novel way by which to optimise cell behaviour (68). Aside from RGD, other adhesion sequences have been studied including IKLLI, IKVAV, LRE, PDSGR and YIGSR from laminin, DGEA from collagen I, and GEFYFDLRLKGDK from collagen IV (78)(79)(80). The IKVAV epitope can be presented at high density within hydrogels of self-assembled amphiphilic peptides created by the Stupp group (43). These injectable gels have been shown to inhibit gliarial scar formation and to promote the regeneration of axons across the site of damage in a mouse compression model of spinal cord injury (42).
2.2.3 Active degradation of scaffold biomaterials

ECM is frequently remodelled by cells during development, homeostasis and healing, a process which involves digestion by a variety of proteases – such as cathepsins and MMPs – followed by deposition of fresh matrix. As discussed earlier, many scaffolds are engineered to degrade by chemical hydrolysis, although this process must somehow be timed to keep pace with the cells' ability to deposit new matrix. Cell-mediated scaffold degradation is more likely to generate a materials temporal profile in tune with the generation of new tissue, and can be engineered via the use of naturally degradable ECM constituents, or their substitution with short peptides. The latter approach is epitomised by the 'synthetic ECMs' pioneered by the Hubbell group. These innovative hydrogel materials are crosslinked by enzyme-degradable peptide sequences, and a combination of cell-mediated degradation and integrin-binding allows the cells to migrate through the gel in a naturalistic process reminiscent of tissue remodelling (5)(18)(35)(81).

In one example from this group, Pratt et al. developed a system whereby a vinyl-sulphate terminated PEG block was crosslinked with a cysteine-containing peptide by a Michael-type addition reaction (35). The crosslinking peptide, GCYKNRCGYKNRCG-NH₂, contained three cysteine residues and two plasmin substrate sites (shown in bold). The rate of invasion of cells into this type of hydrogel can be changed by altering the enzymatic sensitivity of the MMP substrate peptide crosslinkers (by changing the amino acid sequence). Lower Mw PEG gives a higher crosslink density and a finer mesh, which slows down the movement of cells into the gels. At sufficiently high crosslink density it is even possible to exclude cells completely (81). Semi-interpenetrating networks of the thermogelling polymer PNIPAAm and poly(acrylamide) (PAA) crosslinked with MMP-degradable and integrin-binding peptides have been evaluated for bone regeneration. This polymer network allowed osteoblast invasion and supported new bone formation in vivo following injection into a treatment site in rat femora – even without the inclusion of cells or costly growth factors (6).

Cleavage sequences can also be incorporated into multidomain peptides. One example is a recombinant, crosslinkable elastin-like protein which harbours an adhesion motif (REDV) and an elastase sensitive sequence. Cleavage of the latter yields a bioactive VGVAPG fragment intended to stimulate cell proliferation and enhance tissue repair (82). This functionality mimics the multilayered bioactivity of the
ECM, whereby enzymatic remodelling can liberate ‘cryptic sites’ contained within the amino acid sequences of ECM proteins. In fact it is becoming increasingly clear that fragments of many ECM proteins possess bioactivity – with effects ranging from cell migration to differentiation, proliferation and angiogenesis – which only becomes recognisable to the cells when the ECM is modified in some way (83).

2.2.4 Bioactivity of the physical environment

ECM from every tissue of the body presents a unique gallery of molecular features to the cells residing within it, but each also possesses a specific set of physical properties. These encompass architectural features such as local topography and two- versus three-dimensionality; ECM stiffness; and intrinsic and extrinsic stresses and strains which may relate to static and dynamic loading, hydrostatic pressure, and/or fluid flow. As has already been discussed, scaffolds which recreate particular physical properties can potentially contribute towards the mechanical function of the tissue – but additionally, these physical cues may in themselves constitute another layer of biologically relevant information. Indeed, such stimuli may be a critical determinant of some cells’ physiological function. Hence, static and dynamic forces are applied to TE devices in a multiplicity of ways within specialised bioreactors, in order to mature TE blood vessels (pulsatile flow), cartilage (cyclic compression), ligament (tension), and other tissues prior to implantation (84). Equally, it is also possible to optimise cell behaviour by fabricating scaffolds with appropriate stiffnesses and/or architectures. For example, it has been recognised for some time that cells can respond to topographical cues, for example by orientating themselves along micropatterned ridges (85). Scaffold geometry was recently used to align cardiac muscle cells in order to elicit directional contractions, which are essential for efficient blood transfer (86). The crosslinking of microstructured honeycomb poly(glycerol sebacate) sheets was optimised to mimic the anisotropic stiffness of rat ventricular myocardium. These sheets possessed microscale pores in the form of two overlapping squares tilted at 45 degrees, which directed the alignment of neonatal rat heart cells. The resulting constructs displayed anisotropic electrical excitation thresholds as a result of this long-range order.

The phenotypic effects of ECM stiffness have been a topic of intense research over the past few years, exemplified most prominently by the work of the Discher group in Philadelphia. In two studies they concluded that both skeletal and cardiac muscle striate optimally on substrates that mimicked the
stiffness of the native tissue (~10-12 KPa) (87)(88). Thus, only myotubes grown on physiologically stiff substrates formed striations, and cardiomyocytes assembled sarcomeres and beat at a physiological frequency (1 Hz) for longest when the substrate stiffness did not exceed physiological levels – conclusions with obvious implications for TE. In the latter study, the contractile work done by the beating cardiomyocytes peaked at this same level of stiffness. At higher rigidities (>> 10 KPa), the cells were contracting almost isometrically against an inflexible support, leading to high intracellular strains with disruption to the structures of cytoskeletal and docking (e.g. focal adhesion) proteins. On softer supports, however (<< 10 KPa), matrix deformation exceeded cellular deformation, but this movement was inefficient in terms of work done because the substrate itself offered little resistance. In other work, Engler and colleagues famously reported that the expression levels of neurogenic, myogenic, and osteogenic transcription factors by MSCs peaked on substrates with stiffnesses close to those of brain, muscle, and osteoid, respectively (89).

Naturally these and other findings have generated interest in the biochemical bases for the ability of cells to sense and interpret ECM stiffness. It is clear that direct cellular mechanosensing mechanisms do operate, such as the tension-induced unfolding of talin rods: this conformational change exposes cryptic vinculin-binding sites, possibly promoting focal adhesion assembly (90). However ECM stiffness can also influence cells indirectly, for example via force-dependent interactions with ECM macromolecules. As we shall see in Chapter 4, TGF-β can be released from its latent complex in several ways, one of which involves a force-dependent conformational change of the latent complex (91)(92). This requires a minimum ECM stiffness of ~10 KPa to provide resistance against integrin-mediated traction (Figure 2.3). Regardless of how generalisable is this type of mechanism, it is clear that we should expect further research to reveal potent combinatorial effects of soluble, insoluble and mechanical influences on cells.
Figure 2.3: Force-dependent activation of TGF-β. (Left) TGF-β is sequestered in the ECM within a propeptide (black) containing an RGD sequence, to which cell surface integrins can bind. (Middle) Contraction of the actin cytoskeleton against a stiff (> 10 kPa) substrate can cause the propeptide to undergo a conformational change, resulting in TGF-β release. (Right) On a soft (< 5 kPa) substrate, cytoskeletal contraction results in deformation of the matrix rather than the pro-peptide, and the TGF-β is not relinquished. Modified from ref (91). TGF-β latency and activation is discussed in detail in Chapter 4.

Substrate stiffness, and cell adhesion, shape, and contractility appear to be intimately related. Cells will tend to adhere more strongly, spread more extensively, and develop prominent stress fibres on more rigid substrates (~35 KPa and upwards) (89)(17). As can stiff substrates favour osteogenic differentiation, so too can more spread geometries: human mesenchymal stem cells (MSCs) constrained to narrow cross-sectional areas or permitted to spread tend to differentiate towards adipocytes and osteocytes, respectively, when grown in medium that can induce either lineage (93). These findings, however – which derive from experiments conducted on two-dimensional surfaces – may not translate straight-forwardly to 3D cultures. Human MSCs encapsulated within alginate hydrogels are sensitive to gel stiffness despite maintaining a similar, rounded morphology in all conditions (94). On the other hand, more rapidly degrading alginate supports higher levels of bone formation than slowly degrading gels – and it is conceivable that cell shape could be a contributing factor here, for in other gel systems combined degradation and adhesion has been correlated with cell spreading (95)(52). Evidently, it will be challenging to dissect the effects of these inter-related factors, and caution must be taken in interpreting results from materials systems which cannot necessarily separate the effects of different stimuli. These and other physical factors continue to be investigated intensively and provide a complementary approach to the provision of molecular information to cells inside scaffolds. The next section returns to the molecular approach by considering how TE can benefit from scaffolds engineered to deliver growth factors in advanced, biologically-informed ways.
2.3 Bioactive scaffolds II: Growth factors

2.3.1 Motivation

Growth factors, such as TGF-βs, BMPs, vascular endothelial growth factors (VEGF) and platelet-derived growth factors (PDGF), are important signalling molecules in both development and tissue healing. Some of these proteins act as ‘morphogens’, determining the spatial arrangement of cell types within the developing embryo. They may also have profound effects in the control of tissue regeneration – for example, as we have already seen, BMPs can induce the formation of bone ectopically in muscle tissue (56). These observations have found resonance in stem cell research with the result that many growth factors are important components in the differentiation regimes for both adult and embryonic stem cells (2)(96)(97). In TE, the application of growth factors within biomaterials represents a powerful tool for controlling cell differentiation and function. Already, growth factors feature in a handful of commercially available TE products, one of which – Medtronic’s INFUSE® – represents the field’s biggest financial success to date (1)(12). This product attracted nearly USD700 million of sales in 2007 – an order of magnitude more than any of its competitor products. INFUSE® is supplied with powdered recombinant human BMP-2, which is reconstituted in water and added to a collagen sponge immediately prior to use.

The possibility of using growth factors to promote cellular functions such as self-renewal, and the recruitment of endogenous repair cells into implanted materials, is also gaining currency (17). For instance, when murine muscle lacerations were treated by transplantation of myoblasts within RGD-coupled alginate gels, recovery was dramatically improved by the inclusion of hepatocyte growth factor (HGF) and fibroblast growth factor-2 (FGF-2) (19). This combination of growth factors is active in muscle regeneration in vivo, and has the effect of stimulating quiescent satellite cells to undergo mitosis while preventing their terminal differentiation into skeletal muscle. Thus, as well as improving the survival of the transplanted cells, this hydrogel expanded myoblast numbers by stimulating proliferation and inhibiting differentiation until the cells had migrated out of the device. The result was a significantly greater muscle mass, smaller defect size, and greater contribution by the transplanted cells. In a second example, a TGF- β3-infused collagen I gel was deployed as a chemoattractant in a moulded polymer-ceramic scaffold (98). The device was shaped to resemble the entire proximal articular condyle of the rabbit humerus, and was implanted acellularly. At 4 months the scaffold surface was covered with an
aggrecan- and collagen-rich, hyaline cartilage-like matrix. Those constructs containing TGF-β3 were thicker, denser, and became populated with 130% more cells than those with collagen alone, suggesting that circulating progenitor cells may have responded to the released growth factor by homing to the implant site. This approach is completely dependent on the availability and competence of progenitor cells, which in the elderly cannot be relied upon, but the in vivo generation of an entire articular surface from host cells convincingly demonstrates the exciting potential of material-growth factor combinations.

2.3.2 Passive growth factor release strategies

The uncontrolled use of growth factors in materials can lead to rapid release and inactivation, and consequently suboptimal functional outcomes. Furthermore, in developmental pathways and wound healing, different factors become active at different times, and growth factor release profiles that recapitulate these dynamics are likely to provide more leverage over cell behaviour than those that apply growth factors indiscriminately. The development of controlled delivery strategies is therefore a priority in this area of research. Materials schemes based on optimised degradation rates or diffusive properties of polymers have been designed with this in mind (Figure 2.4) (99)(100). Where retarded release of growth factor protein is required, one option is to fabricate loaded microspheres using the double emulsion technique, whereby primary (water in oil) and secondary (oil in water) emulsions generate aqueous pockets of dissolved proteins within polymer droplets (101). The droplets can then be dried into beads, which can be seeded within a scaffold, or else fused together to fabricate an entire scaffold (microsphere sintering) (102)(99). As the microspheres degrade, so the pockets of dissolved morphogens are gradually freed. Alternatively, polymer coatings can be created using chemical vapour deposition or co-electrospinning – a variation on the electrospinning technique which produces core-shell fibres in a one-step process (103)(104). Furthermore, by combining approaches, the sequential release of two or more growth factors may be contrived in order to expose cells to a temporal scheme of events designed to recreate some aspects of developmental pathways or wound healing (99).
2.3.3 Biomimetic growth factor release strategies

Although most efforts to date have concentrated on evaluating the effects of freely diffusible forms of growth factors in solution, most in fact function at interfaces in vivo, bound to ECM components or as part of membrane complexes (105). Whilst concern undoubtedly arises over the cellular accessibility and activity of surface-immobilised proteins, even relatively simple tethering of a growth factor to a biomaterial matrix can elicit desired biological responses (106)(107). For example TGF-β1 covalently tethered to PEG retained its ability to stimulate matrix production in vascular smooth muscle cells over seven days in culture (108). A novel, biomimetic strategy for the delivery of TGF-β within scaffolds is the subject of Chapter 4. More precise, site-specific couplings can be engineered through the use of recombinant proteins into which additional amino acids are introduced at the termini, for example Cysteine-tags or enzyme substrate sequences enabling proteolytic release (109).

More natural mechanisms of growth factor binding and release are also being pursued. In vivo, GAGs – mostly as components of proteoglycans – play key roles in growth factor activity including sequestering them within the matrix, preventing their degradation, and presenting them to cell surface receptors. GAGs are complex molecules with tissue specific distribution and multiple physiological functions. They possess characteristic linear structures of repeating hexosamine-uronic acid disaccharide units (110). Heparin, and heparan-, chondroitin-, keratin- and dermatan-sulphate GAGs (HS, CS, KS, DS, respectively) also have tightly regulated regiospecific sulphation patterns, which determine their specific interactions with proteins (111)(112). These interactions can be essential for growth factors’ physiological effects. FGF-1, for example, requires HS binding for dimerisation and receptor activation (113).

Heparin sequesters numerous morphogens into the matrix by binding them with high affinity until they are liberated by cellular remodelling – and for this reason has been widely incorporated into TE scaffolds as a slow release mechanism for growth factors (Figure 2.4) (114)(115). Pratt et al. have exploited this natural system by attaching peptides with a heparin binding domain to PEG within hydrogels (35). The peptide associates with heparin via electrostatic interactions, and the bound heparin acts as a bridge between the scaffold and growth factor. Thus, a range of different growth factors can be indirectly tethered to the polymer matrix. Once immobilised, the growth factor can be released through the breakdown of heparin by cell-derived enzymes. In this way, release of growth factors can be made to
occur in synchrony with cellular remodelling rather than by simple diffusion. One prominent example in which systems for controlling growth factor release and presentation have been effectively employed is the inducement of blood vessel growth into scaffolds. This application will be used to illustrate the potential of rationally-designed release schemes to improve TE functional outcomes.

Figure 2.4: Presentation and release of growth factors from TE scaffolds. Anticlockwise, from top: growth factors within TE scaffolds may be loaded into polymers whose rate of degradation or diffusive properties can be modulated to tailor release rate, and which may be combined into systems releasing multiple factors with distinct kinetics (99)(100). The exposure of cells to different growth factors with time may therefore imitate developmental pathways and healing responses. An alternative to presenting growth factors in soluble form is to bind them to a surface in either random or specific orientations, with the possible use of a spacer molecule (106)(107)(108). Non-covalent associations with matrix components, particularly glycosaminoglycans (GAGs), can effect slow release and in some cases may potentiate binding to membrane receptors (114)(115). Cell demanded release is based on the presence of protease-sensitive peptide sequences within the growth factor protein (116)(109).
2.3.4 Vascularising scaffolds

Creating a functional vasculature is one of TE’s most fundamental challenges, and consequently TE successes to date have been most notable in thin or avascular structures such as skin, bladder and cartilage. Surgical approaches whereby implants are sited alongside a rich external blood supply are likely to complement materials strategies which attempt to induce or organise vessel formation, either de novo (vasculogenesis) or by sprouting of existing vessels (angiogenesis) (13). Endothelial cells have an inherent ability to form tubular structures but it is essential that these are stabilised if regression is not to occur. Permanent blood vessels are supported by smooth muscle cells and pericytes as well as the endothelial component, and several studies have shown the potential of co-culture with various cell types to improve the longevity of vascular networks (117)(118). Pericytes and endothelial cells in co-culture produce tissue inhibitor of metalloproteinase (TIMP) -3 and -2, respectively, which stabilise vessels by arresting the matrix breakdown that is associated initially with vascular invasion and lumen formation, but also ultimately with regression (Figure 2.5) (119). Several materials-based approaches have employed VEGF, a potent angiogenic factor involved in the early stages of blood vessel formation. VEGF has a narrow therapeutic concentration range, above which capillary formation is prolific but aberrant: the resulting structures are malformed, leaky, and unstable, regressing quickly upon withdrawal of VEGF (120). Furthermore VEGF has a half-life of <90 minutes in the circulation, hence the need for it to be delivered via biomaterials that can release it in low concentrations on a timescale of weeks (121). An interesting scheme devised to this end involved the production of a recombinant VEGF variant, which was enzymatically incorporated into fibrin matrices, and released upon matrix breakdown by cell-produced enzymes (122). The release rate was accelerated by the selection of VEGF isoforms with a plasmin cleavable sequence near the conjugation site. Interestingly, durable vessel formation and in vivo vascularisation was enhanced with this VEGF molecular variant compared to native VEGF in a mouse model despite a lower upregulation of VEGF receptor 2 (the receptor through which VEGF exerts its effects on endothelial cells).
Figure 2.5: Vascularisation of tissue engineering scaffolds. Centre/bottom: new blood vessels form initially by endothelial cell organisation into tubes, which are later stabilised by smooth muscle cells and pericytes. From left: many attempts to induce this process within TE scaffolds have involved the use of vascular endothelial growth factor (VEGF). A low level of sustained release is demanded as burst release results in the formation of unstable, leaky vessels (120). Subsequent release of platelet-derived growth factor-BB (PDGF-BB) helps to recruit smooth muscle cells, which favours vessel maturation (123).

Materials strategies for releasing growth factors are indicated in Figure 2.4. Gene transfer has been used to generate populations of cells that constitutively express either VEGF, or a stabilised variant of hypoxia-inducible factor 1α (HIF-1α). In the latter case, the protein translocates to the nucleus where it initiates a hypoxic response involving the activation of a host of genes and upregulation of angiogenic growth factors (124). Co-culture of different cell types leads to paracrine and membrane interactions that can enhance angiogenesis. Endothelial cells (EC) and pericytes in co-culture increase the production of tissue inhibitor of metalloprotease (TIMP) -2 (EC) and -3 (pericytes), which inhibits tube regression (119).
Other groups have taken a combinatorial approach to growth factor delivery whereby VEGF is released in tandem or sequentially with other growth factors involved in the orchestration of angiogenesis, such as PDGF-BB, FGF-2 and Angiopoietin-1 and -2 (117)(121). PDGF-BB is important in recruiting smooth muscle cells to stabilise nascent vessel walls; when packaged with VEGF-A inside alginate hydrogels the two growth factors show distinct release kinetics, apparently due to their different affinities for alginate (123). Used therapeutically, this material stimulated the formation of mature, smooth muscle cell-associated blood vessels, and improved cardiac function in a rat model of myocardial infarction. Yet another possible avenue is to use gene transfer such that cells constitutively produce low levels of VEGF or other desired proteins. One target is the transcription factor hypoxia-inducible factor 1α (HIF-1α), which holds the key to intracellular detection of hypoxia, which can lead to upregulation of VEGF and other proteins involved in the cascade of angiogenesis (124). A gene was delivered which encoded a stabilised form of HIF-1α, which lacked the oxygen sensitive degradation domain present in the native form and could therefore initiate angiogenic events under normoxic conditions. The plasmid was packaged within designer peptides, one of which incorporated a factor XIIIa substrate sequence as a means to immobilise the DNA within a fibrin gel (Figure 2.5).

Clearly, although vascularisation of scaffolds remains an inadequately resolved challenge, encouraging developments are being made in this area. Overall, these and previous examples demonstrate that current biological understanding and materials technology can be applied to create sophisticated scaffolding biomaterials, loaded with complex physical and molecular information, with leverage over cell behaviour. These expanding possibilities, however, raise the question of whether, in TE, it is always appropriate to aim for the most biomimetic materials solution or whether, in some cases, less is more. This question forms the basis for the final section of this review.
2.4 Bioactive scaffolds III: Complexity

2.4.1 Commercial considerations in tissue engineering

While it is becoming possible to use materials to influence cell behaviour by encoding complex information within their physical and chemical structures, in commercial TE, financial considerations dictate that synthetic and procedural complexity must be kept to a minimum. To compete successfully in the marketplace, TE scaffolds must be not only clinically efficacious, but also cost-effective. This raises the dilemma that over-engineering TE devices may make their translation to clinical use unlikely. An emerging philosophy within TE is that rather than attempting to recreate the complexity of living tissues ex vivo, we should aim to develop synthetic materials that establish key interactions with cells in ways that unlock the body’s innate powers of organisation and self-repair. The following discussion considers ways in which scaffold biomaterials can be made to deliver optimum performance despite limited structural complexity. To begin, examples will be given of how the functions of various types of biomolecule (particularly proteins and polysaccharides) can be distilled into structurally simpler, synthetic mimics.

2.4.2 Simplifying biomolecules

We have already seen that certain features of proteins can be reproduced in short integrin-binding or protease-digestible peptides. Although they may only have a fraction of the activity of the complete protein they can be included at very high concentration and are easy to synthesise and functionalise. This approach can be extended to other protein functional domains, for example in growth factor-mimicking therapeutics. Here, some of the growth factor function is condensed into relatively short peptide fragments, typically of 30-40 amino acids (125)(126), which usually correspond to the receptor-binding region of the protein. The concentrations of these peptides required to elicit biological effects are variable, and in some cases exceed those of the native proteins by orders of magnitude. Some, however, bind their respective receptors with comparable affinities to recombinant growth factors, and can trigger signal transduction leading to appropriate cell responses. Angiogenesis has been induced by one FGF-2 mimetic peptide at similar concentrations to recombinant FGF-2 (126). This molecule contains two 15-amino acid receptor binding domains and a 9-amino acid heparin binding sequence. Furthermore such
Mimetics can have demonstrable effects at the whole-organism level: a 15 amino acid peptide, based on the neural cell adhesion molecule binding site for FGF-receptor-1, imparted to rats a long-lasting improvement in memory upon intracerebroventricular administration (127). A further advantage to these mimetics is their relatively high stability relative to native growth factors, such as BMP-2, that are necessarily used in supraphysiologic doses.

Even active compounds bearing no relation to the primary structures of growth factors can be identified from peptide or small molecule libraries. As many cytokines and growth factors and their receptors are arranged in dimers, bi- or oligovalency can enhance the activity of these compounds. The covalent dimerisation of a 20 amino acid erythropoietin (EPO) mimetic peptide increased its affinity for the EPO receptor 100 fold (128). Mice erythropoiesis assays, which measure the incorporation of radioactive $^{59}$Fe into the blood, revealed a similar increase in in vivo potency of this peptide, although its activity still remained orders of magnitude short of native EPO. More generally, a wide and expanding palette of small molecules and ions such as retinoic acid, dexamethasone and thyroid hormones (to name a few of many) are known to influence differentiation (129)(130). Bioactive glasses such as PerioGlas® can be made to release various ions including calcium and silicon, which can effect upregulation of genetic pathways relevant to bone differentiation (131)(132). The bioactive glass ‘BCT001’ additionally releases strontium to help combat osteoporosis (Chapter 3 of this thesis describes a related strategy using alginate hydrogels).

The ability to bind growth factors and thus modulate cellular functions can be recreated in synthetic GAG mimetics (111)(112). Synthetic, sulphated di- and tetra-saccharides in side chain positions along a polymer backbone can successfully compete with neural CS for growth factor binding, despite non-native molecular architectures. One of these glycopolymers, a polymerised CS tetrasaccharide, had similar potency to neural CS (111). Interestingly, non-specific chemical sulphation of hyaluronic acid, which naturally occurs in a non-sulphated form, induces less extensive structural rearrangements of adsorbed and covalently bound fibronectin, translating into a higher level of cell attachment (133). Alginate can also be chemically sulphated to yield a polymer with binding affinities to growth factors (e.g. VEGF, PDGF-BB, and HGF) comparable to or higher than heparin and with the ability to enhance FGF-induced blood vessel formation (134). A sulphated and carboxylated dextran derivative potentiated VEGF binding to its
receptors, resulting in angiogenic effects, and even incorporating sulphonated monomers (sulphopropyl acrylate potassium) into poly(acrylamide) gels increases the uptake of serum proteins (135)(136). These materials carry the advantages of scalable chemical synthesis, more closely defined material properties, and more abundant starting materials, and are gaining interest as replacements for heparin and CS as modulators of growth factor release and activity.

In addition to binding a broad spectrum of proteins, small oligosaccharide domains present within larger GAG sequences can also regulate cellular function through their involvement in specific structural interactions with their binding partners (112)(137)(138)(139). Tetrasaccharides, for example, represent the minimal CS epitope necessary to stimulate neuronal growth, and the anticoagulant activity of heparin has been localised to a pentasaccharide motif that interacts selectively with antithrombin (140)(141). Thus, in the same way that short peptide sequences have been used to isolate specific protein functions, oligosaccharide fragments may emulate the function of proteoglycans while keeping structural complexity to a minimum (142). Understanding of structure-function relationships within polysaccharides is far behind that of proteins and nucleic acids, due to the difficulty of obtaining structurally defined oligosaccharides in bulk from natural polysaccharides, complicated chemical synthesis, and inadequate sequencing methods (143). The pace of progress in this field is accelerating, but for the time being, the full potential of these advances remains unrealised.
2.4.3 Controlling cells with simple chemistry

As well as introducing defined molecular recognition elements into biomaterials, recent studies have focussed on exploiting more basic chemistry to influence cell fate, potentially paving the way for structurally simple, yet cell-instructive, biomaterials. In the absence of adhesion peptides, cells interact with scaffolds via adsorbed protein, and in this regard hydrophilicity is a key consideration. Whereas hydrophobic scaffolds tend to adsorb protein in sub-optimal configurations (with hydrophobic residues displaced towards the scaffold surface), hydrophilic polymers adsorb protein in a hydrated interfacial phase wherein the proteins are more likely to retain their native conformation (144). In one study, introducing different functional groups to surfaces changed the conformation of adsorbed fibronectin leading to altered integrin binding, such that indices of osteoblastic differentiation (expression of bone markers, alkaline phosphatase activity and mineralization) were significantly upregulated on OH and NH$_2$ functionalised surfaces compared to CH$_3$ and COOH (145). There is considerable interest in utilising such simple chemistry to improve cell responses in favour of particular applications. Recently, a number of studies have described high throughput arrays for the systematic evaluation of large numbers of biomaterials-cell interactions (146)(147)(148). These investigations typically involve robotic spotting of polymer islands (3456 in one example) onto a glass slide coated with a non-adhesive polymer such as poly(hydroxyethyl methacrylate), which prevents cell migration between spots (147). Following cell culture, standard immunohistochemical techniques and microarray scanning can be performed. This provides a way of identifying polymers that support desired responses from specified cell types, for example those that promote human embryonic stem cell (ESC) differentiation to specific lineages (148).

Another approach has been to select chemical functionalities based on their resemblance to characteristic chemical features of particular ECMs. The bio-inspired use of sulphonate groups within hydrogels has already been described, mimicking their presence in GAG chains and increasing protein uptake (136). This approach is well established in bone TE, where there exist many examples of materials incorporating anionic chemical moieties that improve mineral deposition, for example by NaOH treatment of scaffold surfaces or via the incorporation of functionalised monomers such as methacrylated amino acids (GlyMA, SerMA, AspMA, GluMA) (149)(150). This practice stems from the observation that many glycoproteins involved in bone mineralisation display a high proportion of negatively charged amino acids. For example bone sialoprotein possesses two polyglutamic acid sequences, while osteopontin contains a run of 10-12
aspartic acid residues (151). Phosphate groups also nucleate mineral, and although often delivered in soluble form in vitro (as β-glycerophosphate) can be incorporated covalently into scaffolds: Wang et al. describe a photo-crosslinked hydrogel prepared from poly(ethylene glycol)-di-(ethylphosphatidyl (ethylene glycol)methacrylate) and poly(ethylene oxide) diacrylate (152). Osteoblasts (bone forming cells) within the scaffold produce alkaline phosphatase, an enzyme which cleaves the phosphoester linkages in the polymer, releasing phosphoric acid. This promotes mineralisation as the phosphoric acid reacts with calcium in the medium to form insoluble calcium phosphate.

Moreover, these chemical groups may also be instructive to cells. In a recent study, small defined chemical groups were incorporated into PEG gels, and encapsulated human mesenchymal stem cells differentiated towards cells of those tissues which the functional groups chemically resembled (67). Thus, those cells cultured in gels with charged phosphate groups increased expression of RUNX2 (CBFA1; an early bone transcription factor), produced a collagen-rich pericellular matrix, and synthesised osteopontin. Hydrophobic t-butyl groups pushed cells towards an adipocytic (fat cell) lineage, demonstrated by upregulation of the transcription factor PPARγ and the deposition of intracellular lipid deposits. It is unknown (and for practical purposes, arguably irrelevant) whether the role of the chemical modifications was to act directly on the cells, or to cause the preferential accumulation of particular cell-derived molecules, these molecules in turn providing behavioural signals to cells. An example of the latter mechanism in action is the ability of mineral deposits to sequester osteopontin, which improves cell adhesion and viability within phosphate-containing PEG gels (153). Whatever the modes of action, the complexity of biomaterials could be massively reduced if the essential chemical character of ECM influences could be distilled into simple chemical functionalities. A summary of the various ways in which relatively simple molecules can mimic the molecular information within the ECM is given schematically in Figure 2.6.

Figure 2.6 (overleaf): Synthetic mimics of biological structures. Many characteristics of ECM macromolecules have been reproduced in simpler compounds with biologically inspired structures. (a) Certain protein functions, including integrin binding for cell attachment and protease degradability, can be isolated to short amino acid sequences. These sequences can be combined with synthetic polymers or incorporated into complex peptides to enable cells to attach to or break down the material, respectively (43)(68)(69)(154)(81)(82). (b) Some glycoproteins involved in bone mineralization, such as [continued]
(Figure 2.6 continued) bone sialoprotein, possess runs of negatively charged amino acids. Peptides that incorporate these sequences, and synthetic polymers with negatively charged chemical groups, can display improved mineral-nucleating activity (149)(149)(150)(155)(156). (c) Growth factor action has been demonstrated in peptides possessing receptor-binding domains; heparin binding sequences may also be included to aid growth factor sequestration (125)(126). Random peptide libraries have allowed the identification of peptides with affinity to particular receptors, and dimerization of these molecules in some cases can improve receptor binding and physiological response (128). Growth factor action is sometimes potentiated through the actions of glycosaminoglycans (GAGs) such as the binding of heparan sulphate to the FGF-1/FGF receptor 2 complex. This specific interaction can be achieved using short heparin oligosaccharides (137). (d) Furthermore, the protein-binding function of ECM GAGs such as chondroitin sulphate can be mimicked by grouping sulphated oligosaccharides by polymerization, by sulphating natural carbohydrates such as dextran, or by sulphonating synthetic polymers (111)(133)(134)(135)(136)(157).
2.4.4 Discussion and broader implications

The relatively short history of industrial TE has proven that the provision of efficacious products is not in itself sufficient to ensure commercial success; there is a clear need to consider more than just biological and clinical factors. The solutions to this challenge lie at every phase of product development, beginning with identifying the simplest functional performance required to resolve a defined clinical problem.

Organogenesis Inc. and ATS suffered heavily as a result of their overestimating the number of chronic wounds cases that were best solved by high-tech, tissue engineered skin substitutes (respectively, Apligraf®, and Dermagraft®, Dermagraft® now produced by Advanced Biohealing) as opposed to acellular products that facilitate ongoing repair (11)(12).

Evidently, a careful appraisal of the job in hand will reveal that the broader cost and treatment implications for any biomaterials approach vary with several interrelated factors including the form of the device, the mode of delivery, the nature of the cellular component, and regulatory implications. To elaborate briefly, injectable matrices help to tackle problems of surgical invasiveness while TE products in sheet form confront problems related to nutrient supply by limiting diffusional distances. Moreover, materials that can recruit endogenous cells into scaffolds avoid the expense and difficulties associated with culture, storage and distribution of cells, not to mention immune considerations.

In terms of actual materials complexity, simplifying biomolecules as just described is one possible response. Another is to develop adaptable products or systems. A large number of commercially viable products for connective tissues are based on purified ECM components, particularly collagens and CS, representing a relatively generic ECM backdrop compatible with many cell types, which can thus be used in a range of different applications (1). The modularity of the Extracel system mentioned earlier is a particular advantage for niche markets such as vocal fold repair, where the cost of development may be high relative to volume of sales (63). Similarly, the synthetic process used by the Hubbell group allows any thiolated proteins or peptides to be included in PEG hydrogels by reaction with bis(α,ω-vinylsulfone)poly(ethylene glycol) before crosslinking – achieving a modular system in which a bioinert polymer background can be decorated with any combination of bioactive compounds (18). Thus, bioactivity can be conferred onto previously bioinert synthetic polymers, potentially generating materials
that combine versatile material properties and inexpensive production with flexible control over cell behaviour.

Encouragingly, it is clear that in some cases, comparatively simple materials in combination with an appropriate cellular component can support a high level of tissue organisation (4)(72). Simply creating an environment that is permissive for cell-cell interactions may enable cells to self-organise into more complex multicellular structures (such as blood vessels) than we could ever hope to impose ourselves. Furthermore, scaffolds with intelligently optimised mechanical and structural features – stiffness and RGD clustering, for instance – need not necessarily be any more costly to produce than their less well-tuned counterparts. The potential of these features to direct aspects of cell behaviour illustrates that functional sophistication need not necessarily be synonymous with high manufacturing costs. Many laboratories in their own ways are actively pursuing simple but effective solutions to TE problems, such that the TE ideal of structurally simple, yet functionally complex, biomaterials is becoming a plausible possibility for the near future.
2.5 Conclusions

There was much excitement associated with TE when it emerged as a new field in the early 1990s. This positivity led numerous efforts to turn nascent concepts into reality, using technology still in its infancy, with often disappointing results. Now, TE is entering a period of fruition, with many promising animal and human trials published and several products approved (1). Humans are already benefiting from lab-built organs. In constructing their tissue engineered bladders, Atala and his team used relatively simple moulded polymers optimised for mechanical compliancy (13). But as has been discussed, the capabilities already exist to produce far more complex scaffolds. Much is still unknown about the mechanisms by which tissues form and heal, yet already insights from developmental biology and other biological disciplines are actively guiding the development of intelligent materials that work with nature’s own mechanisms of repair.

The last decade has seen an increasing use of biomimetics in scaffold design, aimed at producing matrices which engage cells on a molecular level, coaxing them into remodelling a synthetic implant into genuine tissue. Advanced biomaterial approaches taking advantage of growth factor activity are just beginning to trickle through product development pathways. Growth factors are costly and impose demands on processing and storage conditions, but the enormous success of INFUSE® shows the potential commercial viability of these material/growth factor combinations (12). It is promising that the outcome of growth factor administration can be improved enormously with the use of technically simple slow release schemes, such as delivery using polymers. Such considerations may prove critical for the resolution of complex TE challenges such as that of vascularisation of constructs. The work described in Chapter 4 relates to this theme in that it describes an innovative approach for the delivery of TGF-β, which carries the potential benefits of improved growth factor stability, diminished biological impact of high initial loading and burst release, and the potential for the active release of growth factor by cells. Furthermore, interest is also growing in the exciting possibility of using simple chemistries to influence cell behaviour, and in the development of a range of therapeutics with intrinsic or modulating growth factor activity, including designer carbohydrates. With a biology-driven, but commercially-refined approach, commercial tissue engineering should flourish. In keeping with this philosophy, the next chapter describes a flexible, technically simple and inexpensive strategy for improving osteogenic differentiation within alginate hydrogel scaffolds.
Chapter 3

Strontium- and zinc-alginate hydrogels for bone tissue engineering

3.1 Introduction

Long bone fractures account for an estimated 10% of non-fatal injuries in the USA, and complications are common (20). Delayed union, for example, occurs in 16-100% of all fractures (depending on severity), and nonunion in 4-10% (20). Where proper healing does not take place, the direct treatment costs and social burden of these injuries are compounded by the need for further – sometimes multiple – surgical procedures. Current best-practice for bone induction at the fracture site is to graft autologous bone from the iliac crest, but the supply of this tissue is limited. Enduring pain at the donor site affects 18-31% of patients 24 months postoperatively, and the procedure can also lead to blood loss, infection, haematoma, iliac wing fracture, vascular and nerve injuries, dysesthesia, and scarring (20). The development of bone replacement materials to supersede autografts is thus an important healthcare objective, and tissue engineering substitutes are beginning to show promise in this regard (1).

Alginites are abundant natural polysaccharides with excellent biocompatibility provided they are sufficiently well purified (158). With the addition of calcium, they form hydrogels quickly and reversibly under physiological conditions, which break down with controllable kinetics as crosslinking ions are lost (159)(51). These hydrogels are used across several bioengineering disciplines including tissue engineering and drug delivery, and more recently as a platform system for basic biological research (160)(94) (161). Within tissue engineering, published work details their usage as scaffolds for bone (162), cartilage (163)(164)(165)(160), hepatic (166), cardiac (167), and pancreatic islet repair (168)(169) among others. Part of the reason for this spread of applications is the ability to control the material properties of alginate gels, such as stiffness and degradation, and to modify their chemistry towards particular requirements (170)(171)(51)(159). In its natural form alginate is biologically inert and adsorbs protein poorly, thus some level of modification is usually required to help drive appropriate
cell behaviour. It is readily functionalized via its carboxylic moieties, and the extent of cell interaction can be extrinsically controlled through the addition of macromolecules or peptides.

In the area of bone tissue engineering, alginate has been supplemented with various additives in order to improve the osteogenic response of encapsulated cells. Bioactivity can be improved by providing anchorage sites for cell attachment, for example by incorporating the major structural protein collagen I or its derivative gelatin into the device (32) (162). An alternative but related tactic is to introduce the cell-adhesive motif RGD in the form of short peptides conjugated directly to the alginate chains (172). In one example of this approach, RGD-modified calcium-alginate gels were used to deliver osteoblasts and chondrocytes subcutaneously into mice. This lead to the formation of mature bone, cartilage, and marrow at the implant site, and histological examination revealed multicellular aggregates resembling the growth plate zone of long-bones (72).

Alginate can be blended with various calcium phosphates (32), which are chemically similar to bone mineral, and depending on chemistry and microstructure may be osteoinductive (173). These ceramics may also become involved in non-covalent interactions with ECM proteins and glycoproteins, mimicking those occurring in bone matrix in vivo and helping to retain secreted proteins inside the scaffold (32)(174). A number of groups have investigated strategies for incorporating growth factors or related peptides into alginate gels. Most have involved BMP-2, co-delivered with TGF-β3 (175) or VEGF (176), overexpressed by transfection (177), or replaced by short (~20 amino acids) synthetic peptides derived from the full native sequence (178). Even unmodified alginate alone may support bone formation when injected under the periosteum, a putative source of progenitor cells (4).

One commonality of these studies is that calcium was chosen as the crosslinking ion in all cases, but alginate can be gelled by a number of other divalent and multivalent cations including Sr**, Zn** and Ba** (40)(179). Structurally, alginates are copolymers of (1,4)-linked α-L-guluronate (G) and β-D-mannuronate (M) residues, which occur both in homopolymeric regions – denoted G blocks and M blocks, respectively – and in alternating sequence (MG blocks; Figure 3.1). Gelation of an alginate solution occurs via a non-covalent crosslinking mechanism involving the chelation of divalent or multivalent cations into cavities created by the alignment of two or more alginate chains (39)(180)(181)(182). The assembly of
these ‘egg-box’ structures into larger junctional complexes causes the sol-gel transition (Figure 3.1). The cooperative binding of divalent cations to G blocks is highly selective, with affinity increasing in the order Zn\(^{2+}\)<Ca\(^{2+}\)<Sr\(^{2+}\)<Ba\(^{2+}\) (40). This relationship shows some correlation with ionic radius (Figure 3.2), suggesting that this selectivity is related to the steric fit of ions within ‘egg box’ cavities. Although G blocks have generally been regarded as the predominant crosslinking fraction of alginate, M and MG blocks are also capable of forming gels (180)(183). Again, this property is ion-dependent: barium crosslinks M blocks more effectively than calcium and strontium, whereas the reverse is true of pure MG blocks (179). These distinctive outcomes provide opportunities to tailor the materials properties towards particular applications. The long-term encapsulation of bacteria for fermentation or pancreatic islets for transplantation, for example, both demand long-lived gels. One way to achieve this is to use barium, which has exceptionally high affinity to high G alginate (168)(184)(179)(40).

![Figure 3.1: Alginate structure and gelation.](image)

(Left) Alginate monomers showing (1,4)-linkages, and polysaccharide fragment (159). G = α-L-guluronate, M = β-D-mannuronate. (Right) Idealised schematic of calcium binding to alginate in egg-box dimers and intra-cluster associated multimers. Zigzag lines represent G blocks, wavy lines represent M and MG blocks, and circles represent calcium (or other divalent/multivalent) ions. Note that in the dimer structure, the ratio of calcium to G residues (and hence COOH groups) is 0.25, whereas in the multimer it is 0.5.
Figure 3.2 Correlation between affinity for 92% G and 92% M alginites and ionic radii of some metal ions. Note that 92% G alginate is mostly composed of G blocks and the 92% M of mostly M blocks. Affinity data is from ref. (40), and ionic radii are from ref. (185).

There is furthermore an opportunity – virtually unexplored – of selecting ions based on their known biological effects. Strontium, complexed in the form of strontium ranelate, has recently been licensed in the EU as a treatment for osteoporosis. It has the ability – unique among osteoporosis drugs – to increase bone mass through the concurrent activation of osteoblast (bone forming) activity and suppression of osteoclast (bone resorbing) function (186). We have recently demonstrated that the dissolution products of strontium-substituted bioactive glasses both increased osteoblast proliferation and alkaline phosphatase activity, and inhibited calcium phosphate erosion by osteoclasts (187). Zinc is essential for proper skeletal development and acts as a cofactor for a multitude of enzymes including ALP, which is central to the bone mineralisation process (188). Zinc increases ALP activity in Saos-2 (osteosarcoma) cells and has been investigated as a dopant in β-tricalcium phosphates for bone regeneration, with encouraging results (189)(190).
In this work it is proposed that a simple adjunct to the bone tissue engineering approaches described above could be the substitution of all or a fraction of the calcium usually present in alginate gels for other ions with relevance to bone physiology – in this case, strontium and zinc ions. According to this scheme the alginate gel acts as a slow release reservoir of bioactive ions which help to drive osteoblast activity and promote the deposition of bone matrix within and around the scaffold (Figure 3.3). In this chapter, selected material and biological effects of strontium and zinc substitution in alginate gels are investigated and described. The results demonstrate that important properties of alginate hydrogels such as their degradation rates are strongly influenced by strontium incorporation, but these properties can be controlled independently of the crosslinking ion due to qualitatively different interactions between strontium and calcium with different alginate types. An in vitro model was used to demonstrate how this principle can be employed to optimise cell proliferation. The results suggest that calcium, strontium and zinc alginate hydrogels have improved osteogenic properties compared to two-dimensional tissue culture, and cells grown in strontium-crosslinked gels had the highest levels of ALP activity. This general approach can be adapted to different applications with varying materials and biological requirements.

Figure 3.3: Experimental concept. Alginate solution is crosslinked into a hydrogel by calcium, strontium, and zinc ions. The ions are gradually released as the gel degrades, leading to sustained biological action on encapsulated or nearby cells.
3.2 Materials and Methods

3.2.1 Materials

Two types of sodium alginate were obtained from FMC biopolymer, rich (55-65%) in either G (Protanal LF200DL) or M (Protanal LF200M) residues. These alginates will be referred to as “AlgG” and “AlgM”, respectively. A modified version of AlgG, with the peptide GRGDS covalently attached to the polymer chains via the alginate carboxylic groups, was kindly provided by Luis Rojo Del Olmo. This modified AlgG will be referred to as AlgG-RGD. Millipore 5.0μm Durapore membrane filters were used for casting gels. Inductively coupled plasma (ICP) reference solutions were from MBH analytical Ltd and analytical grade HNO₃ was from VWR. All other chemicals and solvents were from Sigma unless stated. The human osteosarcoma cell line Saos-2 was purchased from ECACC and used at passages < 15. Transwell inserts (12 mm diameter, 3.0 µm pore size) were Corning brand. All cell culture reagents, phosphate buffered saline (PBS), the LIVE/DEAD® viability kit, and alamarBlue® were from Invitrogen (GIBCO). Lactate dehydrogenase (LDH) activity was assayed using Promega Cytotox 96 kits. Except for Trizol reagent (Invitrogen), all RNA extraction and quantitative real-time polymerase chain reaction (qPCR) reagents including primers (Quantitect) were from QIAGEN. For the ALP assay, p-Nitrophenyl phosphate (pNpp) tablets were from Sigma and diethanolamine substrate buffer was from Thermo Scientific.

3.2.2 Preparation of alginate gels for materials characterisation

Autoclaved sodium alginate solutions in 10mM HEPES buffer (150mM NaCl, 10mM KCl, pH 7.4) were dispensed into cylindrical moulds of 16mm diameter and 10mm height, secured at either end by a membrane filter. To ensure complete crosslinking, these were left overnight in stirred 100 mM solutions of CaCl₂ and SrCl₂ alone or in combination as specified in Table 3.1 (160). The filters permitted the diffusion of ions into the mould while preventing alginate release.
Table 3.1: Gel formulations used for materials characterisation, including gel codes referred to in the text.

<table>
<thead>
<tr>
<th>Gel group</th>
<th>Gel</th>
<th>Alginate concentration (% w/v)</th>
<th>Alginate type</th>
<th>Crosslinking solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.Ca</td>
<td>3G.Ca</td>
<td>3</td>
<td>AlgG</td>
<td>100 mM CaCl$_2$</td>
</tr>
<tr>
<td></td>
<td>2G.Ca</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1G.Ca</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M.Ca</td>
<td>3M.Ca</td>
<td>3</td>
<td>AlgM</td>
<td></td>
</tr>
<tr>
<td>G.Sr20</td>
<td>3G.Sr20</td>
<td>3</td>
<td>AlgG</td>
<td>80 mM CaCl$_2$ + 20 mM SrCl$_2$</td>
</tr>
<tr>
<td>G.Sr</td>
<td>3G.Sr</td>
<td>3</td>
<td>AlgG</td>
<td>100 mM SrCl$_2$</td>
</tr>
<tr>
<td></td>
<td>2G.Sr</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1G.Sr</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M.Sr</td>
<td>3M.Sr</td>
<td>3</td>
<td>AlgM</td>
<td></td>
</tr>
</tbody>
</table>

3.2.3 Compressive testing

Within 1 hour of being released from the diffusion chambers, the gels were tested in unconfined compression using an Instron 5866 with a 500N load cell, at a constant rate of deformation (5mm/min). Compressive extension and reactive force were recorded using BlueHill 2 software, and modulus ($E$) was calculated from stress-strain curves over the first 1 mm of deformation (approx. 10% strain).

3.2.4 Swelling, degradation and ion release

Blotted gels were placed in vials and covered with PBS. At intervals the PBS was aspirated and retained for ICP analysis of ion concentrations, the gel was weighed within the vial, and fresh PBS was added. Measurements were taken every 2 hours for the first 8 hours, then at increasing time intervals until complete degradation had occurred, i.e. when the gel mass fell to zero. The day of peak swelling was chosen as the most reliable indicator of gel stability, and will be referred to as the 'lifespan' of the gel. To detect ions released from strontium-containing gels, PBS samples were acidified in 3.5% v/v HNO$_3$ (final concentration) and filtered, and dilutions were made in the same PBS/HNO$_3$ solution. Samples were
analysed for strontium content (plus calcium where applicable) by a Perkin Elmer 2000DV ICP optical emission spectrometer calibrated against reference solutions of 1, 5 and 20 ppm. Samples with an ion concentration above 20ppm were diluted to between 2 and 20ppm and reanalysed. The total ion release from all gels (including calcium where applicable) was corrected for the ions present in the crosslinking solutions and expressed as a ratio of ions to carboxylic acid groups in the alginate. Rates of release were calculated between days 1-5, days 5-10, and day 10 onwards.

### 3.2.5 Cell culture and toxicity of crosslinking solutions

The Saos-2 osteosarcoma cell line was chosen to model osteoblast behaviour, due to their ability to deposit a mineralising collagenous matrix under appropriate inductive stimuli (191)(192). The cells were maintained in serum-containing medium (RPMI + 10% v/v foetal bovine serum, FBS, + 1 mM l-glutamine) and passaged at 60-80% confluence according to standard procedures. Potential crosslinking solutions were assessed for toxicity using alamarBlue® reagent. Confluent Saos-2 in 48-well plates incubated in 1 ml crosslinking solutions for 30 minutes. The solutions tested were: PBS, 100 mM SrCl₂, and ZnCl₂ at 0, 1, 2, and 5 mM plus either CaCl₂ alone, or 20 mM SrCl₂ plus CaCl₂, to a total concentration of 100 mM (all in HEPES buffer). The cells were rinsed once more in PBS and the solutions were replaced with cell culture medium. 24 hours later, medium was exchanged for 10% v/v alamarBlue® reagent in phenol red-free cell culture medium. The plates were incubated for 4 hours and fluorescence was measured at 590 nm emission with 540 nm excitation. The absorbance of the experimental samples (FluorE) was normalised to starting values (FluorC, alamarBlue® solution not exposed to cells) according to the formula:

\[
\frac{\text{FluorE} - \text{FluorC}}{\text{FluorC}}
\]

### 3.2.6 3D cell encapsulation and culture

Double strength AlgG and AlgM solutions in HEPES buffer (as above) were supplemented with AlgG-RGD and autoclaved, which has previously been shown not to affect RGD biofunctionality (75)(193). These were mixed in equal volume with a suspension of Saos-2 in cell culture medium (phenol red-free RPMI + 10% v/v FBS + 1 mM l-glutamine), to final concentrations of 2% w/v alginate and 150 µM RGD and a final cell density of 2.5 x 10⁶ cells.ml⁻¹ (72). This mixture was dispensed into transwell inserts bathed in 100
mM solutions of CaCl\textsubscript{2}, SrCl\textsubscript{2} and/or ZnCl\textsubscript{2} in HEPES buffer (combinations are given in Table 3.2). For viability studies 200 µl gels were cast in 12 mm diameter inserts, and for gene and protein expression experiments 6.5 mm diameter inserts were used to make 40 µl gels. Crosslinking proceeded for 30 minutes at 37°C as previously reported (164), and the solutions were replaced with media, which was changed the following day and every 2 or 3 days. Tissue culture plastic (TCP) controls were cultured alongside alginate gels.

<table>
<thead>
<tr>
<th>Gel</th>
<th>Composition of crosslinking solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CaCl\textsubscript{2} (mM)</td>
</tr>
<tr>
<td>Ca100</td>
<td>100</td>
</tr>
<tr>
<td>Sr100</td>
<td>-</td>
</tr>
<tr>
<td>Sr20</td>
<td>80</td>
</tr>
<tr>
<td>Zn1</td>
<td>99</td>
</tr>
<tr>
<td>Sr20.Zn1</td>
<td>79</td>
</tr>
</tbody>
</table>

Table 3.2: Composition of alginate crosslinking solutions used in cell studies, including gel codes referred to in the text. The alginate concentration was 2% (w/v), and the type of alginate (AlgG or AlgM) is specified in the main text as appropriate.

3.2.7 Cell viability within alginate hydrogels
To visualise Saos-2 within alginate gels, the gels were thinly sliced, stained with LIVE/DEAD® reagent (1 µM calcein + 1 µM ethidium homodimer-1 in PBS) for 30 minutes at room temperature, and then imaged using an Olympus IX51 inverted microscope equipped with a mercury bulb and FITC/TRITC filters. LDH activity and the alamarBlue® assay were chosen as two independent indicators of cell number. AlamarBlue® testing was performed on intact gels at days 1 and 7, as described above but with a 2.5 hour incubation period. Fluorescence was then expressed as a percentage of that of Ca100 gels at 24 hours.

For LDH, gels were frozen in PBS on days 0, 7, 14 and 21. At a later date they were defrosted, placed in 1.2 ml TissueLyser tubes with 5 mm steel balls. They were disrupted in a TissueLyser II bead-mill homogeniser (QIAGEN) for 15 minutes at 20 Hz, and finally treated with 0.1% Triton-X for 30 minutes to
lyse any remaining cells. Samples were then assayed using a CytoTox 96 kit according to the manufacturer’s instructions: briefly, lysate was mixed with an equal volume of substrate solution, incubated at room temperature in the dark for 30 minutes, the reaction was stopped (acidic Stop Solution) and absorbance was read at 490 nm. It was determined in advance through standard curve comparisons that the sample preparation procedure did not compromise the performance of the LDH assay (see Appendix I: Validation of LDH sample preparation).

3.2.8 Osteoblast activity within alginate hydrogels

Osteoblast activity of Saos-2 cells was investigated by qPCR on osteoblast marker genes and ALP protein activity. A hybrid procedure incorporating Trizol extraction with RNeasy purification was used for RNA extraction. At day 21, gels were homogenised in 1 ml Trizol in a QIAGEN TissueLyser II until completely disrupted; typically this took no more than 10 minutes at 30 Hz. The samples were centrifuged at 12,000 g for 10 minutes in a centrifuge cooled to 4 °C. The supernatant was transferred to a new tube and mixed with 150 µl chloroform. The RNA-containing aqueous phase was isolated as per the standard Trizol procedure and mixed well with an equal volume of 70% ethanol. The precipitated RNA was transferred to an RNeasy spin column, and purified and resuspended in RNase-free water according to the standard RNeasy protocol. Some of the troubleshooting steps taken to determine this as an effective means of collecting good quality RNA from alginate hydrogels are summarised in Appendix III: RNA extraction troubleshoot. RNA was quantified using a NanoDrop 1000, diluted to ≤ 80 ng.µl⁻¹, and immediately reverse transcribed using a Quantitect RT kit (manufacturer’s instructions). 10x and 100x dilutions were made in RNase free water. qPCR was performed on a Corbett Rotor-Gene using Rotor-Gene SYBR green PCR master mix and Quantitect exon-spanning primers for RUNX2, COL1A1 and BSP. Primer validation is discussed in Appendix IV: qPCR primer validation. RUNX2 encodes the essential osteoblast transcription factor of the same name (also known as CBFA-1), and collagen I (COL1A1) and bone sialoprotein (BSP) are major bone ECM components. Expression of these genes was normalised against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data was analysed using the delta-delta Ct method, comparing gene expression to that of untrypsinised cells collected at baseline. ALP enzyme activity was assayed using 1 mg.ml⁻¹ pNpp in diethanolamine substrate buffer for 5 minutes.
with absorbance taken at 405 nm. Cell free hydrogels were run alongside samples to correct for non-specific background activity.

### 3.2.9 Statistical analysis

Analysis of Variance with Tukey's post-tests, and Independent samples T-tests were used for compressive modulus, gel lifespan, LDH, and ALP activity data. Non-parametric tests (Kruskal-Wallis and Mann-Whitney) were used for qPCR results. All statistical tests were run on SPSS for Windows, and $P < 0.05$ was considered significant.
3.3 Results and discussion

3.3.1 Strontium substitution does not alter initial stiffness of alginate hydrogels

In the initial phase of this study, calcium- and strontium-alginate gels were characterised in terms of stiffness ($E$), degradation and strontium release in order to evaluate how ion type, alginate type, and alginate concentration influence these properties, and to demonstrate the ability to tailor gel properties. The stress-strain curves of gels tested in unconfined compression were highly linear ($R^2 > 0.99$) over the first 1 mm (~10%) strain (Figure 3.4, Table 3.3).

![Stress-strain plot](image)

**Figure 3.4: Representative stress-strain plot for alginate gels over first 1 mm (~10%) strain.** 3G.Ca gel shown.

**Table 3.3: Average linear correlation factors ($R^2$) values for first 1mm deformation of alginate gels under compression.**

<table>
<thead>
<tr>
<th>Type of gel</th>
<th>$R^2$ over first 1mm deformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1G.Ca</td>
<td>0.9984</td>
</tr>
<tr>
<td>2G.Ca</td>
<td>0.9999</td>
</tr>
<tr>
<td>3G.Ca</td>
<td>0.9998</td>
</tr>
<tr>
<td>3M.Ca</td>
<td>0.9996</td>
</tr>
<tr>
<td>1G.Sr</td>
<td>0.9977</td>
</tr>
<tr>
<td>2G.Sr</td>
<td>0.9999</td>
</tr>
<tr>
<td>3G.Sr</td>
<td>0.9999</td>
</tr>
<tr>
<td>3M.Sr</td>
<td>0.9996</td>
</tr>
</tbody>
</table>

The modulus ($E$) of G.Ca gels (refer to Table 3.1 for gel codes) increased linearly with increasing alginate concentration, from 38 ± 2 kPa (1G.Ca) to 348 ± 8 kPa (3G.Ca; Figure 3.5), in close agreement with published data (160), and was roughly 45% lower for AlgM gels (196 ± 3 kPa, 3M.Ca). $E$ of strontium gels was close to that of calcium gels, both with AlgG (75-109% $E$ of G.Ca gels) and AlgM (97% $E$ of 3M.Ca), suggesting that strontium substitution had little to no effect on stiffness (Figure 3.5). From this we can infer that under the conditions tested, the degree of crosslinking was unaffected by the gelling ion.
Assuming constant crosslinking functionality, crosslinking density is proportional to the effective network chain concentration $v_e/V$ (mol/cm$^3$), which in turn is proportional to $E$ at constant temperature as can be seen from the equation:

$$
\frac{v_e}{V} = \frac{E}{3RT}
$$

Where $E$ is Young's modulus in dyne/cm$^2$, $R$ is $8.314 \times 10^7$ in dyne.cm/mol.K, and $T$ is the temperature in K (194). Hence, $v_e/V$ and by extension crosslinking density (assuming equal functionality) was the same regardless of the ion used. This is unsurprising considering that the cations used to cast these gels were delivered in large excess, and thus it may be supposed that all carboxylic acid groups were saturated with bound ions.

![Figure 3.5: Modulus (E) of alginate hydrogels crosslinked with calcium and strontium over first 1 mm of compressive deformation (~10% strain). Effect of alginate type P < 0.001; Effect of crosslinking ion P = 0.044. Mean ± SD, n ≥ 5.](image)

**3.3.2 Crosslinking ion(s) and degradation behaviour can be independently varied**

Alginate gels immersed in solution gradually lose crosslinking ions, leading first to swelling (as tensile resistance to osmotic swelling becomes weaker), and eventually to degradation as the polymer chains separate. The gels in this study swelled in PBS to 200-300% initial mass (Figure 3.6) before beginning to disintegrate. Under the conditions tested, the 3G.Ca and 3M.Ca gels had a lifespan of around 4 ± 0 and 4 ±
1 days, respectively (Figure 3.7). G.Sr gels were remarkably stable in comparison (effect of ion P < 0.001).

The 3G.Sr composition persisted for 58 ± 5 days before the onset of degradation, which was almost fifteen times as long as the corresponding calcium gels. This behaviour is consistent with the higher affinity for G blocks of strontium over calcium (40)(179). Partial strontium substitution had almost as pronounced an effect: substituting just 20% of the calcium for strontium increased the lifespan to 53 ± 3 in 3G.Sr20 gels, although the actual fraction of strontium within these gels may have exceeded the 20% present in the crosslinking solution due to preferential accumulation of strontium ions in G blocks. Despite this high stability, the lifespan of the G.Sr gels was tailorable down to 4 ± 1 days by reducing the concentration of alginate to 1% w/v. Thus, although strontium substitution left E essentially unchanged, gel lifespan was significantly extended.

The stabilising effect of strontium on AlgG gels was greatly diminished when AlgM was used. Here, lifespan was increased from 4 ± 1 (3M.Ca) to 15 ± 1 days (3M.Sr), which was only around 25% as long as the lifespan of the equivalent AlgG gels. Again this is compatible with work showing that the selectivity of G blocks for larger ions does not apply to M blocks (40). It seems therefore that strontium substitution has a qualitatively different effect on the stability of AlgG and AlgM gels. This interaction is of great practical interest as it allows one to decouple the biological and material effects of the crosslinking ion. Specifically, the favourable effects of strontium on bone cells can be utilised in applications requiring either long or short lifespans, which can be achieved by choosing alginate rich in either G or M residues respectively.
Figure 3.6: Weight profiles of alginate gels swollen in PBS. a) Calcium-alginate gels, b) Strontium-alginate gels. Note that time is plotted on a log axis. Mean ± SEM, n = 6.

Figure 3.7: Gel lifespan (day of peak swelling) for alginate gels of different compositions. Effect of alginate type P < 0.001; effect of crosslinking ion P < 0.001; interaction between ion and alginate type P < 0.001. Mean ± SD, n = 6.
3.3.3 Strontium release from alginate hydrogels is of a physiologically-relevant magnitude

The potential of alginate gels to provide a slow-release reservoir of bioactive ions was investigated using ICP. Strontium was released from the alginate gels in a rapid burst over the first 8 hours (representing the clearance of excess ions from the 100 mM crosslinking solutions) followed by a phase of slower release (Figure 3.8). Strontium release gradually diminished over the first 5-10 days before settling to an approximately constant rate. The rate of steady release was fairly constant, such that linear trend lines fitted from day 10 all had \( R^2 \) values of > 0.92 (Table 3.4), and furthermore was sustained until complete degradation of the gels had occurred (Figure 3.8; compare Figure 3.6). The ratio of total released ions to carboxylic acid groups was between 0.24 and 0.46 in all cases, which fits well with the stoichiometry of calcium binding to alginate, which is 0.25 within egg-box dimers and 0.5 for the repeating units of multimers (39).

![Figure 3.8: Cumulative strontium release from alginate gels as measured by ICP.](image)

The day of maximum swelling, i.e. the onset of gel degradation.

Strontium release from AlgG gels increased with alginate concentration, and the release from the 3G.Sr20 gel was around a third of that of 3G.Sr at days 1-5 and around half at days 5-10 (Table 3.4). The highest rates of strontium release were seen in the AlgM gel, which is consistent with the faster degradation of these gels (Figure 3.7). Here, 5.4 \( \mu \text{M Sr}^{++} \) per gram of gel were released daily from days 5-10, then 1.4 \( \mu \text{M.gram}^{-1}.\text{day}^{-1} \) from days 5-10, and finally a steady rate of 0.5 \( \mu \text{M.gram}^{-1}.\text{day}^{-1} \) was maintained until total degradation had occurred (at day 29; see Figure 3.8). Release rates of calcium were considerably higher than those of strontium over the first 10 days (5.1-9.6 \( \mu \text{M calcium.gram}^{-1}.\text{day}^{-1} \) cf. 1.4-5.4 \( \mu \text{M} \)
strontium gram⁻¹ day⁻¹ for 3G and 3M single ion gels) but these gels were shorter lived and therefore only released ions for the first 10 days.

<table>
<thead>
<tr>
<th>Gel</th>
<th>Ion</th>
<th>Rate of ion release (µM.g⁻¹.day⁻¹; R² in brackets)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Days 1-5</td>
</tr>
<tr>
<td>3G.Ca</td>
<td>Ca²⁺</td>
<td>5.06</td>
</tr>
<tr>
<td>3M.Ca</td>
<td>Ca²⁺</td>
<td>6.75</td>
</tr>
<tr>
<td>3G.Sr20</td>
<td>Ca²⁺</td>
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<td></td>
<td>Sr²⁺</td>
<td>1.28</td>
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<td>2G.Sr</td>
<td>Sr²⁺</td>
<td>3.12</td>
</tr>
<tr>
<td>1G.Sr</td>
<td>Sr²⁺</td>
<td>1.92</td>
</tr>
<tr>
<td>3M.Sr</td>
<td>Sr²⁺</td>
<td>5.40</td>
</tr>
</tbody>
</table>

Table 3.4: Rates of calcium and strontium release from alginate gels, in µM per gram of gel per day. R² values are given in brackets as an indicator of steady release from day 10. Rates of Ca²⁺ and Sr²⁺ release are shown for days 1-5, days 5-10, and from day 10 onward. N/A = not applicable (gels had degraded by day 10).

The figures in Table 3.4 can be converted to daily concentration estimates (in mM) for the cell culture system used by dividing the quoted figures by ten – based on the gels having a density of 1 and comprising 10% of the volume of the culture system. Thus, the concentration of strontium in the medium for the 3G.Sr formulation increased by approximately 0.37 mM daily over the first 5 days, then by 0.15 mM to day 10. The daily release estimates for all strontium gels except 1G.Sr and 3G.Sr20 therefore fall within the in vitro physiologically active range for strontium of 0.1 – 1 mM and above for the first 10 days of culture, falling below this range thereafter (186)(187). 3M.Sr alginate gels, however, released strontium most rapidly (0.54, 0.14, 0.051 mM.day⁻¹ at days 1-5, 5-10, and 10 onwards, respectively), allowing for media changes every 2-3 days and higher local concentrations inside the gels, strontium concentrations probably reached biologically relevant levels even towards the end of the culture period. Indeed, the levels detected in the serum of patients treated with strontium ranelate are towards the lower end of the active range (0.12 mM), suggesting that in vivo strontium may accumulate at the bone surface,
becoming available locally at much higher concentrations (195). These figures may overestimate the release in cell culture medium as alginate gels are less stable in PBS, and still less can they predict the in vivo situation, but they are nonetheless useful as order of magnitude estimates and for assessing differences between formulations. The higher release from AlgM gels and with increasing alginate concentrations immediately suggest two ways of optimising strontium release (further options are discussed below).

### 3.3.4 1 mM ZnCl$_2$ was chosen for three-dimensional culture of Saos-2 in gels

In addition to the calcium and strontium gels previously characterised, zinc was included in two of the gel formulations for the 3D cell culture experiments (refer to Table 3.2 for gel codes). Zinc exerts its biological effects at micromolar concentrations (189) and is acutely toxic at millimolar concentrations, therefore the toxicity of various potential crosslinking solutions was assessed before being used for cell encapsulation. A significant ($p < 0.001$) toxic effect of zinc was apparent at 1 mM concentrations over just 30 minutes (30-40% reduction in activity), and ZnCl$_2$ at 2 and 5 mM was progressively more damaging. 1 mM was chosen for alginate crosslinking because the limited toxic effect seen on TCP was likely to be attenuated by the dilution of the ions as they diffused through the alginate solution. The crosslinking solutions lacking zinc were no more toxic than PBS.

![Figure 3.9: Toxicity of potential crosslinking solutions.](image)

**Figure 3.9: Toxicity of potential crosslinking solutions.** Relative total metabolic activity (alamarBlue® fluorescence) of Saos-2 cells 24 hours after 30 minute exposure to potential crosslinking solutions. Total molarity of crosslinking solutions including ZnCl$_2$ = 100 mM. Effect of zinc $p < 0.001$. * 1 mM ZnCl$_2$ versus no zinc $p < 0.001$. Mean ± SD, n = 6.
3.3.5 **High stability of strontium-AlgG gels impedes proliferation; viability is rescued by AlgM**

The peptide GRGDS, incorporating the cell-adhesive sequence RGD, was incorporated into alginate gels to improve viability by enabling cells to attach to the gel (72). Pilot investigations into Saos-2 viability confirmed that, as expected, the inclusion of GRGDS peptide improved cell survival (these experiments are described in Appendix IV: Pilot experiments in cell viability).

The choice of crosslinking ion(s) significantly affected LDH activity when Saos-2 were cultured in AlgG gels (P < 0.001). LDH activity from Saos-2 cultured in Ca100 and Zn1 gels increased marginally to day 7 and, except for Ca100 gels at day 21, continued to increase thereafter (**Figure 3.10**). Conversely, in all gels containing strontium (Sr100, Sr20, Sr20.Zn1), LDH activity declined during the first week of culture. There was some recovery from day 14 in the Sr20Zn1 and Sr20 groups, and at day 21 in the Sr group. The trends were very different for AlgM gels, which supported higher levels of proliferation in all five ion groups compared to AlgG (p < 0.001). Furthermore the degree of proliferation was similar in all five groups regardless of the crosslinking ion(s) used (effect of ion P = 0.082). For both AlgG and AlgM groups, proliferation was greater on TCP than in any of the hydrogels.

AlamarBlue® reduction by Saos-2 was also dependent on alginate type and crosslinking ion. Within AlgG gels, alamarBlue® fluorescence increased from between 59 ± 8 and 74 ± 9 % of day 1 TCP levels (Sr100 and Zn1 gels, respectively), to between 62 ± 11 and 98 ± 13 % at day 7 (for the same gels; **Figure 3.11**). Again, there was a significant difference at day 7 between gels made with and without strontium, with lower activity occurring in strontium-containing gels (p < 0.01). Conversely, there was no significant difference in day 7 activity between AlgM formulations with and without strontium (p = 0.129), and greater increases in activity were seen than in AlgG gels. Saos-2 increased their activity from between 59 ± 5 and 101 ± 10 % of day 1 TCP activity at day 1 (Sr100, Ca100 gels, respectively), to between 145 ± 23 (Ca100) and 177 ± 27 % (Sr20.Zn1) at day 7. Over the same period, cells grown on TCP roughly tripled in number, which was around 2.3 times the proliferation seen in AlgG gels, and 1.6 times that within AlgM gels.
Figure 3.10: LDH activity of Saos-2 cells encapsulated in 2% w/v RGD-alginate gels and grown on TCP, relative to day 0 gels. Results for AlgG gels, AlgM gels, and TCP are displayed separately for clarity. Effect of crosslinking ion $p < 0.001$; *strontium-containing gels vs non strontium-containing gels at day 7 $p < 0.001$. Effect of crosslinking ion $p = 0.082$. Effect of alginate type at all time points $p < 0.001$. Mean ± SD, $n = 6.$
**Figure 3.11:** AlamarBlue® reduction by Saos-2 cells, encapsulated in 2% w/v RGD-alginate gels and grown on TCP, relative to day 1 TCP (background subtracted). Results for AlgG gels, AlgM gels, and TCP are displayed separately for clarity. Effect of alginate type at 1 day N.S. (p = 0.864) and 7 days p < 0.001. For AlgG gels at day 7, effect of Sr versus no Sr p < 0.01 and effect of ion p < 0.05. * Sr versus Ca and Zn gels at day 7 p < 0.05. For AlgM gels at day 7, effect of Sr versus no Sr N.S. (p = 0.526) and effect of ion N.S. (p = 0.129). Mean ± SD, n = 6.
LIVE/DEAD® imaging at later timepoints revealed that dead cells tended to be isolated from other cells, whereas the majority of live cells were commonly present in large clusters (Figure 3.12). In general, these clusters were first noted at the (softer) periphery of gels, and were less numerous in AlgG gels than AlgM – especially with formulations containing strontium.

Figure 3.12: LIVE/DEAD® images of clusters of Saos-2 cells within 2% w/v AlgM RGD-alginate gels after 21 days of culture. Live cells appear green and dead cells red. Arrows indicate isolated dead cells, and arrowheads point to clusters of live cells. Fluorescent microscopy; 40x magnification; scale = 200 µm.
Scaffold degradation is central to the tissue engineering approach, and allows for cell behaviours such as growth and migration as well as the gradual accumulation of matrix. Indeed, LIVE/DEAD® and LDH results revealed a strong correspondence between the degradation characteristics of the alginate gels and the ability of cells to grow and divide within them. The persistence of strontium-supplemented AlgG gels appeared not only to obstruct growth during the initial 1-2 weeks of culture, but even to cause cell death over the first week (Figure 3.10). This tendency may be related to cell isolation per se (lack of cell-cell contacts and paracrine signalling), limited diffusion of nutrients and signalling factors, or both (196). While LDH activity declined over the first week in strontium-containing AlgG gels, alamarBlue® activity increased slightly – a discrepancy which may be explained by the changing diffusive properties of these gels as they swell over time. The alamarBlue® results somewhat underestimate total metabolic activity due to the relatively slow diffusion of reagent through the gels. This error is greater at day one because the gel structure is more compact early on.

In contrast to the strontium-containing AlgG formulations, the more rapidly degrading Ca100 and Zn1 AlgG gels supported proliferation from the earliest timepoints (Figure 3.10). This was also consistent with the observation that clusters of proliferating cells tended to form initially at the swollen edges of gels. Fortunately it was possible to rescue proliferation in strontium-containing gels by using more rapidly degrading gel formulations: with AlgM the distinction between gels with and without strontium was lost, and neither LDH nor alamarBlue® activity revealed significant differences between the five gel sets. Together, these results indicate that crosslinking alginate hydrogels with strontium and zinc does not inhibit cell proliferation provided that the gels degrade rapidly enough to permit growth. This points towards using AlgM for applications where short term action and cell proliferation is required, such as fracture healing, in which the fracture callous forms rapidly (days to weeks). AlgG gels may be suited to situations in which the materials are delivered acellularly, or where cell proliferation is not required. In previously reported studies, strontium-alginate microcapsules supported significantly higher viability and sulphated GAG accumulation over long term culture of intervertebral disc cells, which within the mature disc are sparsely distributed and rarely divide (197)(198). In the present study, the dual characteristics of faster degradation and higher strontium release suggested that the AlgM gels would be the more suitable choice for the remainder of the experiments.
3.3.6 Osteoblast genes are upregulated in alginate gels; ALP activity is highest in Sr100 gels

Saos-2 were cultured in AlgM gels crosslinked with different ions in order to investigate whether the inclusion of strontium and/or zinc stimulated upregulation of the osteoblast marker genes COL1A1, BSP and RUNX2, and higher ALP protein activity. Saos-2 have remarkably stable gene expression over many passages in culture (199), and indeed expression levels for the TCP group did not deviate far from baseline values (Figure 3.13; relative expressions: COL1A1 0.76 ± 0.23, BSP 3.31 ± 0.94, RUNX2 0.87 ± 0.64). This consistency provides some validation of the data overall. Upregulation of COL1A1, BSP and RUNX2 was seen in all gel groups compared to TCP (p < 0.001), but there were no consistent differences between the five gel compositions (p > 0.17). The mean relative expression of all four genes was very similar in Ca, Sr and Sr20 gels. Zn1 produced the highest mean expression, and Sr20.Zn1 the lowest among gel groups, of BSP and RUNX2, but these differences were not significant (p > 0.64). RUNX2 is a transcription factor central to the process of osteoblast differentiation, with many bone matrix-related and other genes under its control (200). Its upregulation therefore suggests an increase in osteoblast activity of Saos-2 grown in gels, and higher COL1A1 and BSP expression and ALP activity furthermore indicate increased deposition of a mineralising matrix. The apparent osteogenic effect of the alginate gels may be a consequence of three- versus two-dimensional culture and/or of ion release (201)(202)(203).
Figure 3.13: Gene expression of Saos-2 cells grown on TCP or in 2% w/v AlgM RGD-alginate gels at day 21, relative to day 0 TCP. COL1A1, BSP and RUNX2 shown, as indicated. Mean ± full range of values, \( n \geq 3 \) from two independent experiments. * Effect of TCP versus gels \( p < 0.001 \) for all genes; effect of crosslinking ion: COL1A1 \( p = 0.91 \), BSP \( p = 0.53 \), RUNX2 \( p = 0.26 \).
ALP protein activity was then quantified using the pNpp assay (Figure 3.14). Given that this enzyme is membrane bound and thus should only be present in cells, ALP activity was normalised to cell number (determined using LDH activity). In agreement with the general trend of the qPCR data, ALP activity per cell was higher in Saos-2 cultured in gels than on TCP (P < 0.001). Furthermore, activity was highest in Sr100 gels, and this was significant against all other groups except Zn1 (P < 0.05). ALP in Zn1 gels was also significantly higher than Ca gels (P < 0.05). The reduction in normalised ALP activity seen in the TCP group could be due to confluence effects, as ALP activity in Saos-2 is known to decrease post-confluence (192)(204). This phenomenon may have similarities with the reduction in ALP activity seen in postmitotic osteoblasts (205).

Figure 3.14: Alkaline phosphatase (ALP) activity per cell of Saos-2 grown on TCP or in 2% w/v AlgM RGD-alginate gels with different crosslinking ions. The dotted line represents baseline (day 0) activity, determined for Saos-2 in Ca100 gels. Mean ± SD, n = 6. Effect of gels versus TCP P < 0.001. *Sr100 vs Sr20 and SrZn P < 0.05; **Sr100 vs Ca100 and TCP P < 0.001; *Zn versus Ca100 P < 0.05.
In summary, the results indicate that COL1A1, BSP and RUNX2 expression increased in cells cultured within alginate gels compared to TCP, and likewise, higher ALP activity was seen in the gel groups than on TCP (Figure 3.13, Figure 3.14). Cells in this study spread on TCP while remaining more compact in 3D gels (Figure 3.12), but an effect of cell shape seems unlikely: in two dimensions, spread rather than rounded morphologies tend to favour the osteoblast phenotype (at least in MSCs) (93). Although there were no consistent differences in gene expression between the different gel formulations, statistically significant differences in ALP activity were seen between gel groups. The highest activity at both 7 and 21 days occurred in strontium gels and the lowest in calcium (excluding TCP), strongly indicating that strontium alginate gels may have improved osteogenic properties over calcium alginate. It was hypothesised that cocktails of two or three ions might elicit a stronger response than strontium alone, but the activity in mixed ion gels did not appear to improve on Sr100 gels. The higher strontium release from the Sr100 gels compared to the Sr20 gels is a likely explanation for this (Figure 3.8, Table 3.4).

The notion of engineering biomaterials to release osteogenic ions has been explored by several groups including ours, but these attempts have focussed predominantly on delivery via ceramics (187)(206)(190)(207)(208). This study demonstrates that alginate could be considered as an alternative medium for the steady release of bioactive ions, but also highlights the option of choosing alginate as a carrier for osteoinductive ceramics. Ceramics, being sparingly soluble, could also set the gel and could be formulated to achieve a working time appropriate for injectable or moldable systems. Meanwhile the alginate could hold particles or powder together and buffer the escape of dissolution ions to prolong their retention at the implant site. Alternatively, embedding strontium-AlgG microparticles within an AlgM gel could be an attractive way of prolonging the timeframe of strontium release while allowing the bulk gel to degrade quickly to permit cell proliferation or invasion. Another potential strategy might be to use a mixture of ions with synergistic or complementary physiological effects. For example, we are currently evaluating the use of cobalt in tissue engineering scaffolds due to its ability to activate the hypoxia pathway (24). This is an important pathway in many aspects of development and regeneration including skeletal, so to combine cobalt with strontium and/or zinc could be an interesting next step.
3.4 Conclusions

RGD-modified high M strontium alginate gels have favourable strontium release profiles, degrade quickly enough to permit cell proliferation, and support higher alkaline phosphatase activity than calcium gels. This data broadly supports the premise of this work – that alginate hydrogels may be used as slow release reservoirs for bioactive strontium ions to enhance osteogenic activity and/or differentiation – and suggests that strontium alone may be more effective than combinations of more than one ion. Overall, this work presents a novel tissue engineering strategy which is compatible with previous innovations, can be adapted to different tissue engineering needs, and opens several avenues for further development.
Chapter 4

Latent TGF-β hydrogels for cartilage tissue engineering

4.1 Introduction

The human and economic cost of osteoarthritis (OA) is rising. Due to ageing populations and increasing life expectancies, OA is expected to become the world’s fourth leading cause of disability by 2020, up from the sixth leading cause in 1990 (22). The pain and loss of mobility that accompany this debilitating condition mean that a quarter of affected individuals are unable to perform major activities of everyday life (21). In the UK alone in the year 2000, 80,000 hip and knee replacement operations were performed at a cost of over £400 million, and the total cost to the UK of the condition is estimated at around 1% of Gross National Product (21). Disturbingly, many of those suffering from disabling joint pain do not consult their GP – over half in one study – apparently considering OA an inevitable, and intractable, part of ageing (21).

Where conservative treatment fails, surgical options for OA divide broadly into reparative and reconstructive techniques (209). Reparative techniques seek to promote neocartilage formation, for example by bleeding of the joint surface (microfracture), which recruits progenitor cells to the lesion. However, the resulting tissue is frequently found to be fibrotic and mechanically inadequate. Reconstructive techniques such as mosaicplasty, in which an osteochondral plug is transplanted from an unaffected area, are technically challenging and superior clinical outcomes are as yet unproven (209). Likewise, the utility and cost-effectiveness of autologous chondrocyte implantation is still under question (209) (23). Furthermore, these approaches are not amenable to the repair of large scale defects such as occur in OA, where the entire joint may be affected. Thus far, whole or partial joint replacement is the only option in such circumstances, carrying major surgical risks as well as drawbacks related to fixation of the implants to bone, and aseptic loosening – which may require corrective surgery. There exists therefore an urgent unmet need for scalable regenerative solutions to OA, which can reliably induce the
formation of functionally acceptable neocartilage, and resolve difficulties of integration at the donor site (209). The development of ‘smart’ biomaterials is one area showing huge promise in terms of optimising the regenerative response of host or transplanted cells, and doing so in a cost-effective manner (210).

Commercially available biomaterials-based products for cartilage repair based on alginate, collagen, fibrin, HA, chitosan, poly(glycolic acid)/poly(lactic acid) (PGA/PLA), and self-assembling peptides are currently in clinical or preclinical trials (211)(1). For example, crosslinkable esterified and thiolated derivatives of HA are marketed as HYAFF® (Fidia advanced biopolymers) and Glycosil™ (Glycosan Biosystems, Inc.), respectively. HA is a major constituent of cartilage and synovial fluid, and these products appear to assist cartilage repair when transplanted with primary chondrocytes, MSCs, or ESCs (2)(212)(213).

Yet to break through into clinical use include biomaterials strategies that attempt to mimic the zonal organisation of articular cartilage, for instance by zoning of articular chondrocytes into discrete deep, middle and superficial layers of a PEG hydrogel (214). Also in development are scaffolds incorporating growth factors or other bioactive compounds to enhance cellular response. Proteins or compounds of interest have included insulin-like growth factor 1, basic FGF (FGF-2), parathyroid-related protein, BMPs and Dexamethasone; but especially, the three transforming growth factor betas (TGF-β1-3; Box 1) (215)(216)(217)(211)(218). Within cartilage TE, TGF-β is of particular interest due to its central role in chondrogenesis, and for its tendency to increase differentiation (at the expense of proliferation) and to stimulate ECM deposition (219). In one recent study, large-scale cartilage regeneration was achieved within a cell-free bioreactor system by incorporating TGF-β3 into PCL-hydroxyapatite composite scaffolds moulded to resemble the entire articular surface of rabbit humeral condyles. The addition of TGF-β3 resulted in enhanced recruitment of progenitor cells and the establishment within four months of a uniform covering of articular cartilage (57).
### 4.1.1 Box 1: TGF-β superfamily and signalling

TGF-β1-3 are members of the TGF-β superfamily of signalling proteins, which encompass the BMPs, activins and inhibins, and Nodal and Nodal-related proteins among others (220). They are produced by and act on almost all cell types, with highly pleiotropic effects. They help to regulate growth, proliferation, differentiation, apoptosis, matrix production, and epithelial-to-mesenchymal transitions, and play central roles in embryogenesis, wound healing, immunological functions and carcinogenesis. Loss of one isoform may be partially compensated for by the remaining variants; nonetheless, null mutations in any one TGF-β gene are lethal either perinatally or neonatally (57).

The canonical signalling pathway for TGF-β1-3 involves ligand binding to a type II cell surface receptor (TβRII), which recruits and phosphorylates a type I receptor (TβRI, alternatively Alk5). This sets in motion a chain of events culminating in the nuclear localisation of phosphoryated Smad protein and transcription factor complexes, as shown schematically in Figure 4.1. Within the nucleus these complexes bind to Smad-binding elements or other DNA recognition sequences determined by the co-transcription factors, eliciting phenotypic effects that depend on the cell type, timing, and physiological context (57).

![Figure 4.1: TGF-β signal transduction](image)

**Figure 4.1: TGF-β signal transduction.** TGF-β binding to TβRII leads to phosphorylation of TβRI (Alk5). Phosphorylated TβRI in turn phosphorylates Smad proteins (Smad2 and Smad3). These are thought to associate in a trimer with Smad4, also known as the co-Smad. Further association with transcription factors (TF) and transport through nuclear pores result in transcriptional regulation (221).
As already discussed in some detail, sophisticated growth factor delivery strategies have been shown to improve the functional outcome of biomaterials interventions, in cartilage TE and elsewhere. Recall from Chapter 2 that fine control over concentrations and temporal release profiles of VEGF and PDGF were essential to optimise the quality of neovessel formation \textit{in vivo} (page 36) (222)(122)(123). Sustained release strategies are likely to important for \textit{in vivo} TE because a single loading of growth factor must lead to sustained action over days to weeks, in contrast to \textit{in vitro} studies in which medium can be changed as often as desired. Controlled growth factor delivery strategies for cartilage TE have included loaded microparticles of poly(lactic acid-co-glycolic acid), alginate or gelatin, polion nanoparticles, heparin to retard release of TGF-β3, and the use of self-assembling peptides carrying a binding sequence for TGF-β1 which enhanced growth factor retention; this delivered better histological outcomes at 12 weeks than peptides without the binding motif (215)(223)(216)(217)(224).

Although undoubtedly promising, there is a marked contrast between the relative simplicity of these approaches and the exquisite control exercised over TGF-β bioavailability \textit{in vivo}. TGF-β is secreted by cells as part of an inactive latent complex, allowing for several layers of regulation (Figure 4.2). The ~100 KDa small latent complex (SLC) derives from a single gene product, the TGF-β propeptide, which assembles homodimerically within the cell via several disulphide bridges. During intracellular processing, the propeptide is cleaved into N-terminal and C-terminal fragments, the LAP and mature TGF-β respectively (219)(225)(226). The LAP has high affinity for TGF-β and thus the two remain in close non-covalent association as the SLC. Binding occurs at a hydrophobic interface which masks the receptor binding region of TGF-β; this association stabilises TGF-β conformation and confers latency until activation occurs via one of several cell-dependent processes (see below) (227)(225). Four mannose-6-phosphate residues are added to LAP as part of extensive glycosylation before export, and are required for association with TGF-β. An RGD motif within LAP is indispensible for binding to integrin receptors (91).
**Figure 4.2: TGF-β latent complex and ECM association.** Latency associated peptide (LAP) and transforming growth factor beta (TGF-β) comprise the small latent complex (SLC). The large latent complex (LLC) additionally includes latent TGF-β binding proteins (LTBPs). Based on a figure from (91).

The SLC may be exported directly or, more usually, further bound to LTBPs via a single disulphide bond on LAP Cysteine-33 (Cys-33), constituting the large latent complex (LLC). LTBPs share a high level of homology with fibrillins 1 and 2 – ECM proteins capable of forming fibrils on their own, or else contributing to large elastic fibres together with tropoelastin (219)(228). LTBPs can thus be targeted to fibrillin fibrils or elastic fibres, as well as structurally unrelated ECM components such as fibronectin. In this way, ECM provides a ready supply of sequestered latent TGF-β, which only becomes active under appropriate physiological (or pathological) conditions.

TGF-β activation is a complex, tightly regulated process as would be expected for a cytokine with such multitudinous and potent effects. It occurs via at least two distinct mechanisms *in vitro* – protease and integrin-dependent – although there is considerable overlap between these two modes. Proteolytic cleavage of LAP can be effected by numerous serine proteases and MMPs, unmasking receptor binding sites and releasing TGF-β with or without some residual portion of LAP (220). Integrin binding to the RGD motif on LAP, combined with tractional forces exerted by the cell, can enable receptor binding either by liberating TGF-β completely or by inducing a conformational change in the SLC that exposes TGF-β. A relatively stiff (> 10 KPa) substrate is necessary in this case to resist cell-generated tension: with overly compliant substrates, the force exerted by the cell is accommodated by deformation of the matrix rather than of LAP, and the activating conformational change does not occur (Figure 2.3) (229)(92).

Interactions are thought to exist between protease- and integrin-dependent mechanisms, including situations whereby integrin binding promotes cleavage by bringing the SLC and membrane-bound MMPs...
into apposition (91). Interactions with the matrix glycoprotein thrombospondin-1 can induce conformational changes in LAP which permit receptor binding. Also known to cause activation are physical stimuli such as heat, reactive oxygen species, and extremes of pH (220)(230). A summary of these factors and the molecular players involved are given in Table 4.1.

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Table 4.1: Physical and molecular factors involved in latent TGF-β activation. Also shown (in grey) are integrins known to bind, but not necessarily activate, latent TGF-β. Compiled from information in (91), (220), and (230).
Despite the existence of these elaborate biological means of controlling TGF-β availability, no attempt to present TGF-β in its latent form within a biomaterial has yet been described. At least three generic and procedural benefits to using latent TGF-β within TE scaffolds can be postulated:

- Enhanced stability over free TGF-β, which is a ‘sticky’, hydrophobic protein tending to adhere to tissue culture plastic in vitro and raising concern over possible protein fouling/aggregation.

- Higher loading and improved exposure kinetics due to the inactivity of latent TGF-β and the potential for active release of TGF-β by cells according to local demand. The provision of TGF-β in an inactive form implies that the high doses required to sustain chondrogenesis for days to weeks may be loaded into TE scaffolds without exposing cells to excessive quantities of active growth factor, with TGF-β bioavailability then being controlled via gradual activation by cells.

- The option to conjugate the TGF-β indirectly (e.g. via LAP) rather than directly to the TGF-β, which is likely to diminish its biological activity.

This work describes novel poly(ethylene glycol)-hyaluronic acid-latent TGF-β (PEGDA-HA-LTGF-β) hydrogel scaffolds for cartilage TE. Recombinant human commercial SLC (henceforth referred to as latent TGF-β, LTGF-β) was shown to have a lower tendency to adsorb to TCP than free TGF-β, and its relative inactivity was demonstrated using Smad3 phosphorylation as an indicator of TGF-β signalling in human foreskin fibroblasts (HFF-1). To assess the potential of this strategy for cartilage TE, LTGF-β was PEGylated via lysine residues using Traut’s reagent and PEGDA, and the resulting PEGylated TGF-β (PEG-LTGF-β) was biologically active. Bovine chondrocytes were encapsulated within PEGDA-HA-LTGF-β hydrogels. It was found that a single high loading of PEG-LTGF-β can stimulate metabolic activity of bovine chondrocytes over almost five weeks in culture and lead to higher protein accumulation than gels without TGF-β over the same timescale, although total LDH activity at 34 days was similar to that of TGF-β-free hydrogels. Several ways are suggested in which this strategy could be developed in order to maximise the functional benefits for cartilage TE.
4.2 Methods

4.2.1 Materials

Recombinant human SLC (TGF-β1 plus LAP-β1; hereafter referred to as latent TGF-β or LTGF-β), recombinant human free TGF-β1, and TGF-β1 Quantikine ELISA kits were purchased from R&D Systems Europe Inc. (Abingdon, Oxfordshire, U.K.). Free TGF-β was reconstituted in 4 mM HCl + 0.1% w/v BSA, and aliquoted and frozen immediately at -20°C as directed by the supplier. LTGF-β was stored as supplied (250 μg.ml⁻¹ in glycerol) at -20°C. PEGDA (Mw 4000) was synthesised by Helena Chia (University of California, Los Angeles; work-shadowed by Elsie Place), who also kindly provided the photoinitiator Irgacure 2959. Glycosil™ (thiolated HA) was obtained from Glycosan Biosystems, Inc. (Salt Lake City, UT, U.S.A.). Bovine serum albumin (BSA), lysozyme from chicken egg white, and cytochrome C from bovine heart were all from Sigma-Aldrich (Dorset, U.K.).

For sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, 30% acrylamide/bis 29:1, polyvinylidine difluoride membranes (PVDF), filter papers, and secondary antibody (Goat anti-Rabbit IgG-horseradish peroxidase conjugate) were all from Bio-Rad Laboratories, Inc. (Hemel Hempsted, Hertfordshire). N,N,N',N'-Tetramethylethylenediamine (TEMED), ammonium persulphate (APS), Tween, double strength laemmli sample buffer, RIPA buffer and phosphatase inhibitors were purchased from Sigma-Aldrich. The 5x loading buffer and PageRuler™ prestained protein ladder were from Fermentas (York, U.K.); and Imperial protein stain, and Pierce Enhanced Chemiluminescence (ECL) Western Blotting Substrate, and Restore Western Blot Stripping Buffer were from Thermo Scientific Inc. (Cramlington, Northumberland, U.K.). Methanol was from VWR (Lutterworth, Leicestershire, U.K.), and sodium dodecyl sulphate (SDS), glycine and Tris Base were from Fisher Scientific (Loughborough, Leicestershire, U.K.).

Human foreskin fibroblasts (HFF-1) were obtained from ATCC-LGC (Teddington, Middlesex, U.K.) and used at passages <8. All cell culture reagents, antibiotics, PBS, and alamarBlue® were from GIBCO (Invitrogen Ltd., Paisley, Renfrewshire, U.K.), except the Insulin-Transferrin-Selenium Acid + Linoleic Acid premix (ITS+ premix) which was from VWR. Pronase from streptomyces griseus and EDTA-free Complete Mini protease inhibitors were from Roche Ltd. (Basel, Switzerland), and the protease inhibitors
Marimastat and CP471474 were from Tocris Bioscience (Bristol, U.K.). The TGF-β receptor inhibitor SB-431542 was purchased from Ascent Scientific Ltd. (Bristol, U.K.), and RGD peptide was from Bachem AG (Bubendorf, Switzerland). Collagenase type I from clostridium histolyticum, aprotinin, leupeptin, manganese chloride, blebbistatin, neutral buffered formalin (NBF), and 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) were from Sigma-Aldrich. Rabbit polyclonal antibodies against Smad3 (ab28379), Collagen I (ab34710), Collagen II (ab34712), and Collagen VI (ab6588), and rabbit monoclonal antibody against phosphorylated Smad3 (phosphoSmad3; ab52903) were purchased from Abcam (Cambridge, U.K.). ProLong Gold antifade coverslipping medium and fluorescent secondary antibody were from Invitrogen (Goat anti-Rabbit IgG-AlexaFluor 488). Promega (Southampton, U.K.) Cytotox 96 kits were used to assay LDH activity, and Bradford reagent was from Bio-Rad. All other chemicals and reagents were purchased from Sigma-Aldrich unless stated.

4.2.2 Stability of free and latent TGF-β

All plasticware (6-well plates and microcentrifuge tubes) was coated overnight in sterile 1% w/v BSA in PBS and air-dried before use. Solutions of free or latent TGF-β (2 ng.ml⁻¹ in DMEM) were incubated at 37°C in 6-well plates (5 ml per well). 200 µl aliquots were snap frozen in liquid nitrogen at 1, 2, 4, 8, 24, and 48 hour timepoints. TGF-β concentrations were determined by ELISA according to the manufacturer’s instructions. This assay specifically recognises uncomplexed (free) TGF-β1, and gives very limited signal when LAP is bound. LTGF-β samples were therefore activated with 20% v/v 1M HCl for 10 minutes to dissociate the TGF-β dimer from the LAP, and neutralised using the same volume of 1.2 M NaOH + 0.5 M HEPES, as directed by the manufacturer. The ELISA was initiated immediately after neutralisation and within 30 minutes of defrosting the samples. Concentrations were normalised using a log-log plot of concentration versus absorbance for TGF-β1 standards of 0-2000 pg.ml⁻¹.

4.2.3 Activity of free, latent and PEGylated latent TGF-β

To investigate fibroblast signalling response to TGF-β, HFF-1 were grown to 50-80% confluence on 8-well glass chamber slides for immunocytochemistry, or 100 cm² petri dishes for Western blotting. Cells were serum-starved in DMEM with 0.2% w/v BSA for 24 hours ahead of all signalling experiments. Negative
(no TGF-β) and positive (free TGF-β at either 1 or 10 ng.ml⁻¹) controls were included with every experiment, and the results were only considered if these wells showed clear and appropriate responses, i.e. weak to no staining in negative controls and strong nuclear staining in positive controls. Where necessary, ELISA was performed on activated samples ahead of the experiments to precisely determine the TGF-β content of the LTGF-β formulations. Where applicable this will be described as ‘free equivalent’ concentrations. HFF-1 were exposed for 1 hour to free TGF-β, LTGF-β, or PEGylated LTGF-β (PEG-LTGF-β) in DMEM at the specified free equivalent concentrations. LTGF-β samples requiring acid activation were prepared at 10x final concentration, acidified and neutralised as described in the previous section, brought to the final volume and used immediately. Plasmin digestion of LTGF-β was carried out at 37°C using 0.01 U.ml⁻¹ plasmin for either 90 or 180 minutes, then arrested by the addition of 7.5 µg.ml⁻¹ aprotinin. Where indicated, manganese chloride was included at 500 µM.

4.2.4 Inhibition of LTGF-β activity

Cells (HFF-1, chondrocytes) were serum starved for 24 hours prior to experimentation. To assess signalling responses to varying doses of ligand, they were exposed to a ten-fold dilution series of free or latent TGF-β in DMEM, from 0.001-10 ng.ml⁻¹ (or free equivalent), for 1 hour. To investigate inhibition of LTGF-β activity, free or latent TGF-β were used at concentrations of 10 ng.ml⁻¹ (or free equivalent) in DMEM for 1 hour, with or without the following treatments in combinations specified in the relevant sections. For inhibition of protease action, either Roche protease inhibitors (1 tablet per 10 ml) plus Marimastat and CP471474 (both at 1 µM) (PIMC), or a custom mixture of aprotinin (7.5 µg.ml⁻¹), leupeptin hemisulphate (25 µM), Marimastat and CP471474 (ALMC) were used. The TGF-β receptor I (Alk5) inhibiting molecule SB-431542 was used at 10 µM, soluble RGD peptide at 5 mM, and the nonmuscle myosin IIa inhibitor blebbistatin was used at 100 µM. Cells were pretreated with inhibitors for 1 hour before, as well as during, stimulation.

4.2.5 Immunocytochemistry

For immunostaining, slides were rinsed in Tris buffered saline (TBS), fixed in 10% v/v NBF for 15 minutes at room temperature, permeabilised in 0.1% v/v Triton-X for 10 minutes, and blocked in 3% w/v
BSA for 1 hour (all solutions in TBS). Slides were rinsed three times in TBS between each step. Samples were stained overnight at 4°C in primary antibody solution (anti-phosphoSmad3, 1:100 in TBS), rinsed three times in TBS, then stained with Goat anti-rabbit IgG-AlexaFluor 488 at 1:2000 in TBS. Nuclei were counterstained with DAPI (1.8 µM in water) and air dried before coverslips were applied.

4.2.6 Confocal microscopy

Imaging of antibody-stained sections was performed on a Leica SP2 upright confocal microscope running Leica LAS software, under a 20x objective lens. Offset and gain were set manually at the beginning of each session. AlexaFluor 488 was excited at 488 nm with an Argon laser and emission was collected between 500-550 nm; DAPI was excited under UV (351 nm) and collected at 430-490 nm. The two fluorophores were excited separately and images were acquired between frames to avoid bleed-through of DAPI to the green channel. Stacked images were collected at 1.5 µm intervals with four times line averaging and reconstructed as maximum intensity projections in Volocity Demo software (Perkin Elmer Inc., Waltham, MA, U.S.A.).

4.2.7 Western blotting

Cells were rinsed twice in cold PBS, and the petri dishes were placed on ice. Cells were lysed in 100 µl RIPA buffer containing protease and phosphatase inhibitors, for 10 minutes with occasional agitation with a cell scraper. The lysate was collected and spun at 10,000 g in a cooled (4°C) centrifuge for 10 minutes. The supernatant was transferred to a fresh tube and snap frozen. Later, protein was quantified using Bradford reagent (800 µl water + 1 µl sample + 200 µl Bradford reagent), with absorbance measured at 595 nm against BSA standards in RIPA buffer. Samples were prepared by heating for 10 minutes at 95°C in laemmli sample buffer, and typically 20-30 µg total protein was loaded per well.

To make the acrylamide gels for SDS-PAGE, 10% v/v acrylamide solutions were prepared in running gel buffer (375 mM Tris Base, 3.5 mM SDS, pH 8.8) and crosslinked with 8.7 mM TEMED and 8.8 mM APS between 1.5 mm Bio-Rad casting plates. Stacking gel solution (5% v/v acrylamide, 125 mM Tris Base, 3.5 mM SDS, pH 6.8) was crosslinked with 13.4 mM TEMED and 4.4 mM APS. 20 µl sample, or 7 µl protein
ladder, was loaded per lane, and separation typically proceeded for 1-1.5 hours at 100 V and 400 mA powered by a Bio-Rad PowerPac Basic™ (running buffer 25 mM Tris Base, 192 mM glycine, 3.5 mM SDS). PVDF membranes were activated in methanol (20 minutes) and 1 hour was allowed for the transfer at 100V and 400 mA (transfer buffer 20% v/v methanol, 25 mM Tris Base, 192 mM glycine). The membrane was blocked in 5% w/v BSA in TBS with 0.1% v/v Tween (TBS-T) and probed overnight at 4°C on a shaker with anti-phosphoSmad3 antibody at 1:1000 in TBS-T. The membrane was rinsed four times for 10 minutes in TBS-T before the addition of secondary antibody (Goat anti-Rabbit IgG-HRP, 1:3000 in TBS-T) for 1 hour at room temperature. The membrane was rinsed again (4 x 10 minutes in TBS-T), and chemiluminescence was performed according to the manufacturer’s instructions. Membranes were wrapped in clingfilm and imaged in a Biospectrum® 500 Imaging System (Ultra-Violet Products Ltd., Cambridge, UK) with VisionWorksLS software (version 6.8), for ten minutes in the dark for chemiluminescence, and 0.1 seconds under transillumination to capture the protein ladder. Membranes were stripped in stripping buffer for 10 minutes, rinsed in TBS-T (4 x 10 minutes), blocked as before, and imaged to confirm removal of antibody. The membranes were then reprobed with anti-Smad3 antibody (1:1000 in TBS-T) and imaged as already described. Chemiluminescence and transilluminated images were superimposed in Adobe Photoshop CS5 to ascertain the positions of the protein marker bands, and adjusted using the histogram function (‘levels’) to better visualise the chemiluminescent signal.

4.2.8 PEGylation of model proteins and LTGF-β

LTGF-β and the model proteins BSA, lysozyme, and cytochrome C were modified by reaction with Traut’s reagent and PEGDA. The model proteins were prepared as 4 µM solutions in sodium phosphate buffer (SPB; 3 mM sodium phosphate monobasic, 89 mM sodium phosphate dibasic, pH 8.0) and the LTGF-β was used as received. Traut’s reagent was prepared as an 8 mM working solution and PEGDA dissolved to 14 mM, both in SPB. The protein of interest, PEGDA and Traut’s reagent were combined in BSA-coated microcentrifuge tubes in a 4:11:3 ratio and left for 1 hour at room temperature. In negative control samples, Traut’s reagent was replaced by SPB. For the model proteins, EDTA was included in the SPB at either 5 mM (= SPEB) or 100 mM (= SPEB+) where indicated. The final concentrations of reactants, for LTGF-β, were Traut’s reagent 1.3 mM, PEGDA 8.5 mM (acrylate endgroups 17 mM), and LTGF-β 55.5 µg.ml⁻¹. SDS-PAGE was performed as described in the previous section, and gels were stained with
Imperial protein stain for 1 hour and destained overnight in 20% v/v methanol, 10% v/v acetic acid.

Aliquots of the reacted LTGF-β (PEG-LTGF-β) were either measured by ELISA (manufacturer’s instructions) or frozen immediately at -80°C.

### 4.2.9 TGF-β release from PEGDA hydrogels

LTGF-β, PEG-LTGF-β, or LTGF-β negative control reaction samples (no Traut’s reagent) were added to PEGDA solutions (10% w/v plus 4.5 mM Irgacure 2959 photoinitiator in PBS) to approximately 10 ng.ml⁻¹ or free equivalent. The polymer solutions were pipetted between glass slides separated by a 1 mm glass spacer and polymerised 3 cm from an 8-watt, 365 nm UV source (UVP, Cambridge, UK) for 15 minutes. 6 mm diameter gels were cut using a biopsy punch, rinsed in PBS to remove unpolymerised material, and placed under 270 µl 1% w/v BSA solution in PBS, in BSA-coated microcentrifuge tubes. The gels were left at room temperature, and the BSA solution was exchanged at 0.25, 1, 2, 4, 8, and 24 hours. At 24 hours, the solutions were acidified with 60 µl 1 M HCl to dissociate the free TGF-β dimer from the LAP, and final samples were collected at 48 hours. Concentrations of released TGF-β were determined by ELISA according to the manufacturer’s instructions. Total (starting) TGF-β content was determined using the unpolymerised PEGDA solutions diluted 20x in BSA/PBS. Weight measurements were recorded throughout the experiment in order to correct for gel swelling when calculating the amounts of released TGF-β.

### 4.2.10 Cell culture and isolation of bovine chondrocytes

HFF-1 were grown in serum-containing medium (DMEM + 10% FBS + 1 mM glutamine) and passaged at 60-80% confluence according to standard cell culture procedures. Bovine articular chondrocytes were harvested from calf metacarpophalangeal joints within 8 hours of sacrifice. Briefly, distal fore- and hindlimbs were disinfected thoroughly, skinned, and dissected in a class II biological safety cabinet. Cartilage from the articulating surfaces was removed, diced finely, and digested for 1 hour in pronase (2 mg.ml⁻¹ in high glucose DMEM with GlutaMax + 10 mM HEPES + 1% v/v antibiotic/antimycotic) at 37°C. The pronase solution was then exchanged for collagenase (0.4 mg.ml⁻¹ in high glucose DMEM with GlutaMax + 10 mM HEPES + 1% v/v antibiotic/antimycotic + 5% v/v FBS) and the tissue was maintained
at 37°C overnight under constant (rolling) agitation. Cells were counted the following morning, plated at 5 x 10^4 cells.cm⁻² and cultured in high glucose DMEM with GlutaMax plus 10% FBS, 284 µM ascorbic acid, and 1% v/v antibiotic/antimycotic. Cells were either used the following morning (passage 0), or frozen and grown for 2 passages at a later date, as indicated.

4.2.11 Chondrocyte encapsulation in LTGF-β hydrogels

Bovine chondrocytes were encapsulated in LTGF-β hydrogels for cartilage tissue engineering. Two experimental repeats were performed, with minor procedural differences as indicated. Glycosil™ (HA) was reconstituted in sterile water to 50% final volume, and PEGDA dissolved separately in PBS to 50% final volume; the PEGDA solution was then filter sterilised and separated into two vials, one to include PEG-LTG-F-β. In the first experimental repeat, a previously reacted aliquot of PEG-LTG-β was defrosted, whereas for the second repeat, the LTGF-β was reacted on the day as described in the section ‘PEGylation of model proteins and LTGF-β’. Bovine chondrocytes were harvested, counted, and suspended in the PEGDA solutions, the PEG-LTG-β was added to one of the two vials, and the HA and PEGDA/chondrocytes (with or without PEG-LTG-β) were mixed in equal volumes. The polymers crosslink by reaction between acrylates (PEGDA) and thiols (HA); the free acrylates on PEG-LTG-β also react with the thiolated HA, tethering the protein in place. For the first experimental repeat, the final concentrations were 1% w/v HA, 2.5% w/v PEGDA, 50 ng.ml⁻¹ PEG-LTG-β (free equivalent), and 5 x 10^6 chondrocytes.ml⁻¹, and for the second experimental repeat final concentrations of 0.75% w/ HA, 2% w/v PEGDA, 500 ng.ml⁻¹ PEG-LTG-β (free equivalent), and 4.5 x 10^7 chondrocytes.ml⁻¹ were attained. This mixture was immediately dispensed into transwell inserts of pore size 5.0 µm: 150 µl per 12 mm diameter insert (first experimental repeat) or 50 µl per 6.5 mm diameter insert (second experimental repeat). Two sets of gels were prepared without TGF-β and one set with PEG-LTG-β. After approximately 5 minutes, 2 ml serum-free chondrogenic medium (high glucose, phenol red-free DMEM + 1 mM sodium pyruvate + 1% v/v ITS+ premix + 0.1 µM dexamethasone + 284 µM ascorbic acid + 1% v/v antibiotic/antimycotic; (218)) was added to each well. Two groups (no TGF-β and PEG-LTG-β) received serum-free medium as described, whereas for the third group (free TGF-β) the medium was supplemented with 10 ng.ml⁻¹ free TGF-β, which was added freshly at each medium change. The medium
was changed the following day and every two days thereafter. Gels were cultured for either 28 days (first experimental repeat) or 34 days (second experimental repeat).

### 4.2.12 Biochemical analysis of chondrocyte activity

To investigate cell death over the culture period, used medium was collected and frozen at -80°C until the end of the experiment. The amount of LDH released into the medium by dead cells was measured using a Cytotox 96 assay according to the manufacturer’s protocol. Total metabolic activity was assayed at approximately 5 day intervals using alamarBlue® reagent according to the manufacturer’s instructions. Briefly, 10% v/v alamarBlue® in serum-free medium was left in wells for 4 hours (2 ml) or 2 hours (1 ml) for the first and second experimental repeats respectively, and the absorbance of the samples was read in duplicate at 570 nm with background subtraction at 600 nm. The absorbance of the experimental samples ($AbsE$) was normalised to starting values ($AbsC$, alamarBlue® solution not exposed to cells) according to the formula:

$$\frac{AbsE - AbsC}{AbsC}$$

At day 34 of the second experimental repeat, LIVE/DEAD® staining was performed, and gels were assessed for total protein content and LDH activity to estimate cell number. For LIVE/DEAD® staining, an approximately 1 mm thick section was cut from the periphery of one gel of each group, and incubated at room temperature in LIVE/DEAD® reagent ($1 \mu M$ calcein + $1 \mu M$ ethidium homodimer-1 in PBS) for 15 minutes before imaging. Confocal microscopy was as described above (page 86) except that 488 nm excitation with 500-550 nm emission were used for calcein, 561 nm excitation with 580-700 nm emission were used for ethidium, the two fluorophores were captured simultaneously, and 5 μm steps were used between stacked images. For LDH and total protein, gels were weighed and then homogenised in 0.5 ml 150 mM NaCl for 15 minutes at 30 Hz in a QIAGEN TissueLyser II. Aliquots for LDH were lysed in 0.1% v/v Triton-X in 150 mM NaCl, diluted 10x in water, and assayed according to the manufacturer’s instructions against standards of known cell numbers with cell free hydrogel samples run as blanks. Aliquots for the Bradford protein assay were digested in papain (7 mM cysteine, 5 mM EDTA, 62.5 mU.ml⁻¹ papain, 150 mM NaCl) for 8 hours. 20 μl sample was then mixed with 780 μl water, 200 μl Bradford reagent was added and the tubes were vortexed well. Within 30 minutes the absorbance was read at 595
nm alongside BSA standards and a cell-free hydrogel sample (all in identical solutions including the papain buffer).

4.2.13 Histology

Gels were analysed by histology at timepoints specified in the results section. The gels were fixed for 1 hour in 10% v/v NBF and sliced into approximately 1 mm sections. For immunostaining of cartilage matrix constituents, fixed sections were permeabilised for 20 minutes with 0.1% v/v Triton-X, and blocked for 2 hours in 3% w/v BSA. All solutions and rinsing steps were in TBS, all steps were carried out on an orbital shaker, and the gels were rinsed for 20 minutes between each step. Gels were probed overnight at 4°C on a rocker in primary antibody solutions (Rb anti-Collagen II at 1:100, Rb anti-Collagen I at 1:100, Rb anti-Collagen VI at 1:200). Gels were rinsed for 1 hour then incubated for 1 hour at room temperature in secondary antibody solution at 1:2000 (Gt anti-Rb IgG-AlexaFluor 488 and Gt anti-Ms IgG-AlexaFluor 568). After rinsing for 30 minutes, gels were stained in DAPI (1.8 µM in water) for 10 minutes and stored in TBS. Gels were imaged by confocal microscopy as described on page 86. Negative control (no primary antibody) gels ensured that the fluorescent signal was due to specific staining. Single fields or stacks at 5 µm intervals were collected, as appropriate for each set of samples. Additionally, DAPI fluorescence was later imaged at 4x objective magnification on an Olympus IX51 inverted microscope equipped with a mercury lamp and DAPI filter.

Eosin staining was performed on 1 mm thick fixed gel sections by soaking for a few minutes in eosin stain, then destaining overnight in several changes of TBS. Gels were embedded in paraffin wax, 10 µm sections were cut, and slides were stained with picrosirius red and haematoxylin and eosin (performed by Ms. Lorraine Lawrence, Imperial College Histology service). Samples were imaged on an Olympus IX51 inverted microscope using 4x, 10x and 20x objective lenses. Images were cropped and resized using Adobe Photoshop CS5.
4.2.14 Statistics and data manipulation

Analysis of Variance with Tukey’s post-tests, and Independent samples T-tests were used except for all data except the free and latent TGF-β stability experiment and the timed release of TGF-β from PEG hydrogels, for which (non-parametric) Kruskal-Wallis and Mann-Whitney U tests were used. All statistical tests were run on SPSS for Windows version 12.0, and \( p < 0.05 \) was considered significant. All data manipulation including chart generation was performed using Microsoft Excel.
4.3 Results and Discussion 1: Adsorption and biological response to LTGF-β

4.3.1 LTGF-β adsorbs to surfaces less readily than free TGF-β

To explore the possible benefits of using LTGF-β within TE scaffolds, first of all the concentrations of free and latent TGF-β were measured over 48 hours in vitro, as an indicator of the tendencies of free and latent TGF-β to adsorb to TCP surfaces. Measured concentrations of free and latent TGF-β in BSA-coated well plates differed significantly ($p = 0.05$) after 8 hours incubation and beyond. At this point LTGF-β concentrations were unchanged, but free concentrations had diminished by 25% (Figure 4.3). By 48 hours the amount of free protein had fallen to 61% of the starting concentration, whereas no decrease was seen for LTGF-β (105% of starting values).

![Figure 4.3: In vitro concentrations of free and latent TGF-β over time.](image)

Concentrations of free and latent TGF-β over 48 hours in DMEM on BSA-coated 6-well plates, as determined by ELISA. n = 3, mean and range of values shown. * Free versus latent TGF-β concentrations $p = 0.05$.

TGF-β is a hydrophobic, ‘sticky’ protein tending to adhere strongly to surfaces (manufacturer’s instructions), raising concerns over protein handling during scaffold fabrication or loading, and possible fouling or denaturing during slow release over days to weeks. However, LAP binding to TGF-β induces extensive structural rearrangements in both proteins, burying several hydrophobic residues to create a hydrophobic interface stabilised by van der Waals interactions and π-bonding (227)(225)(231). These results suggest that the use of LTGF-β should allow greater confidence over protein integrity during processing steps and over long periods in culture or in the body.
4.3.2 LTGF-β is relatively inactive; implications for burst release from scaffolds

In dosing TE scaffolds with growth factors, the desire for long-term action must be balanced against the need to limit the exposure of cells to high concentrations. Recall from Chapter 2 that burst release of high doses of VEGF is associated with aberrant vasculature (page 36) (232). Dysregulated TGF-β activity is implicated in OA and other disorders of joint tissues (233). In an extreme example, intra-articular injection of high doses of TGF-β (three deliveries of 200 ng into murine knee joints) induced OA-like changes including zone-specific changes to proteoglycan levels, osteophyte formation, and macroscopic cartilage lesions (234). Elsewhere, elevated TGF-β signalling stemming from increased TGF-β bioavailability was found to cause a transition from growth to apoptosis of bone marrow stromal cells in biglycan and decorin deficient mice, with consequently decreased bone mass (235). Therefore, it was of great interest to illustrate the diminished potency of LTGF-β compared to free TGF-β.

Cellular response to TGF-β was assessed by immunostaining for the phosphorylated form of Smad3 (phosphoSmad3) in human foreskin fibroblasts (HFF-1). A dose response was performed at TGF-β concentrations (free or free equivalent) between 0.001 ng.ml⁻¹ and 10 ng.ml⁻¹. Free equivalent concentrations were determined in advance by ELISA on activated LTGF-β samples. In cells treated with free TGF-β, a robust signalling response was seen for concentrations of 0.01 ng.ml⁻¹ and upwards, as indicated by strong nuclear staining for phosphoSmad3 (Figure 4.4). Meanwhile cells exposed to LTGF-β remained unresponsive at concentrations below 10 ng.ml⁻¹, at which point nuclear fluorescence was observed. Therefore, LTGF-β prior to activation appears to have around 0.1% of the activity of the full protein, which is consistent with the dose-dependent inhibition of TGF-β bioactivity (growth inhibition and luciferase assays) by LAP (231)(225). Our approach therefore is of interest as a means by which to load high levels of TGF-β into scaffolds without subjecting cells to excessively high doses of active growth factor. Ideally for TE, the encapsulated cells would then activate the TGF-β over a period of days to weeks, thereby achieving sustained release without an initial burst of excessive activity. Therefore, having confirmed the relative inactivity of the commercial LTGF-β, the next task was to explore its activation by cells.
Figure 4.4: Signalling response to different concentrations of free and latent TGF-β by human foreskin fibroblasts (HFF-1). Cells were incubated in the presence of free or latent TGF-β at the indicated concentrations for 1 hour prior to fixing. Confocal microscopy; scale = 200 µm.
4.3.3  **Response to LTGF-β was not diminished by inhibition of protease activity, integrin binding, or contractility**

As already discussed, latent TGF-β can be activated by cells in a variety of protease- and/or integrin-dependent pathways. Cell-mediated release of growth factors from fibrin and PEG hydrogels has been shown to result in high quality blood vessel formation (232)(122)(236). Therefore the positive signalling response of HFF-1 to LTGF-β at free equivalent concentrations of 10 ng.ml⁻¹ was investigated further, to ascertain whether this reflected cell-mediated LTGF-β activation. HFF-1 were stimulated with 10 ng.ml⁻¹ TGF-β (free or free equivalent) in the presence of a selection of inhibitors. Protease-dependent pathways were targeted by a custom cocktail of MMP and serine protease inhibitors (ALMC; **Table 4.2**), and integrin-dependent pathways by a competitive RGD peptide and blebbistatin, a potent inhibitor of nonmuscle myosin II (hence cellular contractility). A small molecule inhibitor of TβRI, SB-431542 (TGFβRI), was run as an additional control, and inhibitor treatments were performed on negative (no TGF-β) and positive (free TGF-β) control wells in case they perturbed Smad signalling independently of any effects on LTGF-β activation.

<table>
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<tr>
<th>Protease</th>
<th>Inhibitor</th>
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**Table 4.2: TGF-β-activating proteases and pharmacological inhibitors used.** List of proteases compiled from (91) and (220); specificity of proteases based on manufacturer’s information.
As before, stimulation of HFF-1 with 10 ng.ml\(^{-1}\) free or latent TGF-β for 1 hour led to the nuclear accumulation of phosphoSmad3 (Figure 4.5). The addition of soluble RGD, or the protease inhibitor cocktail ALMC, or both treatments in combination with blebbistatin, did not diminish the intensity of staining. In contrast, the TGFBR1 completely abolished the phosphorylation response, both with free and latent TGF-β.

A similar experiment was performed on bovine chondrocytes to confirm and extend these observations. Freshly isolated chondrocytes showed strong nuclear phosphoSmad3 staining even after serum starvation, therefore cells were used at passage 4. In addition to the custom ALMC cocktail, a commercial protease inhibitor cocktail was used. EDTA-free tablets were used because EDTA interfered with cell attachment to the slide. EDTA is included in such cocktails to inhibit MMPs (by chelating their namesake zinc ions), therefore the commercial formulation was additionally supplemented with the MMP inhibitors Marimastat and CP471474 (Table 4.2) to form a novel cocktail, PIMC. Some non-specific staining was seen on immunostained samples although a clear positive signalling response to TGF-β was seen for both latent and free TGF-β at 10 ng.ml\(^{-1}\) (Figure 4.6). The Western blot membranes showed bands close to the expected molecular weight of 48 KDa (manufacturer’s information; apparent M\(_{w}\) 55 KDa). On both slides and Western blot membranes no difference in Smad3 phosphorylation was seen between samples treated with LTGF-β with or without ALMC. Although some loss in fluorescence/band intensity was seen with the PIMC cocktail, this occurred with both free and latent TGF-β-treated wells, indicating an inhibitory effect via a mechanism other than TGF-β activation. Again, the TGFBR1 abolished Smad3 phosphorylation, as seen from the Western blot membrane.
Figure 4.5: Signalling response by human foreskin fibroblasts (HFF-1) to 10 ng.ml⁻¹ free and latent TGF-β in the presence of inhibitors of TGF-β activation. - = no inhibitor; RGD = soluble RGD peptide; ALMC = aprotinin, leupeptin, Marimastat, CP471474; TGFBRI = TGF-β receptor inhibitor. Scale = 200 µm.
Figure 4.6: Signalling response by passage 4 bovine articular chondrocytes to 10 ng.ml⁻¹ free and latent TGF-β in the presence of protease inhibitors. (Top) Immunostaining for phosphoSmad3. Scale = 200 µm. (Bottom) Western blot membrane showing phosphoSmad3 and total Smad3 after stripping. Abbreviations as per Figure 4.5, plus PIMC = Roche protease inhibitor cocktail, Marimastat, CP471474.
The failure of any of the inhibitor treatments to diminish the phosphoSmad3 signal intensity suggests that the Smad3 phosphorylation response seen at high concentrations of LTGF-β may not be due to cell-mediated activation of the complex. The HFF-1 dose response (Figure 4.4) indicated that the response threshold for the LTGF-β was somewhere between 1 and 10 ng.ml⁻¹, therefore the assay should have been relatively sensitive to inhibition. Alternative possibilities are that the formulation may include a small amount of impurity (i.e. free TGF-β), or that the TGF-β retains some limited ability to bind its receptor even when associated with the LAP, which only becomes apparent at high doses. ELISA on unactivated LTGF-β samples typically gave concentration readings of around 1% of the activated total, although this is difficult to interpret without information regarding the recognition sites of the antibodies in this kit.

Whether or not the signalling response to 10 ng.ml⁻¹ LTGF-β represents bona fide activation by HFF-1 and chondrocytes, however, is of limited concern for the present application. Indeed, for cartilage TE it is preferable for the TGF-β to become active only gradually, over far longer timescales than the 1 hour exposure used here. Furthermore, stretch-dependent activation of LTGF-β delivered in solution would not be expected: tractional force across the SLC is required for this to occur, which may conceivably be enabled by tethering of the LTGF-β to a surface or hydrogel matrix (229)(91). The work described below aimed to discover whether LTGF-β tethered within a three-dimensional hydrogel scaffold would stimulate chondrogenic activity over four to five week culture periods. The general approach was to prepare PEG-HA hydrogels with tethered LTGF-β, for the encapsulation of bovine chondrocytes. The first step was to develop the means by which to link the LTGF-β to the hydrogel matrix.
4.4 Results and discussion 2: Conjugation of PEGDA to LTGF-β

4.4.1 PEGylation of proteins was achieved using Traut’s reagent

To immobilise LTGF-β within hydrogels it was necessary to introduce a reactive group(s) to LAP that would enable subsequent reaction with the hydrogel polymer. The hydrogels used for TE (vide infra) consisted of thiolated HA crosslinked with PEGDA, which occurs via a Michael-type addition reaction. Therefore the chosen strategy was to modify the LTGF-β with PEGDA (PEGylation), leaving a free acrylate to react with the HA using the same chemistry as the crosslinking reaction.

Traut’s reagent was chosen as a linker between LTGF-β and PEGDA. According to this scheme, the Traut’s reagent targets primary amines belonging to amino acid sidegroups. Both lysine (K) and arginine (R) residues carry amine sidegroups, but only lysines are expected to react due to charge delocalisation on arginine sidechains. This introduces a free thiol to the LTGF-β (SH-LTGF-β), which combines with the PEGDA acrylate endgroups via Michael addition (shown schematically in Figure 4.7). A high acrylate:thiol ratio (13:1) was used to favour Michael addition between SH-LTGF-β and PEGDA over disulphide bond formation between SH-LTGF-β.

![Chemical reaction diagram](image)

**Figure 4.7: Conjugation chemistry.** (Top) Traut’s reagent reacts with primary amines (lysines) to introduce thiols to proteins. (Bottom) Co-reacting a protein (LTGF-β shown) with Traut’s reagent and PEGDA causes the SH-LTGF-β to react with PEGDA, producing a PEGylated protein.
The model proteins BSA, cytochrome C, and lysozyme were chosen to develop the reaction conditions. Although Mw of the LAP dimer is similar to that of BSA (LAP ~80 KDa before glycosylation, versus BSA ~70 KDa), the two smaller proteins are closer in terms of the number of lysines (LAP 20, BSA 60, cytochrome C 7, lysozyme 14; Table 4.3). It should be noted that the TGF-β dimer itself contains in total 16 lysines, which are also potential unintended targets of the PEGylation reaction.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Approx. Mw (kDa)</th>
<th>Lysines</th>
<th>NCBI Reference Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin</td>
<td>Bos taurus</td>
<td>70</td>
<td>60</td>
<td>NP_851335.1</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>Bos taurus</td>
<td>12</td>
<td>7</td>
<td>NP_001002891</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Gallus gallus</td>
<td>15</td>
<td>14</td>
<td>NP_990612.1</td>
</tr>
<tr>
<td>Latency associated peptide β1 (dimer)</td>
<td>Homo sapiens</td>
<td>40 x 2</td>
<td>10 x 2</td>
<td>NP_000651.3</td>
</tr>
<tr>
<td>TGF-β1 (dimer)</td>
<td></td>
<td>12.5 x 2</td>
<td>8 x 2</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3: Protein information for the model proteins bovine serum albumin, cytochrome C, lysozyme, LAP-β1, and TGF-β1. Approximate molecular weight (Mw) and number of lysines shown. Note that LAP-β1 and TGF-β1 come from the same gene product and are dimeric. (237)(238)(239)(240)

The influence of EDTA on the PEGylation reaction was investigated because the manufacturer’s instructions for Traut’s reagent stipulate the inclusion of EDTA at 2-5 mM to chelate metal ions. This prevents the oxidation of sulphydryl groups and hence disulphide bond formation between SH-LTGF-β. However, since deprotonation also favours Michael addition it was possible that EDTA might inhibit coupling to PEGDA. Therefore, three conditions were tested: EDTA was either left out of the pH 8.0 sodium phosphate (SPB) reaction buffer, or included at either 5 mM (SPEB) or 100 mM (SPEB+). The high EDTA (SPEB+) buffer was tested because BSA preparations often contain high levels of iron from haemoglobin, which may overwhelm small amounts of EDTA; 0.1 M EDTA is sometimes recommended when working with BSA to maintain sulphydryl stability (241).

SDS-PAGE results for all three model proteins confirmed the efficacy of the PEGylation procedure (Figure 4.8). As expected according to the reasoning above, the most extensive modification occurred in SPB
buffer (no EDTA), but paradoxically, less modification happened with 5 mM EDTA than 100 mM. The reason for this is not clear. Michael addition is sensitive to pH, but buffer pH was corrected after the addition of EDTA, so this is unlikely to have been an issue.

Figure 4.8: PEGylation of model proteins. (Top) attempt 1; (Bottom) attempt 2. Unmodified protein (-) and reactions carried out in sodium phosphate buffer (SPB), SPB with 5 mM EDTA (SPEB), or SPB with 100 mM EDTA (SPEB+).

SPB buffer was chosen for the LTGF-β PEGylation reactions due to the higher efficiency of the reaction. Under these conditions BSA showed an increase in Mw from ~70 KDa to a range of Mws of around 100-170+ KDa. This indicated the addition of at least 7-25 PEGDA molecules, which is a sizeable proportion of the total number of lysines (60). Reacted lysozyme showed discrete bands corresponding to around 20, 25, and 30-35 KDa, from an initial known Mw of 15 KDa (apparent Mw ~13 KDa). These bands presumably represent mono-, di-, and tri-PEGylated protein (from 14 total lysines). At the first attempt, Cytochrome C was modified to the extent of 35-170 KDa from 12 KDa, which was cause for concern as it far exceeded the ~30 KDa Mw increase expected if all 7 lysines were modified. A repeat under the same
conditions yielded Mw increases to around 20-45 KDa, although the staining was weak and the bands difficult to read in this gel. This emphasised the importance of inspecting PEGylated protein batches before use. The different susceptibilities of the model proteins to PEGylation also demonstrates the importance of factors other than the total number of lysines – for example, the steric accessibility of lysines, and the chemical nature of adjoining sidegroups.

4.4.2 Latency associated peptide was PEGylated using Traut’s reagent

As indicated in Table 4.3 and detailed in Figure 4.9, each LAP monomer contains 10 lysine residues, some of which may be available to react with Traut’s reagent leading to LTGF-β PEGylation. LAP additionally carries several endogenous cysteine residues, most of which are involved in disulphide linkages. However, the cysteine at position 33 (within a STCKT sequence near to the N terminus of the peptide; Figure 4.9) normally mediates linkage to LTBPs, raising the intriguing possibility that within the isolated SLC it may be available to react directly with PEGDA. Furthermore, the pKa of cysteine SH groups (~8.1-8.9) is reduced when the cysteine is flanked by positively charged amino acids (242). The +1 charge on the adjacent lysine is thus expected to increase the likelihood of reaction with PEGDA by favouring deprotonation. Coupling specifically and solely to this cysteine residue would preserve the full functionality of the TGF-β, and presumably ensure presentation of the TGF-β in a physiological orientation. To address this possibility, therefore, LTGF-β samples were reacted with PEGDA alone (no Traut’s reagent), in addition to those reacted with Traut’s reagent as described above.

**Figure 4.9**: Primary sequence of TGF-β1 propeptide. Grey letters correspond to signal peptide (residues 1-29), blue letters to LAP (residues 30-278; 249 amino acids), and black to TGF-β1 (residues 279-390; 112 amino acids). The eighteen lysine residues (K) are highlighted in red, and cysteines (C) in gold. (240)
Unmodified LTGF-β run on SDS-PAGE under reducing and denaturing conditions produces bands at 12.5 KDa and 40-45 KDa, corresponding to the TGF-β and LAP monomers, respectively (Figure 4.10). Where LTGF-β was reacted with PEGDA but no Traut’s reagent, no Mw increase was apparent in the LAP. If anything, the LAP band appeared to have travelled further than in control samples (see batch a) suggesting that the presence of PEG may affect the movement of protein through the gel, compromising interpretation.

**Figure 4.10: PEGylation of LTGF-β.** Positions of LAP and free TGF-β are indicated. LTGF-β was run either alone or after reacting for 1 hour with PEGDA +/- Traut’s reagent (Traut’s). Batches (a-c) are indicated.

LAP reacted with PEGDA and Traut’s reagent showed clear Mw increases, indicative of PEGylation, in accordance with the results for the model proteins. Three batches were run (a-c), and once more the extent of modification was seen to vary from batch to batch, in the order b>a>c. Moderate changes were seen in batches a and c, of up to around 30 KDa and 15 KDa respectively. Final Mws of >>100 KDa were seen in batch b, which may indicate protein aggregation, for example by disulphide bridging between SH-LTGF-β, or uncontrolled reaction of Traut’s reagent, perhaps with arginine residues. Free TGF-β was too faint to resolve following the reactions with PEGDA and Traut’s reagent (*vide infra*).
To confirm LTGF-β PEGylation and investigate further the possibility of specific coupling via LAP Cys-33, LTGF-β was incorporated into UV photopolymerised PEGDA hydrogels and diffusional release of TGF-β measured by ELISA; PEGylated protein should become covalently tethered inside the gel via free acrylates. Acid was added at 24 hours to dissociate the TGF-β dimer from the LAP, and further release was measured at 48 hours.

Over the first 8 hours of swelling, 61% of unreacted LTGF-β diffused out of the gel (Figure 4.11). By 24 hours 70% had been released, and acid treatment liberated a further 31%, so that by 48 hours all of the TGF-β was accounted for. LTGF-β reacted with PEGDA but no Traut’s reagent (to target Cys-33) showed significantly (p < 0.05), but only marginally (54% released versus 70%), greater retention over 24 hours; and by 48 hours 98% of the TGF-β had been released. It therefore seems possible that the PEGDA had reacted with the Cys-33 in a minority of LAP molecules, but if so, this was not enough to prevent most of the protein being lost from the gel within a day. The possibility of hijacking endogenous cysteine residues to tether the LTGF-β in a defined, biologically relevant orientation was therefore not well-supported by the SDS-PAGE and ELISA timed release results. This apparent failure may be due to incorrect pairing of LAP Cys-33 with a free cysteine in TGF-β, which has been suggested to occur when the SLC is exported without LTBPs (225).

![Figure 4.11](image.png)

**Figure 4.11:** Release of latent TGF-β from UV photopolymerised PEGDA hydrogels over time. Acid was added at 24 hours (arrow) and concentrations measured by ELISA. LTGF-β was either unmodified, or reacted with PEGDA for 1 hour +/- Traut’s reagent (Traut’s). LTGF-β from the batch c reaction was used. Effect of reaction conditions p < 0.05 from 0.25 - 24 hours. * LTGF-β versus LTGF-β + PEGDA p < 0.05.
In contrast, only 12% of the LTGF-β reacted with Traut’s reagent diffused out of the gel within 24 hours, confirming that PEGylation had occurred, and that the PEG in turn was linking the protein directly to the polymer scaffold. Furthermore, upon acidification another 58% of the total TGF-β diffused out of the gel, to a total release of 70%. This was very encouraging, because if the TGF-β itself had become extensively PEGylated, it too would have remained tethered inside the gel following acid treatment. In light of these promising findings, it was decided that the Cys-33 coupling approach would be discarded, and the cell work described in the following sections would proceed using LTGF-β PEGylated using Traut’s reagent (PEG-LTGF-β).
4.4.3 Latent TGF-β is either lost or modified during PEGylation reaction

Consistently, there was a loss in staining intensity of TGF-β and LAP after the conjugation reactions, both with and without Traut’s reagent (Figure 4.10). In addition, post-reaction ELISA readings gave diminished signals (Table 4.4), suggesting that TGF-β concentrations had fallen to 0.09-0.20 of original levels. This suggests that during the PEGylation reaction, TGF-β protein is either lost – for example by adsorption to the sides of the tube – and/or modified – i.e. PEGylated. The aim of the cell work described in the next section was to clarify how the PEGylation reaction affected TGF-β biofunctionality, using PEG-LTGF-β batches a and b. The relative measured concentrations of 0.15 and 0.20 after PEGylation with Traut’s reagent were used to derive correction factors (i.e. 6.7 and 5 times original concentrations) for loss of protein or of protein activity when Traut’s reagent was used. Conversely, for LTGF-β reacted with PEGDA alone (no Traut’s reagent), the concentration readings were essentially unchanged in two of three batches. The TGF-β dimer was assumed not to have been modified in these reactions, therefore the relative concentration readings for this condition (Table 4.4) were used to correct for possible protein adsorption to the sides of the tube during the reaction. The concentrations so calculated will be denoted HIGH and LOW respectively (see Table 4.4), and it will be made explicit in the text and diagrams which batches and concentration estimations were used.

<table>
<thead>
<tr>
<th>Batch</th>
<th>Relative amount PEGDA + Traut’s</th>
<th>Correction factor HIGH</th>
<th>Relative amount PEGDA</th>
<th>Correction factor LOW</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>0.15</td>
<td>6.7</td>
<td>0.50</td>
<td>2</td>
</tr>
<tr>
<td>b</td>
<td>0.20</td>
<td>5</td>
<td>1.03</td>
<td>1</td>
</tr>
<tr>
<td>c</td>
<td>0.09</td>
<td>N/A</td>
<td>0.90</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 4.4: Relative apparent concentrations of LTGF-β after 1 hour reacting with PEGDA +/- Traut’s reagent. LTGF-β was reacted either with PEGDA or with PEGDA and Traut’s reagent for 1 hour, and the concentrations before and afterwards were read by ELISA (‘Relative amount = concentration after reaction/concentration before reaction’). The inverse of these ratios defines a correction factor for each condition, used to calculate concentrations (denoted HIGH and LOW as indicated) for use in selected experiments in the next section. N/A – batch c was not used in subsequent cell work.
4.5 Results and discussion 3: Biological activity of PEG-LTGF-β

4.5.1 PEG-LTGF-β induces Smad3 phosphorylation in fibroblasts, but with some loss of activity

Immunostaining and Western blot for phosphoSmad3 and total Smad3 were used to investigate whether PEG-LTGF-β retains its biological activity. In the first experiment, free equivalent concentrations of TGF-β were uncorrected for loss or modification of TGF-β during the reactions. HFF-1 were stimulated with stock LTGF-β and PEG-LTGF-β (batch b) at 1 and 10 ng.ml⁻¹, and additionally two modes of LTGF-β activation were attempted: acid treatment was performed on samples at 0.1 and 1 ng.ml⁻¹; and manganese chloride (MnCl₂, 500 µM) included with LTGF-β at 1 ng.ml⁻¹. Low pH causes dissociation of TGF-β from LAP, as already discussed, while MnCl₂ promotes integrin binding and can be used to enhance integrin-mediated activation of TGF-β (229).

As before, 10 ng.ml⁻¹ (free equivalent) unmodified LTGF-β led to Smad3 activation while 1 ng.ml⁻¹ failed to elicit a strong response (Figure 4.12). However in this instance there did seem to be a very low level of Smad3 phosphorylation at 1 ng.ml⁻¹. When the same doses of PEGylated protein were used, nuclear phosphoSmad3 staining appeared less intense than control, indicating that the protein was still functional but that some loss of activity had occurred. Acid activated, unmodified LTGF-β at 0.1 and 1 ng.ml⁻¹ elicited a robust phosphorylation response. Convincing nuclear staining was also seen for PEG-LTGF-β under the same conditions, confirming that some functionality was unquestionably retained. This was especially encouraging given that SDS-PAGE results for the batch used (b; Figure 4.10) had revealed very large Mw increases, suggesting that this had been more extensively PEGylated than the other batches. MnCl₂ did not appear to increase the response to 1 ng.ml⁻¹ latent TGF-β in either the control or PEGylated group.
Figure 4.12: Signalling response to LTGF-β and PEG-LTGF-β by HFF-1. Cells were exposed to LTGF-β and PEG-LTGF-β (batch b) at the indicated concentrations for 1 hour. + ACID = acid activation of LTGF-β; + Mn<sup>++</sup> = 500 µM MnCl<sub>2</sub>. Free equivalent concentrations of LTGF-β are shown, without correction for loss or modification of protein during PEGylation reactions. Confocal microscopy; scale = 200 µm.
The next two experiments attempted to assess whether the concentration corrections described earlier (Table 4.4) could provide a good approximation of the activity of unmodified protein. Initially, a high concentration (10 ng.ml\(^{-1}\)) of LTGF-β was chosen and both HIGH and LOW concentration corrections were used as per Table 4.4. Immunostaining for phosphoSmad3 of HFF-1 treated with PEG-LTGF-β batch b for 1 hour appeared brighter than control (unmodified LTGF-β) when the HIGH concentration correction was used, and less intense than control when the LOW concentration was used (Figure 4.13).

Western blotting was performed for HFF-1 treated with batches a and b at HIGH and LOW concentrations (Figure 4.13). No staining for phosphoSmad3 was seen in the no TGF-β control lanes. Free and latent TGF-β controls produced clear bands at 55 KDa, although the staining was more intense for free TGF-β. The loading control of total Smad3 was similar for all samples. As expected, the intensity of staining for the experimental samples was weaker when the LOW correction was used than for the HIGH correction. For batch a, staining for the LOW correction was roughly similar to that for the unmodified LTGF-β while the HIGH correction led to more intense staining than control. Conversely for batch b, staining for the HIGH concentration correction was similar to control while the signal from the LOW concentration correction was weaker. These results suggest that the higher Mw seen on SDS-PAGE for batch b may be associated with greater loss of activity than for batch a (Figure 4.10). The lack of agreement over whether the HIGH or LOW correction would give a similar response to control suggested that attempts to use ELISA readings to approximate biological activity were likely to prove inaccurate.
Figure 4.13: Signalling response to PEG-LTGF-β by human foreskin fibroblasts (HFF-1) and comparison to unmodified LTGF-β at different concentration corrections. HIGH and LOW refer to concentration corrections detailed in Table 4.4 and explained in the accompanying text. Estimated concentrations (free or free equivalent) were 10 ng.ml⁻¹ for all conditions. (Top) Immunostaining for phosphoSmad3, PEG-LTGF-β batch b used. Confocal microscopy; scale = 200 µm. (Bottom) Western blot membranes – two repeats from the same experiment. (a) and (b) refer to PEG-LTGF-β batches. For consistency, the lane order of the top right membrane was altered in Photoshop. Membranes are also shown after being stripped to remove phosphoSmad3 antibody.
Finally, it was of interest to determine whether PEG-LTGF-β retained its ability to be activated by cells, for example by proteases. The attempt to stimulate integrin-mediated TGF-β activation by adding MnCl₂ to the medium had not led to increased signalling in either unmodified or PEGylated LTGF-β samples (Figure 4.12), therefore a protease-dependent mechanism of activation was explored (plasmin digest). Following Pedrozo et al, LTGF-β was digested for 90 and 180 minutes in 0.01 U.ml⁻¹ plasmin and stopped using 7.5 µg.ml⁻¹ aprotinin (243). Smad3 phosphorylation in response to control and PEGylated LTGF-β was then assessed as before. Acid treatment was also performed as a control. Batch b was used with the HIGH concentration correction to ensure a strong response, and for all conditions 1 ng.ml⁻¹ (free or free equivalent) TGF-β was used.

Immunostaining and Western blotting confirmed that unmodified LTGF-β had limited to no activity (Figure 4.14). Exposure to acid activated samples, however, led to a strong Smad3 phosphorylation response, which in the immunostained samples was seen to be localised to the nucleus. The level of staining was similar for unmodified and PEGylated LTGF-β. Plasmin-digested samples, however, failed to activate Smad3 signalling in both control and PEGylated samples, indicating that the conditions used by Pedrozo et al may not have been directly applicable to this experimental system. The experimental samples in their case were prepared from decellularised ECM and therefore contained naturally deposited LTGF-β, which would have largely been present as part of the LLC.

In conclusion, PEG-LTGF-β retained some level of biological activity (ability to stimulate Smad3 phosphorylation), although some loss of activity was seen following PEGylation with Traut’s reagent. This is not surprising given that the reaction randomly targets lysine residues, of which TGF-β contains 16, some of which are likely to become involved in the reaction. The activity of batch b – which showed a more extensive modification than batch a (Figure 4.10) – appeared to be lower than batch a. Correcting PEG-LTGF-β concentrations based on ELISA readings, to compensate for loss of activity post-reaction, did not match the activity of unmodified protein in a consistent way, therefore the activity of PEG-LTGF-β cannot be quantified with confidence from these results. Attempts to stimulate integrin- and plasmin-dependent activation of stock and experimental samples were unsuccessful. Having confirmed that PEG-LTGF-β is biologically active, the potential of LTGF-β for cartilage TE was assessed by culturing bovine chondrocytes in PEG-HA hydrogels, as described in the following section.
Figure 4.14: Signalling response to PEG-L TGF-β by human foreskin fibroblasts (HFF-1) [continued]
[Figure 4.14 continued] with acid activation and plasmin digestion. Estimated concentrations (free or free equivalent) were \(1 \text{ ng.mL}^{-1}\) for all conditions, and the PEG-LTG\(\beta\) (batch b) was corrected using the HIGH concentration estimation (Table 4.4). + acid = acid activation; + P90 = plasmin digestion for 90 minutes; + P180 = plasmin digestion for 180 minutes. (Top) Immunostaining for phosphoSmad3. Confocal microscopy; scale = 200 \(\mu\text{m}\). (Bottom) Western blot membranes – two repeats from the same experiment. Membranes are also shown after being stripped to remove phosphoSmad3 antibody.
4.6 Results and discussion 4: Cartilage tissue engineering

4.6.1 No increase in proliferation or matrix synthesis was seen with 50 ng.ml\(^{-1}\) PEG-LTGF-β

To evaluate the potential of LTGF-β for cartilage TE, bovine chondrocytes at passage 2 were encapsulated into PEG-HA hydrogels and cultured for 4 weeks. Gels were prepared with or without 50 ng.ml\(^{-1}\) PEG-LTGF-β. As previously mentioned, the remaining free acrylate groups on the conjugated PEG molecules can react with thiols in Glycosil\(^\text{TM}\), tethering the PEG-LTGF-β to the hydrogel. 'PEG-LTGF-β' and 'no TGF-β' groups were fed with serum-free chondrogenic medium minus TGF-β. The 'free TGF-β' positive control group (gels prepared without LTGF-β) received medium supplemented with 10 ng.ml\(^{-1}\) free TGF-β, which was added freshly at each medium change (every two days) for the duration of the study.

The alamarBlue\(^\text{®}\) assay for metabolic activity was performed at several timepoints to assess chondrocyte proliferation. Cells provided with free TGF-β doubled their activity over the first seven days in culture (Figure 4.15). Cell number declined thereafter until, at day 28, activity was similar to starting levels. Metabolic activity of 'no TGF-β' chondrocytes peaked at 3 days with approximately a 45% increase on day 1 values. Activity then declined steadily to day 21 (~30% of starting values), but showed some minor recovery at day 28. Activity of the 'PEG-LTGF-β' group closely resembled that of the 'no TGF-β' control group. From days 10 to 21, activity was significantly \((p < 0.05)\) higher for PEG-LTGF-β than for no TGF-β, but these differences were small (15-23% of no TGF-β starting values) and of questionable biological relevance.

Used medium was collected and assayed for LDH released by dead cells. Cell death in the 'free TGF-β' group peaked around days 1, 12, and 24-26 (Figure 4.15). The initial peak was shared by the other two experimental groups and presumably reflects stresses associated with the encapsulation procedure and adaptation to new environmental conditions. The onset of the latter two peaks, at days 6 and 21, correspond to peaks in metabolic activity (Figure 4.15); increasing LDH release thus closely coincides with declining metabolic activity. The cause of this decline may be related to metabolic demands, possibly indicating that nutrient diffusion into the gels could not keep pace with consumption, and/or that the volume of medium used was insufficient to sustain these cells for two days. Aside from a difference at day 1, the pattern and extent of cell death was virtually identical in the 'no TGF-β' and 'PEG-LTGF-β' groups,
showing a gradual decline from peak activity at days 1 and 3. LDH release was very low over the final two and a half weeks, presumably reflecting low total cell numbers at this point.

**Figure 4.15:** Viability of bovine chondrocytes cultured for 28 days in PEG-HA gels with and without TGF-β. (Top) Total metabolic activity as indicated by alamarBlue® reduction, reflecting cell proliferation. Effect of gel $p < 0.001$; effect of ‘free TGF-β’ versus ‘no TGF-β’ and ‘PEG-LTGF-β’ $p < 0.001$; effect of ‘PEG-LTGF-β’ versus ‘no TGF-β’ $p = 0.72$. * ‘no TGF-β’ versus ‘PEG-LTGF-β’ $p < 0.05$; ** ‘no TGF-β’ versus ‘PEG-LTGF-β’ $p < 0.001$. (Bottom) LDH release from cultured hydrogels, reflecting cell lysis. Effect of ‘free TGF-β’ versus ‘no TGF-β’ and ‘PEG-LTGF-β’ $p < 0.001$. Experimental groups here and for all subsequent figures are as described on page 116. Mean ± SD, n = 5.

Various histological analyses of hydrogel samples were carried out at days 7, 14, 21 and 28, to evaluate ECM deposition by chondrocytes. The general collagen stain picrosirius red, and an anti-collagen II antibody were used on wax embedded and hydrated gel samples, respectively (Figure 4.16). Marked differences were seen in collagen staining between, on the one hand, ‘free TGF-β’ gels, and on the other, ‘no TGF-β’ and ‘PEG-LTGF-β’ gels. After seven days of treatment with free TGF-β, chondrocytes were
large, round, and laden with collagen as indicated by vivid staining with picrosirius red. At day 13 abundant pericellular collagen II was seen, and by day 28 this had formed a relatively uniform, continuous matrix. Conversely, ‘no TGF-β’ and ‘PEG-LTGF-β’ chondrocytes at day 7 were small and weakly stained; collagen II at days 13 and 28 was still confined to the immediate cell environment, with virtually no interstitial staining.

**Figure 4.16:** Collagen staining of PEG-HA hydrogels with encapsulated chondrocytes cultured for up to 28 days. (Top) Picrosirius red staining of wax-embedded sections fixed on day 7 of culture. Brightfield microscopy; 10x magnification; scale = 200 µm. (Bottom) Confocal images of hydrogels immunostained with collagen II (green) plus DAPI (blue). 20x magnification; scale = 100 µm.

Eosin Y staining was performed on fixed, hydrated gels to highlight general features. In accordance with the previous observations, ‘free TGF-β’ gels contained larger chondrocytes and stained more intensely than ‘no TGF-β’ and ‘PEG-LTGF-β’ gels (Figure 4.17). Nuclei stained with DAPI were brightest and most numerous in ‘free TGF-β’ gels at all timepoints, indicating higher cell numbers (Figure 4.18).
Additionally, DAPI binds to RNA, albeit with a weaker fluorescent signal than with DNA (quantum yield DAPI/RNA ~20% that of DAPI/dsDNA, manufacturer’s information), so the brighter fluorescence of ‘free TGF-β’ nuclei may in part reflect higher transcription in these cells. ‘Free TGF-β’ gels after 28 days of culture were semi-opaque, firm in consistency and could easily be handled, whereas ‘no TGF-β’ and ‘PEG-LTGF-β’ gels were transparent and friable (Figure 4.19). Taken together, the alamarBlue®, LDH, and histology results indicate that the inclusion of PEG-LTGF-β under these experimental conditions did not stimulate cellular proliferation, or enhance cartilage matrix synthesis by the encapsulated chondrocytes.

Figure 4.17: Eosin staining of PEG-HA hydrogels with encapsulated chondrocytes cultured for 21 and 28 days. Brightfield microscopy; 4x and 10x magnification as indicated; scale = 500 µm for 4x magnification, and 200 µm for 10x magnification.
Figure 4.18: DAPI staining of PEG-HA hydrogels with encapsulated chondrocytes cultured for up to 28 days. Fluorescent microscopy; 4x magnification; scale = 500 µm.

Figure 4.19: Macroscopic appearance of PEG-HA hydrogels after 28 days of culture. Some residual colour is left over from the alamarBlue® assay. Membrane diameter = 12 mm.
4.6.2 High loading of PEG-LTGF-ß stimulated metabolic activity over 5 weeks

Given that no improvements in viability nor matrix synthesis were detected with PEG-LTGF-ß compared to no TGF-ß under the previous experimental conditions, the following modifications were made to the experimental setup.

- A higher density of cells was seeded into the gels \( (4.5 \times 10^7 \text{ cells.ml}^{-1} \text{ versus } 5 \times 10^6 \text{ cells.ml}^{-1}) \). It was reasoned that higher concentrations of secreted proteases might promote TGF-ß release from LTGF-ß.

- The scaffolds were loaded with a higher dose of PEG-LTGF-ß than before \( (500 \text{ ng.ml}^{-1} \text{ versus } 50 \text{ ng.ml}^{-1}, \text{ free equivalent}) \). In accordance with the dose responses shown in Figure 4.4, the biological activity of the LTGF-ß would have been only a fraction of that of the free growth factor, and as discussed in the section 4.5 Results and discussion 3: Biological activity of PEG-LTGF-ß, some loss of activity would have occurred post-conjugation.

- A longer experimental timeframe was chosen \( (34 \text{ days versus } 28 \text{ days}) \). In the first experiment, PEG-HA hydrogels began to liquefy between weeks 3 and 4. During this period the hydrogels began to lose their integrity and became unable to support their own weight (Figure 4.19). It seemed possible that gel degradation should increase the availability of tethered PEG-LTGF-ß, therefore the experiment was extended to include more time for gel breakdown to occur.

- Finally, as a general procedural improvement bovine chondrocytes were freshly harvested and used 24 hours post-plating (i.e. 48 hours post-isolation, passage 0, versus passage 2, 48 hours post-thawing). It is well-known that chondrocytes quickly lose their rounded morphology and undergo phenotypic changes on two-dimensional TCP (244). Using the cells freshly and before spreading had occurred ought to promote more physiological behaviour.

Similarly to the earlier findings, chondrocytes receiving free TGF-ß proliferated over the first nine days of culture \( (\text{alamarBlue}® \text{ assay, Figure 4.20}) \). Metabolic activity climbed to 65% over day 1 levels by day 9, then stabilised at 43-55% over day 1 levels from day 14 onwards. Activity in the ‘no TGF-ß’ group was stable up to day 15, but dropped during the third week to a new plateau of around 53-65% of day 1 values. Comparing this profile to the precipitous decline seen in the first experimental repeat \( (\text{alamarBlue}®, \text{ Figure 4.15}) \) suggests that the use of freshly isolated chondrocytes led to a more vigorous culture. Beneficial paracrine signalling may also have been enhanced by the higher cell seeding density.
Figure 4.20: Viability of bovine chondrocytes in PEG-HA gels with and without TGF-β. (Top) Total metabolic activity (alamarBlue® assay) of bovine chondrocytes encapsulated in PEG-HA hydrogels over a 5 week culture period. Effect of gel $p < 0.001$ (all intergroup comparisons $p < 0.001$). * ‘PEG-LTGF-β’ versus ‘no TGF-β’ $p < 0.01$; ** ‘PEG-LTGF-β’ versus ‘no TGF-β’ $p < 0.001$. (Bottom) Total LDH activity of chondrocytes cultured in hydrogels for 34 days, reflecting number of live cells. * $p < 0.001$ versus ‘no TGF-β’, $p < 0.01$ versus ‘PEG-LTGF-β’. Mean ± SD, $n = 8$ (alamarBlue®), $n = 7$ (LDH).

Interestingly, chondrocytes in ‘PEG-LTGF-β’ gels exhibited an apparently bimodal response in metabolic activity, which peaked at days 5-9, and again at day 27, and overall was significantly higher than in ‘no TGF-β’ gels ($p < 0.001$). One interpretation of this data is that chondrocytes responded to and possibly activated the LTGF-β present in their local environment during the first week and a half of culture. Once exhausted, a period of relative quiescence ensued until the gel began to break down. At this point more distant stores of LTGF-β were released, becoming newly available to the cells. Despite these effects, total LDH at day 34 – reflecting total cell number – was no different between ‘PEG-LTGF-β’ and ‘no TGF-β’ gels (Figure 4.20). On average over twice as many chondrocytes were ultimately present in the ‘free TGF-β’ group than in the other gels. This suggests that the putative period of TGF-β starvation during the late second and third weeks was decisive in determining final cell number.
4.6.3 High loading of PEG-LTGF-β led to altered matrix synthesis

Matrix synthesis by encapsulated chondrocytes was next evaluated by histology. Collagen II was intra- and pericellularly distributed in all three groups at day seven, with very limited staining between cells (Figure 4.21). More protein was seen between cells at day 21, although staining was still more intense in the immediate vicinity of the cells. The pericellular staining was brightest in the ‘PEG-LTGF-β’ gels and least intense in the ‘no TGF-β’ group, suggesting highest and lowest production of collagen II, respectively. Interstitial collagen II was more homogeneously distributed in ‘free TGF-β’ gels than in the other groups, and by day 34 in this group, positively stained chondrocytes populated lacunae within a dense, uniform matrix of collagen II. The collagen II laid down by ‘no TGF-β’ chondrocytes was less abundant, largely confined to interstitial regions, and in general was no longer associated with the cells. The cells were surrounded by concentric zones empty of collagen II, suggesting that the cartilage matrix may have been displaced at later timepoints by more recently deposited material. The distribution of collagen II in ‘PEG-LTGF-β’ gels was very similar to that of ‘no TGF-β’ gels, but apparently present in greater quantities (i.e. brighter fluorescence); this was later confirmed by conventional histology (vide infra).

![Figure 4.21: Collagen II staining of PEG-HA hydrogels with encapsulated chondrocytes](continued)
Collagen I was probed as an indicator of the hyaline versus fibrocartilagenous nature of the neocartilage matrix. Collagen I distribution at day 7 in all groups was similar to that of collagen II, being mostly present within chondrocytes and/or their immediate surroundings (Figure 4.22). ‘Free TGF-β’ and ‘PEG-LTG-F-β’ gels, however, stained more brightly than ‘no TGF-β’ gels, which may reflect generally higher levels of protein synthesis, in association with their higher metabolic activity over the first ten days of the experiment (Figure 4.20). By day 34, however, very little collagen I was detected, and this was mostly confined to interstitial regions – suggesting that production of collagen I had ceased, and possibly that some of the protein deposited earlier had been remodelled. Overall, the presence of collagen I in the matrix suggests that the neomatrix may more closely resemble fibrocartilage than hyaline cartilage, although more in-depth biochemical analysis is needed to fully characterise the generated tissue.

Figure 4.22: Collagen I staining of PEG-HA hydrogels with encapsulated chondrocytes cultured for up to 34 days. Collagen I = green, DAPI = blue; confocal microscopy; 20x magnification; scale = 100 µm.

[Figure 4.21 continued] cultured for up to 34 days. Collagen II = green, DAPI = blue; confocal microscopy; 20x magnification; scale = 100 µm.
Collagen VI was also investigated as in normal cartilage it has a restricted and well-defined distribution, within the chondron. It is considered an essential component of the pericellular niche, and is quickly (2-3 days) deposited by chondrocytes upon their introduction to a new environment – including TE scaffolds (245). Indeed by day 7, chondrocytes in all three groups had elaborated a collagen VI-rich pericellular matrix (Figure 4.23). By day 21 the protein had spread interstitially, which in human articular cartilage is generally considered a hallmark of osteoarthritic changes to the middle and deep zones (246)(247). On the other hand, the distribution of collagen VI in bovine articular cartilage undergoes age-related changes and in young animals it is widely present throughout the interstitial matrix of the superficial zone (245).

The chondrocytes in the present study were derived from a young calf, which may account for the abundance of collagen VI in these TE constructs. Not until day 34 did clear differences emerge between the experimental groups. ‘Free TGF-β’ gels displayed a relatively uniform interstitial distribution of collagen VI throughout the gel, with higher pericellular concentrations as expected. The abundance of collagen VI may be related to the known stimulatory effect of TGF-β on its production (248). The appearance of ‘no TGF-β’ gels was highly abnormal, with concentrated foci of collagen VI displaying no obvious relationship to chondrocytes, and large (tens to hundreds of micrometers) regions devoid of collagen VI. This distribution recalled the appearance of the collagen II staining (Figure 4.21), again giving the appearance of having been displaced by more recently deposited material. ‘PEG-LTGF-β’ gels were similar in appearance to ‘no TGF-β’ gels, but showed less extensive interstitial accumulations, and overall greater co-localisation of collagen VI with cells.
Figure 4.23: Collagen VI staining of PEG-HA hydrogels with encapsulated chondrocytes cultured for up to 34 days. Collagen VI = green, DAPI = blue; 20x magnification; scale = 100 µm.

Further histological and biochemical analyses were performed at the end of culture (day 34). Macroscopically, all gels were firm and semi-opaque, in contrast to the fragile state of ‘no TGF-β’ and ‘PEG-LTGF-β’ gels at day 28 of the first experimental repeat (Figure 4.24 versus Figure 4.19). Again, the use of a high density of fresh, passage 0 chondrocytes appeared to have promoted a healthier culture.

Figure 4.24: Macroscopic appearance of PEG-HA hydrogels after 34 days of culture. Base (membrane) diameter = 6 mm.
Conventional histology confirmed that the intense staining for collagen II in the day 34 ‘PEG-LTGF-β’ gel (Figure 4.21) genuinely reflected more abundant protein (Figure 4.25): both picrosirius red and haematoxylin & eosin highlighted more concentrated interstitial protein deposits than were seen in either of the other groups. Protein within the ‘free TGF-β’ gel was in general less concentrated but more evenly distributed than in the ‘PEG-LTGF-β’ gel. Both stains suggested increasing protein content in the order ‘no TGF-β’ < ‘free TGF-β’ < ‘PEG-LTGF-β’. Results from the Bradford assay for total protein confirmed these findings, as ‘PEG-LTGF-β’ gels were found to contain a similar amount of protein as ‘free TGF-β’ gels, and significantly ($p < 0.01$) more than the ‘no TGF-β’ group (Figure 4.26). Taken together, the results suggest that matrix deposition was favoured by loading the PEG-HA hydrogel scaffolds with high doses of tethered LTGF-β.

Despite these promising findings, abnormal cell morphology was observed within day 34 ‘PEG-LTGF-β’ and ‘no TGF-β’ gel samples. These cells were irregularly shaped, some were elongated, and many displayed large protrusions – as opposed to the evenly rounded form of the ‘free TGF-β’ chondrocytes (Figure 4.27, Figure 4.25). Furthermore the neutral red counterstain to alcian blue revealed that the ‘no TGF-β’ and ‘PEG-LTGF-β’ chondrocytes were diffusely surrounded by a basophilic substance(s) which may represent either necrotic cell debris such as leached nucleic acids, or alternatively secretory products such as sulphated carbohydrates (Figure 4.25) (249). The positioning of this substance and of collagens II and VI (Figure 4.21, Figure 4.23, Figure 4.25) appear inverted with respect to the cells, again suggesting that the initial cartilage matrix produced in ‘no TGF-β’ and ‘PEG-LTGF-β’ gels was displaced by material deposited later on. Whatever the precise identity and origin of this substance, chondrocyte viability within all gels at this timepoint was excellent: numerous live chondrocytes and very few dead cells were visualised by LIVE/DEAD® reagent (Figure 4.27).
Figure 4.25: Histology of PEG-HA hydrogels with encapsulated chondrocytes cultured for 34 days (wax-embedded sections). Stains are as labelled; brightfield microscopy; 4x and 10x labels indicate magnification; ‘zoom’ shows close-ups of 4x images; scale = 500 µm for 4x magnification, 200 µm for 10x magnification, and 125 µm for zooms of 4x images.
Figure 4.26: Total protein in PEG-HA hydrogels after 34 days of culture. * $p < 0.01$ versus ‘no TGF-β’. Mean ± SD, n = 7.

Figure 4.27: LIVE/DEAD® staining of bovine chondrocytes within PEG-HA hydrogels after 34 days of culture. Live cells appear green and dead cells red. Confocal microscopy; scale = 100 µm.
Overall, these results clearly demonstrate a biological effect of PEG-LTGFL-β on chondrocyte response to encapsulation in PEG-HA hydrogels. Based on the collected data, this response may be interpreted as consisting of three phases:

- An initial period of TGF-β responsiveness, lasting for approximately nine days. This period was associated with increased metabolic activity, and possibly with increased ECM protein synthesis (see e.g. collagen I staining at day 7, Figure 4.22).
- A period of declining metabolic activity from around days 9-21, which may have set in as local stores of PEG-LTGFL-β were exhausted. Despite this decline, day 21 chondrocytes stained more intensely for collagen II than either control group suggesting that matrix synthesis remained high.
- A period of relative metabolic recovery (days 21-34), which may be related to greater mobility of tethered PEG-LTGFL-β as the PEG-HA hydrogels began to break down. This recovery did not fully compensate for the cell death seen during the previous phase, as cell number at day 34 was similar to the ‘no TGF-β’ group. Some of the previously accumulated matrix was displaced by basophilic material during this phase, leaving collagens II and VI confined to interstitial regions. The hydrogels were populated by numerous live chondrocytes at day 34, and overall more protein had accumulated in this group than in ‘no TGF-β’ gels.¹

The data presented in this section therefore demonstrates that LTGF-β can be deployed in TE scaffolds to biological effect. The success of the PEG-LTGFL-β chondrogenesis did not match that of the ‘free TGF-β’ group, but this was not expected given the stringency of this control (fresh free TGF-β provided continuously throughout the culture period), and any improvement over the negative control is to be taken as a success. Although the data is encouraging, a number of key questions remain which should be a priority in future work, as detailed shortly.

¹The apparent discrepancy between the results for day 34 LDH activity (‘PEG-LTGFL-β’ ~ ‘no TGF-β’) and total protein (‘PEG-LTGFL-β’ > ‘no TGF-β’) is resolved by the fact that the protein assay accounts for cumulative matrix production whereas LDH activity (with the protocol used here) derives only from cells which were alive at the final timepoint.
4.7 Summary and future directions

4.7.1 Latent TGF-β offers several possible benefits for tissue engineering

Returning to the potential benefits to TE of LTGF-β identified in the introduction to this chapter, the results presented herein serve to illuminate some of these points.

- It was proposed that the in vitro handling and long-term conformational stability of TGF-β would be improved were the TGF-β to be used in its latent form, as this is how TGF-β is stored long-term in ECM. Consistent with this was the observation that the concentration of free TGF-β on BSA-coated tissue culture plastic was depleted by around 25% after eight hours, and almost 40% by 48 hours, presumably due to adsorption to surfaces (Figure 4.3). Meanwhile the concentration of LTGF-β was essentially unchanged.

- The HFF-1 Smad3 phosphorylation responses to a range of doses of free and latent TGF-β confirm the relative inactivity of LTGF-β (Figure 4.4). This supports the proposition that high doses of LTGF-β could be loaded into scaffolds – to ensure that enough growth factor is present to support chondrogenesis for many days to weeks – while avoiding exposing cells to excessive amounts of active TGF-β.

- It was suggested that stores of inactive LTGF-β loaded within scaffolds could gradually become liberated by the actions of cells. Evidence was sought for cell-mediated LTGF-β activation through protease- and integrin-dependent means by treatment with inhibiting compounds, but none of the treatments diminished the signalling response to LTGF-β (Figure 4.5, Figure 4.6). This may be due to the LTGF-β requiring longer periods of time, tethering to an immovable substrate, or particular environmental stimuli, to become active.

- LTGF-β provides an opportunity to tether TGF-β within TE scaffolds without directly modifying the TGF-β itself, thereby preserving more of its biological activity. The PEGylation procedure used in this study somewhat diminished the potency of the LTGF-β (Figure 4.12, Figure 4.13), but nonetheless the TGF-β was able to diffuse out of hydrogels following dissociation from tethered LAP (Figure 4.11). This suggests that the TGF-β dimer can be held in place following indirect (to LAP) rather than direct (to itself) covalent modification.

Taken together with the TE results reported in the previous section, these findings qualify this novel TE approach for further exploration. To confirm and more fully explore the potential advantages of LTGF-β,
several important channels of investigation should be pursued. These opportunities are elaborated in the next section under the topics of, respectively: biological effects of burst exposure to free and latent TGF-β; characterisation of LTGF-β activation by cells; LTGF-β conjugation strategy; and optimising chondrocyte response to LTGF-β for cartilage TE.

4.7.2 Several key questions are a priority for future research

Exposing cells to high concentrations of TGF-β is arguably best avoided due to the association of TGF-β dysregulation with several disease states including OA (234)(233). However, the diminished signalling response to latent as compared to free TGF-β (Figure 4.4), and the compounding effect of PEGylation on biological activity (Figure 4.12, Figure 4.13) were taken as justification for incorporating a high dose of PEG-LTGF-β (500 ng.ml⁻¹ free equivalent) into TE scaffolds without undue concern over an aberrant cellular response. This was judged necessary as no improvement had been seen when 50 ng.ml⁻¹ was used (free equivalent). One area for further investigation, then, is in the comparative responses by chondrocytes and/or other cell types to varying doses of free and latent TGF-β over longer timeframes (several days) in culture. The effects of exposure to high and low concentrations of either could easily be assessed in terms of proliferation (e.g. total DNA, LDH, alamarBlue®), ECM synthesis (e.g. hydroxyproline incorporation, total protein), and apoptosis (e.g. staining for Caspase, Bcl-2, or Terminal deoxynucleotidyl transferase dUTP nick end labelling – TUNEL).

As reported in the section 4.3.3 HFF-1 response to LTGF-β was not diminished by inhibition of protease activity, integrin binding, or contractility, attempts to inhibit the Smad3 phosphorylation response of HFF-1 and bovine chondrocytes were unsuccessful. Although not central to the study, it would be reassuring to demonstrate activation of the commercial SLC used in these studies. Time-dependency of activation might be explored, for example. In the present project, preliminary experiments were attempted in which HFF-1 were exposed to free and latent TGF-β at 1 ng.ml⁻¹ (or free equivalent) for up to 24 hours to determine whether the LTGF-β would become active over this period. Smad3 phosphorylation was seen at all timepoints for cells receiving free TGF-β, but no activation was apparent in cells exposed to LTGF-β (data not shown). If further observations were to confirm these findings, then the possibility that tension-dependent activation is required could be explored (Figure 2.3). In vitro schemes to enable tension-dependent activation are easily envisaged, and would require fixation of the
LAP to a relatively immovable surface – for example a glass slide or tissue culture plastic, or even a stiff hydrogel (> 10 KPa) – in a manner that preserves the RGD site on LAP so that coupling to integrins can occur. Controls should include treatments to enhance or abolish contractility (e.g. ATP, blebbistatin, cytochalasin D, nocodazole). Beyond this, digestion of LTGF-β could be attempted with different proteases from Table 4.1 with varying concentrations and digest times, in an extension of the work described earlier (Figure 4.14). Liberation of free TGF-β could be measured by ELISA and confirmed with biological assays (because antibody binding sites could still be masked by bound fragments of LAP post-digestion). Such an approach was attempted in the current study using plasmin, but active TGF-β was not detected by the ELISA. Ultimately, however, proof of concept relies on detecting a positive effect in cartilage TE experiments. It may be that, under these conditions, only a small fraction of the LTGF-β need be activated at any one time in order to achieve a favourable final outcome. This suggestion is based on the fact that, as seen in Figure 4.4, a signalling response was elicited by just 0.01 ng.ml⁻¹ free TGF-β. Hence, future efforts should be directed at optimising the conjugation strategy, hydrogel properties, and culture conditions for TE.

The conjugation strategy described in this work, while effective, gave variable results from batch to batch (Figure 4.10) and did not allow for precise control over PEGylation sites and by extension the orientation of the tethered complex. As discussed in the section 4.4 Results and discussion 2: Conjugation of PEGDA to LTGF-β, it was hoped that an endogenous cysteine residue (Cys-33) within LTGF-β could be directly reacted with PEGDA, but the experimental findings were unpromising (Figure 4.10, Figure 4.11). Strategies to access Cys-33 or to target other specific residues could potentially be beneficial both in terms of preserving the function of the TGF-β and larger complex, and presenting the LTGF-β within hydrogels in a defined orientation. Zisch and colleagues in the Hubbell group tackled a similar issue by introducing an extra cysteine residue at the C-terminus of a recombinant VEGF variant; the purified protein was then tethered within PEG-vinylsulphone hydrogels via Michael addition (236). Alternatively, this difficulty might be circumvented if the LLC were to be used in place of the SLC as per this study. LTBP5s are immobilised within ECM via transglutaminase linkages as shown in Figure 4.2. As it happens, the transglutaminase action of factor XIIIa has been utilised by the same group to incorporate a mutant VEGF variant into fibrin hydrogels, suggesting that a similar approach with the LLC may be feasible (109).
Use of the LLC would also dispel concern over possible unphysiological processing and export of the isolated SLC (225).

As the TE results stand, it is encouraging that the chondrocytes within PEG-LTGF-β hydrogels showed increased metabolic activity and protein deposition compared to 'no TGF-β' controls (Figure 4.20, Figure 4.26). When fully optimised, this strategy may prove to be an efficient way of supporting cartilage formation, by using a single initial loading which only gradually becomes available to the cells. The total amount of TGF-β loaded into these scaffolds (25 ng per gel, free equivalent; 500 ng.ml⁻¹ final concentration) was close to the amount of free TGF-β (20 ng per well) added to the ‘free TGF-β’ gels at every single medium change, of which there were eighteen. For comparison, in the study by Lee and colleagues described earlier (page 77) in which cartilage was generated over an entire articular condyle by recruiting host cells, a single loading of 10 ng.ml⁻¹ free TGF-β was used prior to in vivo implantation. On the other hand, in recently published work from the Burdick group free TGF-β was released from nanofilm-coated alginate microspheres into HA hydrogels in a dose equivalent to 2 µg.ml⁻¹ (100 ng per 50 µl hydrogel) – four times higher than in the present work (223). When MSCs were encapsulated in the hydrogel, this led to the in vitro formation of neocartilage with similar Young’s modulus, and collagen, DNA and GAG content to hydrogels receiving TGF-β thrice weekly in the culture medium. When implanted subcutaneously in mice, TGF-β delivered via the microspheres supported convincing neocartilage formation, whereas very little matrix was produced when the TGF-β was simply blended with the HA before gel crosslinking. Considering the doses used in each, the gain from PEG-LTGF-β in the present work was less striking than in these two examples, therefore it seems there is room for optimisation of the LTGF-β approach if maximum benefit is to be attained.

A role of scaffold degradation in modulating exposure of the chondrocytes to TGF-β was inferred from the temporal profile of chondrocyte metabolic activity in Figure 4.20, alongside observations over hydrogel degradation. This suggests that engineering continuous exposure to TGF-β by controlling scaffold degradation could offer one plausible route to optimising performance. Although HA is degradable by hyaluronidase enzymes, the PEG-HA hydrogel scaffolds degrade relatively slowly, beginning to liquefy between weeks 3 and 4 (Figure 4.19). In order to effect more precise control over this important property, Chung and co-workers have recently experimented with hydrolytically degradable
methacrylated caprolactone hyaluronic acid hydrogels for cartilage TE, finding that matrix synthesis and ultimate mechanical properties were superior in the hydrolytically degradable gels (250). Their system allows for tunable control over hydrogel degradation, suggesting one possible way to investigate the effect of degradation dynamics on chondrocyte response to PEG-LTGF-β. Another important factor to explore in future work is the conditioning of seeded hydrogels with cyclic mechanical loading. The potential benefits of this treatment are threefold: cyclic loading may enhance the stretch-induced activation of LTGF-β; published data suggests that it may act synergistically with TGF-β in stimulating matrix synthesis; and finally it has been reported to stimulate the expression of catabolic enzymes (229)(251)(252)(253). This treatment may therefore not only improve the sensitivity of chondrocytes to TGF-β, but also increase its activation by both proteolytic- and integrin-dependent mechanisms. Overall, the results presented in this chapter illustrate several potential benefits of LTGF-β for cartilage TE, and suggest several intriguing avenues for the further development of this concept.
4.8 Conclusions

Latent TGF-β adsorbs to TCP less readily than free TGF-β and is relatively inactive, with a concentration threshold for Smad3 phosphorylation (in HFF-1) around a thousand times higher than free TGF-β. Cell-mediated release of TGF-β via protease- and/or integrin-dependent mechanisms was not demonstrated but may be dependent on longer timeframes and/or optimised materials properties and culture conditions. PEG-LTGF-β enhanced proliferation and matrix synthesis by chondrocytes encapsulated in PEG-HA hydrogel scaffolds, and several means of optimising this novel TE system are immediately apparent. Ultimately LTGF-β may offer the advantages of improved stability over long culture periods, diminished cellular response to initial high growth factor concentrations, gradual release by cells, and indirect immobilisation in scaffolds, with preservation of TGF-β biological activity.
Chapter 5

Conclusions

5.1 Bioactive scaffolds and commercial tissue engineering

As we have seen from the numerous examples throughout this thesis, TE has the potential to restore function in a wide range of medical conditions involving tissue loss or dysfunction. Tremendous progress has been made over the past decade towards not only generating functional tissue substitutes, but also doing so in a commercially viable manner. The stream of products currently proceeding through clinical trials, and the patients already benefiting from TE technology, are cause for celebration. Yet the TE literature details many innovative new scaffold materials that go beyond anything currently being used clinically. Here, biomimetic principles – with inspiration coming principally from natural ECM, tissue homeostasis, and development – are being used to inform the design of biologically instructive scaffolds. The promise is of a new generation of materials that can help to unlock and augment the body’s own capacity for repair.

Materials can now be made to engage with cells in biologically-meaningful ways, for example by proliferating or differentiating in response to cues encoded in the physical and chemical fabric of the scaffold. It is now possible for completely synthetic materials to participate in processes akin to natural tissue remodelling: PEG hydrogels crosslinked with synthetic peptides containing protease-digestible sites can be degraded by cellular enzymes (18); the inclusion of integrin-binding (or other cell surface receptors) peptides to such materials enables cell attachment, which may generate survival signals as well as tractional forces for migration (35). A simple RGD-modified alginate hydrogel was seen in one study to facilitate the organisation of co-transplanted osteoblasts and chondrocytes into growth plate-like structures (Figure 2.2) (72). In another exciting example, spinal cord regeneration was facilitated by a hydrogel presenting an epitope from laminin (IKVAV), a prominent glycoprotein involved in axon guidance as well as being found in basement membranes throughout the body (42). Furthermore it
appears that a few key ECM biomolecules can be adaptable to many TE contexts, as demonstrated by the Extracel system from Glycosan (63). The recognition of the importance of physical factors such as topography, strain, pressure, and matrix stiffness has also opened new avenues for eliciting desirable responses from cells.

The simplification of ECM and biomolecular information is being taken to extremes in some groups, with growth factor activity, certain functions of glycosaminoglycans (such as growth factor binding and modulation of activity), and cell-cell adhesions being recreated in some way via purely synthetic approaches (126)(254). Even basic chemical considerations such as hydrophilicity, or simple chemical functional groups can significantly affect the adsorption and conformation of proteins, or influence stem cell differentiation by as yet poorly understood means (145)(67). This approach has been taken to the extreme in studies which have systematically explored cellular responses to literally hundreds or even thousands of synthetic polymers at once, with the aim of identifying those that strongly favour particular behaviours (148).

Even certain ions can strongly influence cell activity. Chapter 3 described the evaluation of strontium- and zinc-alginate hydrogels for bone tissue engineering applications. Strontium was selected because it can promote bone formation by osteoblasts while inhibiting resorption by osteoclasts – leading to decreased fracture risk in osteoporotic patients (186)(187). Zinc acts as a cofactor for alkaline phosphatase, an enzyme important (although mysteriously so) for bone mineralisation (188). Initially, stiffness, swelling and degradation, and ion release were compared between calcium- and strontium-alginate gels. Calcium- and strontium-alginate had similar initial stiffness (170 ± 7 KPa for 2G.Ca versus 186 ± 7 KPa for 2G.Sr; gel codes are given in Table 3.1) indicating that the degree of crosslinking was unchanged between conditions. In stark contrast, 3G.Sr gels had a lifespan almost fifteen times longer than that of 3G.Ca gels: 3G.Ca gels persisted for just 4 ± 0 days in PBS before beginning to lose mass, whereas the lifespan of the corresponding strontium gel was 58 ± 5 days. This was expected given that published work describes strontium as having a higher affinity for alginate with a high proportion of G residues than calcium (40). Although the lifespan of strontium gels could be reduced to 4 ± 1 days by using 1 % w/v gels instead of 3% w/v, further flexibility came in the form of an interaction between the crosslinking ion and the alginate type. Although G blocks are selective for strontium over calcium, this relationship does not
extend to M blocks (40). Thus, switching AlgG for AlgM virtually abolished the enhancement in gel stability seen with strontium substitution (3M.Sr lifespan 15 ± 1 days). This interaction is of considerable practical utility as it means that the biological and materials effects of the crosslinking ions can be decoupled. Specifically, the desirable effects of strontium can be exploited in applications requiring both high and low gel stabilities by using high G or high M alginate, respectively. A final finding from the gel characterisation phase was that strontium release from the gels appeared to be of a physiologically relevant magnitude (although the release estimates cannot directly predict release in *in vitro* cell culture or *in vivo*). Strontium was released from AlgM gels at a higher rate than from AlgG, which correlated with the faster degradation of these gels.

Three-dimensional culture of Saos-2 cells within RGD-containing AlgG and AlgM gels demonstrated that the persistence of the AlgG strontium-containing gels impeded proliferation, as revealed by slight decreases in LDH activity to day 7 and confirmed by alamarBlue® data. Proliferation within strontium-containing gels was rescued by using AlgM, which supported Saos-2 proliferation from the earliest timepoints, and viability was sustained for the full three weeks of the study. qPCR on cells cultured within AlgM gels revealed upregulation of the osteoblast marker genes COL1A1 (collagen I), BSP (bone sialoprotein) and RUNX2 (the transcription factor) at day 21. Although these genes were more highly expressed than on TCP, there were no significant differences between groups. ALP activity at days 7 and 21, however, was highest in Sr100 gels (*p* < 0.05 compared to all other gel groups). Although inconclusive (especially with respect to the qPCR data), overall these results seem promising and demonstrate the potential of alginate hydrogels – and perhaps of other polysaccharides such as pectin, which have different selectivities for cations – to provide slow release reservoirs for bioactive ions including calcium, strontium, zinc, and cobalt (40). Furthermore this strategy is technically simple and inexpensive, and is amenable to being combined with existing strategies for bone TE – such as blending with collagen I or ceramics, which in themselves may be engineered to have favourable ion release profiles (187)(190).

Another major theme of this thesis has been the controlled release of growth factors within scaffolds. These potent molecules operate key roles during development, wound healing, and normal tissue homeostasis. Thus they have become standard tools in differentiation protocols for adult and embryonic stem cells, and a huge volume of literature documents their use in TE to improve cell survival,
proliferation, differentiation, matrix production, and homing. The soaring commercial success of Medtronic’s INFUSE® demonstrates the potential impact of these schemes (12). However, due to this same potency, they must be used with care. Convincing evidence suggests that the manner of growth factor delivery can have a decisive influence on TE success. This is well illustrated by two examples pertaining to angiogenesis. Here, stable blood vessels formed in response to, in one case, a recombinant VEGF variant which could be immobilised into fibrin hydrogels and released by cellular proteolytic activity; and secondly by dual delivery of VEGF and PDGF-BB from alginate hydrogels (232)(122)(236)(123). In the latter study sequential release was achieved, apparently due to different passive affinities of the two growth factors for alginate.

Other documented strategies for growth factor release include the tailoring of polymer diffusional properties and degradation kinetics, release from polymer microspheres, the inclusion of glycosaminoglycans such as heparin (or synthetic mimetics thereof, as described in the Chapter 2 section ‘simplifying biomolecules’) and tethering of the protein, perhaps via a spacer arm such as PEG (100)(106)(108)(114)(236). The experiments reported in Chapter 4 made use of a covalent conjugation strategy (PEGylation) as part of the evaluation of a novel approach to TGF-β presentation and release. In this work, TGF-β was loaded into hydrogel scaffolds as part of the SLC, which consists of the TGF-β dimer wrapped in its own pro-peptide, the LAP (91). One advantage of this approach is the superior stability of latent TGF-β compared to free TGF-β. In a simple experiment to assess the tendencies of each to adsorb to TCP, the concentration of LTGF-β was unchanged from starting levels after 48 hours incubation, whereas the concentration of free TGF-β fell by 39%. Another possible benefit is that high doses can be loaded into scaffolds, becoming available to the cells only gradually via active processes. Advanced growth factor presentation and release strategies often lead to better TE outcomes: for example, blood vessels formed in response to the controlled release of VEGF in a chick chorioallantoic membrane model of angiogenesis were of higher quality than the malformed and leaky blood vessels formed in response to burst release of VEGF (236). As described on page 134, the controlled release of free TGF-β from coated alginate microspheres vastly improved the quality of neocartilage compared to simple mixing with the hydrogel polymer (223). To confirm the relative inactivity of the LTGF-β, a dose response evaluation was performed using human fibroblasts (HFF-1). The cells’ response to TGF-β was evaluated by immunostaining for the phosphorylated form of Smad3, which becomes active downstream of TGF-β.
binding to its receptor. Evidence of a signalling response was found at concentrations of 0.01 ng.ml\(^{-1}\) with free TGF-\(\beta\), but only 10 ng.ml\(^{-1}\) with LTGF-\(\beta\), confirming the relative inactivity of the latent protein and dispelling concern over exposing cells to high concentrations of growth factor during encapsulation and the early stages of culture. It was also of interest to investigate whether the response seen with 10 ng.ml\(^{-1}\) LTGF-\(\beta\) derived from LTGF-\(\beta\) activation by cells. Attempts to perturb proteolytic and integrin/contractility-dependent activation pathways led to no discernible effect on the HFF-1 response – a finding that was confirmed (for inhibition of proteases) with bovine chondrocytes, leading to the conclusion that the observed responses did not stem from active release mechanisms. However, it was possible that the TGF-\(\beta\) would become active over longer timeframes (days to weeks), under different culture conditions, or by different cell types.

To assess LTGF-\(\beta\) for cartilage TE, bovine chondrocytes were seeded into PEGDA-HA hydrogels with tethered LTGF-\(\beta\). Thiolated HA (Glycosil\™) was crosslinked by PEGDA to form the hydrogels, but could also react with monoacrylates left over after protein modification with PEGDA. LTGF-\(\beta\) was PEGylated in a reaction optimised using the model proteins bovine serum albumin, lysozyme, and cytochrome C. A one pot reaction between the protein of interest, PEGDA, and Traut’s reagent was used, leading to Mw increases observable by SDS-PAGE. Timed release of growth factor from PEG gels demonstrated that 88% of PEG-LTGF-\(\beta\) was retained within the gel over 24 hours, but that 58% of this TGF-\(\beta\) diffused out of the gel upon treatment with acid (which disrupts the association with LAP). Thus, the use of LTGF-\(\beta\) may allow conjugation via the LAP rather than the growth factor itself, which diminishes biological activity (108). To investigate the biological action of PEG-LTGF-\(\beta\), phosphoSmad3 immunostaining and Western blotting was carried out on HFF-1 stimulated with unmodified and PEGylated LTGF-\(\beta\). The PEG-LTGF-\(\beta\) was demonstrated to retain its biological activity, but some loss of activity had occurred, and the extent of loss of activity was batch-specific. Bovine chondrocytes encapsulated in PEG-HA hydrogels with 50 ng.ml\(^{-1}\) (free equivalent) tethered PEG-LTGF-\(\beta\) showed no increase in metabolic activity or matrix deposition over control hydrogels containing no TGF-\(\beta\). When a higher dose (500 ng.ml\(^{-1}\) free equivalent) was loaded, benefits in metabolic activity and ECM synthesis were seen over an almost five week period. Specifically, alamarBlue\® activity showed two peaks in comparison to the negative control group, around days 5-10 and during the late fourth to early fifth week of culture. These peaks in activity may correspond to, initially, an effect of PEG-LTGF-\(\beta\) in the local cell environment, and subsequently, an effect caused by
the liberation of stored PEG-LTGF-β during gel breakdown. Total LDH at 34 days was no different to that of the negative control group, but total protein was similar to the positive control group (free TGF-β delivered continuously over the entire culture period). This was confirmed histologically, although the distribution of ECM protein differed between groups: whereas treatment with free TGF-β led to a homogeneously distributed collagenous matrix, PEG-LTGF-β gels showed intensely stained accumulations of protein. In the future it will be important to optimise the hydrogel culture conditions in order to fully realise the potential benefits of this novel TE approach. Overall, the innovations described in this thesis correspond to current TE trends, which seek to use biomimetic principles to evoke regenerative responses from transplanted or host cells, but to do so using technically and commercially feasible means.
Appendices to Chapter 3

I. Validation of LDH sample preparation

Due to the high stability of AlgG strontium gels (Figure 3.7), conventional methods of dissolving alginate gels, to enable biochemical analysis, were not effective on these formulations. For example, the strontium gels were resistant to incubation with sodium citrate, EDTA, and high phosphate buffers. Mechanical disruption in a bead mill homogeniser (QIAGEN TissueLyser II) followed by treatment with Triton X-1000 was more effective at breaking down gels than purely chemical means. However, such harsh treatment may expose proteins to shear forces with possible deleterious effects on protein conformation. Additionally, because gel consistency varied considerably between different groups and timepoints, systematic errors seemed possible. LDH standard curves were therefore constructed with known cell numbers in gelled or ungelled (by calcium) alginate, homogenised for 15 minutes at 30 Hz (Figure A.I).

![Figure A.I: LDH standard curve results for Saos-2 homogenised in alginate solution and gels.](image)

Two important findings arose:

- A linear relationship between cell number and absorbance was maintained ($R^2$ for crosslinked alginate 0.95, ungelled alginate 0.88), indicating that the method can reliably be used to estimate cell number.

- The consistency of the gel (here taken to the extreme with gelled versus ungelled alginate) did not seem to influence the result: the gradients of the curves were 0.0005 in both cases, and closely matched to the eye.

These findings were taken as validation of the sample preparation method.
II. RNA extraction troubleshoot

Prior to the qPCR experiment described in the main chapter, investigations were made into RNA extraction techniques from alginate hydrogels to ensure that good quality RNA was available for this work. RNA quantity and quality was assessed by taking UV absorbance spectra on a Nanodrop 1000. Pure RNA has an absorbance maximum at 260 nm and a 260/280 nm ratio of ~2.0, and the 260/230 nm ratio should additionally be around 2 (255). Figure A.II shows examples of high and low quality RNA preparations, and Table A.I lists some common contaminating compounds and their effects on RNA absorbance spectra and ratios.

Figure A.II: UV absorbance spectra of (left) pure RNA and (right) a contaminated preparation. The pure sample has a 260/280 ratio of 2.02 and a 260/230 ratio of 2.06, and the contaminated sample has 260/280 and 260/230 ratios of 2.02 and 0.27 respectively. Note the absorbance maxima at 260 nm (vertical lines). Screenshots are from NanoDrop software.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Characteristic absorbance</th>
<th>Effect(s) on spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>280 nm</td>
<td>260/280 ratio &lt; 2.0 (~1.8 for pure DNA)</td>
</tr>
<tr>
<td>Protein</td>
<td>280 nm (tryptophan, tyrosine) 220 nm (peptide bond)</td>
<td>Low 260/280 ratio  Low 260/230 ratio</td>
</tr>
<tr>
<td>Trizol reagent, phenol</td>
<td>270 nm 224 nm</td>
<td>Overestimation of RNA quantity  Absorbance maximum shifted towards 270, minimum shifted to 240 nm</td>
</tr>
<tr>
<td>Guanidine salts</td>
<td>230 nm</td>
<td>Low 260/230 ratio</td>
</tr>
</tbody>
</table>

Table A.I: List of common contaminating compounds in RNA preparations and their characteristic absorbances. Guanidine salts are common in RNA isolation buffers, for example guanidine thiocyanate (absorbance at ~260 nm) is a component of the RNeasy buffers RLT and RW1. (255)
For these investigations, 200 µl 2% w/v AlgG gels containing, typically, 2 million Saos-2 cells.ml⁻¹ were swollen for 1 hour or overnight before RNA extraction was attempted. Initial isolation attempts generally resulted in poor RNA yield (< 20 ng.µl⁻¹) and very low 260/230 ratios (< 1). Various troubleshooting steps were performed, the results of which are described below and summarised in Table A.II along with briefly described methods.

Initially, a conventional method for dissolving alginate gels was attempted, followed by RNeasy extraction on the pelleted cells. Although this method worked well for the calcium gels, the higher stability of the strontium gels meant that cell recovery, and consequently RNA yield, was low (Method 1, Table A.IIa). Proper homogenisation is a critical step in isolating RNA from tissues, so from this point onwards the gels were disrupted in a bead-mill homogeniser (TissueLyser II, QIAGEN), and several means of improving gel breakdown were tested (Methods 2-4, Table A.IIa). Although 10-15 minutes in the TissueLyser ultimately proved effective at homogenising gels, the RNA at this stage was generally of low abundance and quality (consistently, contamination was detected at 230 nm). It was also confirmed that similar problems were found with photopolymerised PEG hydrogels (PEG molecular weights 4000 and 700; Methods 5-6, Table A.IIa), although in one instance more promising samples were collected (discussed below).

Next, possible ways of removing contamination were pursued. Homogenised gels were centrifuged to precipitate insoluble matter including particles of gel, and various means of removing contaminants were tested (Methods 7-9, Table A.IIb). It was noted at this stage that the extractions from strontium gels generally produced higher quality samples than calcium gels. It seemed likely that this was related to the higher stability of strontium binding to alginate (Figure 3.7, Figure 3.2), as crosslinked gel particles sedimented better during centrifugation leading to more complete removal of the alginate. Further evidence in favour of this notion was the observation that the low molecular weight PEG gels (PEG700), produced more compact, firmer pellets than the PEG4K gels (Method 5, Table A.IIa), and also better RNA. Attempts were therefore made to remove alginate from the lysate by adding strontium to aggregate the polymer (Methods 10-11, Table A.IIb) – but to no avail.
With these findings in mind, the homogenisation was next performed in Trizol reagent. Both alginate and PEG gels were less soluble in Trizol than in RLT buffer, and sometimes required longer homogenisation times, but the corollary of this was that centrifugation was more effective at removing gel debris. RNA isolated from PEG gels using the standard Trizol procedure had low 260/280 and 260/230 ratios, and apparently was not abundant enough to suffer some inevitable loss during the RNeasy cleanup procedure (Methods 12-13, Table A.IIIc). However, when combined with on-column RNeasy purification the purification from both ungelled alginate and strontium-alginate gels appeared satisfactory. Method 14 was ultimately used in the qPCR experiment described in Chapter 3.
<table>
<thead>
<tr>
<th>Method, rationale (notes)</th>
<th>Gel</th>
<th>[RNA] (ng.mL⁻¹)</th>
<th>260/280</th>
<th>260/230</th>
<th>Verdict</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1: Homogenisation</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1 Incubate gels in dissolution buffer (55 mM Ca₃(PO₄)₂, 150 mM NaCl, 55 mM EDTA; 37°C, 15 min), push gels several times through 19G needle, pellet cells (10 min, 500 rcf), discard supernatant, resuspend cells in PBS, pellet again, discard supernatant, vortex in 350 μl buffer RLT and use standard RNeasy protocol. Typical procedure for dissolving calcium alginate gels (strontium gels did not dissolve very well)</td>
<td>Ca-Alg</td>
<td>42.8</td>
<td>2.03</td>
<td>1.85</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sr-Alg</td>
<td>16.6</td>
<td>1.85</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>28.0</td>
<td>1.89</td>
<td>0.96</td>
</tr>
<tr>
<td>2 Homogenise in TissueLyser (2 min, 20 Hz, RLT buffer), then use standard RNeasy protocol</td>
<td>Ca-Alg</td>
<td>3.4</td>
<td>1.86</td>
<td>0.20</td>
<td>✗</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sr-Alg</td>
<td>5.5</td>
<td>2.17</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.1</td>
<td>2.32</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.7</td>
<td>3.39</td>
<td>0.26</td>
</tr>
<tr>
<td>3 Homogenise in TissueLyser (3 min, 30 Hz, RLT buffer), pass through QiAshredder, centrifuge lysate (5 min, full speed), then use standard RNeasy protocol on supernatant. Use of both QiAshredder and TissueLyser to homogenise gel; centrifugation to remove gel debris from sample</td>
<td>Ca-Alg</td>
<td>12.5</td>
<td>2.19</td>
<td>1.17</td>
<td>✗</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sr-Alg</td>
<td>41.0</td>
<td>2.06</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>31.0</td>
<td>2.64</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>13.0</td>
<td>2.08</td>
<td>0.50</td>
</tr>
<tr>
<td>4 Add 50 mM K₂HPO₄ to RLT buffer, homogenise in TissueLyser (3 min, 30 Hz), centrifuge lysate (5 min, full speed), then use standard RNeasy protocol on supernatant. Phosphate was added to improve gel dissolution (unsuccessful)</td>
<td>Ca-Alg</td>
<td>7.6</td>
<td>2.18</td>
<td>0.34</td>
<td>✗</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sr-Alg</td>
<td>3.6</td>
<td>2.21</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.2</td>
<td>0.21</td>
<td>0.02</td>
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<td></td>
<td></td>
<td></td>
<td>26.1</td>
<td>0.07</td>
<td>0.05</td>
</tr>
<tr>
<td>5 Homogenise in TissueLyser (60 mins, 30 Hz, RLT buffer), centrifuge lysate (15 min, 14,000 rcf), then use standard RNeasy protocol</td>
<td>PEG4K</td>
<td>10.4</td>
<td>1.91</td>
<td>0.65</td>
<td>✗</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PEG700</td>
<td>34.6</td>
<td>1.93</td>
<td>2.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>34.2</td>
<td>1.92</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>32.0</td>
<td>1.89</td>
<td>1.36</td>
</tr>
<tr>
<td>6 As per no. 5 except use QiAshredder after centrifugation step</td>
<td>PEG4K</td>
<td>12.5</td>
<td>1.80</td>
<td>0.46</td>
<td>✗</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PEG700</td>
<td>79.0</td>
<td>2.13</td>
<td>0.39</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>83.0</td>
<td>2.17</td>
<td>2.48</td>
</tr>
</tbody>
</table>

Table A.II: Summary of troubleshooting steps for RNA extraction from alginate and PEG hydrogels. (a – above) Troubleshooting steps in gel homogenisation. (b – page 148) Troubleshooting steps in removing gel debris and other contaminants. (c – page 149) Extractions using Trizol reagent. Brief methods and reasons for taking each step are given. Bold typeface highlights the notable points for each attempt. Alg = ungelled alginate; Ca-Alg = Ca100 gel; Sr-Alg = Sr100 gel; PEG700 = photopolymerised PEGDA Mw = 700; PEG4K = photopolymerised PEGDA Mw = 4000. RNA concentration, and 260/280 and 260/230 nm ratios are stated, and an overall judgement on the success of the extraction is indicated in the far right column: ✓ = method has significant potential (typical yield > 20 ng.ml⁻¹, 260/280 ratio ~ 2, 260/230 ratio >> 1); ✗ = RNA does not meet the required standard; ~ = RNA does not meet the required standard, but some samples or parameters show promise. Sources: manufacturer’s information (Trizol, RNeasy) and reference (255).
<table>
<thead>
<tr>
<th>Method, rationale (notes)</th>
<th>Gel</th>
<th>[RNA] (ng.µl⁻¹)</th>
<th>260/280</th>
<th>260/230</th>
<th>Verdict</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stage 2: Removal of contaminants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 Homogenise in Tissuelyser (10 min, 30 Hz, RLT buffer), centrifuge lysate (5 min, 8,000 rcf), <strong>sterile filter supernatant</strong>, then use standard RNeasy protocol. Longer homogenisation performed; supernatant was filtered to remove any remaining debris</td>
<td>Ca-Alg</td>
<td>13.2</td>
<td>1.98</td>
<td>0.91</td>
<td>✗</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.8</td>
<td>2.03</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sr-Alg</td>
<td>15.9</td>
<td>1.96</td>
<td>1.12</td>
<td>✖</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.6</td>
<td>1.86</td>
<td>1.22</td>
<td></td>
</tr>
<tr>
<td>8 Homogenise in Tissuelyser (10 min, 30 Hz, RLT buffer), centrifuge lysate (5 min, 8,000 rcf), use standard RNeasy protocol on supernatant, but with <strong>extra wash steps</strong> (3 washes per buffer instead of 1 x RW1, 2 x RPE). Extra washes may help to remove contamination</td>
<td>Ca-Alg</td>
<td>15.4</td>
<td>1.83</td>
<td>0.85</td>
<td>✗</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.8</td>
<td>1.90</td>
<td>0.77</td>
<td></td>
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<tr>
<td></td>
<td>Sr-Alg</td>
<td>32.1</td>
<td>2.03</td>
<td>1.32</td>
<td>✗</td>
</tr>
<tr>
<td></td>
<td></td>
<td>38.5</td>
<td>1.98</td>
<td>1.48</td>
<td></td>
</tr>
<tr>
<td>9 Homogenise in Tissuelyser (15 min, 30 Hz, RLT buffer), centrifuge lysate (3 min, full speed), use standard RNeasy protocol on supernatant, but <strong>invert tube several times and leave for 2 min in each buffer during wash steps</strong>. Longer and more thorough washes may help to remove contamination</td>
<td>Ca-Alg</td>
<td>7.7</td>
<td>2.20</td>
<td>0.64</td>
<td>✗</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.2</td>
<td>1.96</td>
<td>1.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sr-Alg</td>
<td>20.9</td>
<td>2.00</td>
<td>1.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>40.3</td>
<td>2.01</td>
<td>1.86</td>
<td></td>
</tr>
<tr>
<td>10 Homogenise in Tissuelyser (10 min, 30 Hz, RLT buffer), centrifuge lysate (3 min, 10,000 rcf), <strong>add SrCl₂ (final concentration 7 mM)</strong>, vortex, centrifuge (3 min, 10,000 rcf), then use standard RNeasy protocol on supernatant. Use strontium to aggregate alginate, then remove by centrifugation</td>
<td>Alg</td>
<td>45.6</td>
<td>2.02</td>
<td>0.37</td>
<td>✗</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29.4</td>
<td>1.98</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>11 As above (12) except, after strontium addition and centrifugation, incorporate proteinase K digestion according to instructions in RNeasy Fibrous Tissue kit (10 min, 55°C, after homogenisation). <strong>Recommended for tissues with high protein content, to help release RNA</strong></td>
<td>Alg</td>
<td>31.9</td>
<td>1.96</td>
<td>1.10</td>
<td>✗</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19.7</td>
<td>1.95</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.8</td>
<td>1.71</td>
<td>0.05</td>
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<td>6.5</td>
<td>1.68</td>
<td>0.11</td>
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</table>

Table A11b: Troubleshooting steps in removing gel debris and other contaminants. (See full legend on page 153)
<table>
<thead>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 3: Trizol reagent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 Homogenise in TissueLyser (10 min, 30 Hz, in Trizol), use standard Trizol extraction procedure including the optional centrifugation step after homogenisation (to remove insoluble debris) Trizol procedure may prove more suitable with gels (PEG gels became very tough in Trizol)</td>
<td>PEG4K</td>
<td>38.5</td>
<td>1.76</td>
<td>0.38</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36.0</td>
<td>1.88</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PEG700</td>
<td>52.2</td>
<td>1.76</td>
<td>0.27</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45.7</td>
<td>1.75</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>13 Perform RNeasy cleanup procedure on samples from (8) above RNeasy cleanup procedure may complement Trizol extraction</td>
<td>PEG4K</td>
<td>19.5</td>
<td>2.25</td>
<td>0.17</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36.3</td>
<td>2.18</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PEG700</td>
<td>6.3</td>
<td>2.42</td>
<td>1.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.3</td>
<td>2.48</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>14 Homogenise in TissueLyser (30-60 min, 30 Hz), Trizol, centrifuge lysate (10 min, 12,000 rcf, 4°C), transfer supernatant to new tube and proceed with standard Trizol procedure from addition of chloroform to isolation of aqueous phase, mix aqueous phase with equal volume 70% v/v EtOH, vortex, transfer to RNeasy column and proceed with standard RNeasy isolation Hybrid Trizol-RNeasy procedure – similar to RNeasy lipid protocol</td>
<td>Alg</td>
<td>91.6</td>
<td>2.08</td>
<td>1.99</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>121.7</td>
<td>2.08</td>
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<td></td>
<td></td>
<td>108.1</td>
<td>2.00</td>
<td>0.94</td>
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<tr>
<td></td>
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<td>108.1</td>
<td>2.00</td>
<td>0.94</td>
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<tr>
<td></td>
<td></td>
<td>106.6</td>
<td>2.07</td>
<td>0.67</td>
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<td>2.05</td>
<td>1.93</td>
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<td>56.9</td>
<td>1.96</td>
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<td></td>
<td>65.0</td>
<td>2.05</td>
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<td>15 As per (14) except mix aqueous phase with 0.5 vol 100% v/v EtOH, 0.5 vol high salt buffer (0.8 M C₆H₁₂Na₃O₁₂, 1.2 M NaCl; instead of 1 vol 70% v/v EtOH) Salt can inhibit carbohydrate binding to RNA; may therefore help to remove alginate (no clear benefit over no. 15)</td>
<td>Alg</td>
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<td>2.09</td>
<td>1.47</td>
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<td>2.06</td>
<td>1.47</td>
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</table>

Table A.IIIc: Extractions using Trizol reagent. (See full legend on page 153)
### III. qPCR primer validation

Quantitect (QIAGEN) exon spanning primers were purchased for the human genes GAPDH, COL1A1, BSP, and RUNX2. Standard curves were performed on these primer sets using 10x serial dilutions of cDNA from the cell type of interest (Saos-2), followed by agarose gel electrophoresis on the reaction products. All primers had efficiencies close to 1, indicating almost perfect doubling of DNA with each cycle (Table A.III). Melt curve analysis showed a single peak in all cases, and electrophoresis revealed a single reaction product. Representative examples are shown in Figure A.III (for BSP).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Working range (Ct)</th>
<th>Working range (dilution)</th>
<th>Efficiency</th>
<th>R²</th>
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<tr>
<td>GAPDH</td>
<td>Up to 25-30</td>
<td>10 – 10⁶</td>
<td>1.04</td>
<td>&gt; 0.99</td>
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<tr>
<td>COL1A1</td>
<td>Up to 25-30</td>
<td>10 – 10⁴</td>
<td>1.09</td>
<td>&gt; 0.99</td>
</tr>
<tr>
<td>BSP</td>
<td>Up to 30-35</td>
<td>10 – 10⁵</td>
<td>1.01</td>
<td>&gt; 0.99</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Up to 30-35</td>
<td>10 – 10⁴</td>
<td>0.99</td>
<td>&gt; 0.99</td>
</tr>
</tbody>
</table>

*Table A.III: Standard curve results for qPCR primers.* Working range and dilution indicate the Ct and dilution ranges over which the indicated efficiency and R² values were generated.
Figure A.III: Primer validation for BSP. (Top) Raw (cycling) fluorescence for serial dilutions of cDNA, showing intervals of ~ 3.3 Cts between 10x dilutions. (Middle) Melt curve analysis of samples, showing a single peak (amplicon). (Bottom) Agarose gel electrophoresis of reaction products, showing a single specific band (amplicon). NTC = no template control (water), No RT = no reverse transcriptase control; numbers indicate ten fold dilutions of cDNA.
IV. Pilot experiments in cell viability

Prior to the viability studies reported in the main chapter (Figure 3.10, Figure 3.11, Figure 3.12), pilot experiments were conducted in order to assess and optimise Saos-2 viability within alginate gels. Saos-2 were encapsulated at a density of 2.5 x 10⁶ cells.ml⁻¹ in 2% and 3% w/v AlgG hydrogels, using Ca100, Sr20 and Sr100 crosslinking solutions (Table 3.2). AlgG was supplemented where indicated with a commercial GRGDS-coupled alginate (NOVATECH-RGD; Novamatrix, Commonwealth Biotechnologies, Inc.) to a final RGD concentration of 150 µM RGD (72). All other reagents and the encapsulation procedure were as described in the main chapter. Gels were cultured for one and five days. On the day of analysis, gels were sliced thinly, and stained with LIVE/DEAD® reagent and imaged as described. Five random fields per gel were photographed after determining exposure times using a negative control (Triton-X treated cells). Live and dead cells were counted using ImageJ software (NIH), after manual thresholding. The particle size range was set based on the ability to detect only those cells in the plane of focus while excluding smaller or larger objects such as distant or out of focus cells.

Saos-2 seeded in unmodified AlgG displayed excellent (88 ± 3% to 95 ± 3%) viability at day 1 (Figure A.IV). This was followed by a large decline to day 5 (p < 0.001), at which point viability was estimated at around 29 ± 3% to 54 ± 12%, with no significant differences between groups. The lesser stability of the Ca100 gels (Figure 3.7) led to sample breakage and probably an underestimate of cell number compared to the Sr20 and Sr100 formulations, so the data on number of cells per field are not to be interpreted too literally. However the qualitative finding of drastically reduced cell numbers corroborates the viability data and is likely to reflect a genuine trend (Figure A.IV). No significant differences in viability between 2% and 3% w/v gels were detected. 2% w/v gels were chosen for the following experiments as this is close to standard concentrations used in the literature and the lower concentration may permit freer metabolite transport (72)(256)(168).
Figure A.IV: Viability and numbers of Saos-2 in alginate gels at days 1 and 5 of culture, as determined by quantification of LIVE/DEAD® stained cells. 2% and 3% w/v unmodified AlgG gels.

(Top) Percentage live cells within gels. Effect of day 1 versus day 5 $p < 0.001$. (Bottom) Total number of cells per image. Mean ± SD, $n = 6.5$ random fields were analysed per gel.

NOVATECH-RGD alginate was included in the next experiment with the aim of improving survival by providing integrin attachment sites (72). In addition, the ImageJ particle parameters were set more rigorously than before to ensure exclusion of particulate debris – hence, lower cell numbers were recorded. Therefore the two data sets cannot be directly compared, particularly with respect to the estimated cell numbers per field. Despite this, some helpful and sound observations can be made, based on differences between days 1 and 5 within each experiment.

The drastic decline in cell viability and cell number seen between days 1 and 5 in the original study was not replicated. Day 1 viability in this study was estimated at $76 \pm 5\%$ to $80 \pm 4\%$, and viability at day 5 remained relatively robust over approximately a 10 percent range, from $64 \pm 7\%$ to $73 \pm 7\%$ (Figure
A.V). This supported the hypothesis that the inclusion of RGD peptide would sustain higher cell viability than unmodified AlgG, in accordance with the findings of others (72). Cell number again declined to day 5, but less catastrophically than before. Therefore an in-house RGD-coupled alginate was used for all subsequent experiments.

Figure A.V: Viability and numbers of Saos-2 in alginate gels at days 1 and 5 of culture, as determined by quantification of LIVE/DEAD® stained cells. 2% w/v AlgG + NOVATECH-RGD gels. (Top) Percentage live cells within gels. Effect of day 1 versus day 5 p < 0.001. (Bottom) Total number of cells per image. Five random fields were analysed per gel. Mean ± SD, n = 6.
References


List of Figures

**Figure 1.1: TE approaches.** In traditional TE, cellularised scaffolds are given time to mature *in vitro* before being introduced into the patient, while for *in vivo* TE, the scaffold is implanted directly into the patient, with or without cells. These approaches are currently being applied in therapy for a range of tissues and organs, including those indicated (not an exhaustive list).

**Figure 1.2: Tissue engineering timeline.** TE gained in profile through the nineties, hitting a peak around the turn of the millennium, but several early commercial ventures failed and large-scale private financing was withdrawn. Improved business planning and a sounder scientific base have since propelled TE towards a new era of success (7)(13)(15)(12)(16).

**Figure 2.1: TE scaffolds, and hydrogel crosslinking.** (Top) Hydrogel, fibrous and porous scaffolds are used in TE. Custom morphologies can be created using SFF techniques. (Bottom) Permanent, covalent links between polymer chains can be created by UV irradiation or through the use of reactive groups, with or without initiators, creating ‘chemical’ gels (bottom left). Physical crosslinks are reversible bonds based on a variety of non-covalent interactions, and this approach can be extended through the use of biological agents such as complementary oligonucleotides and oppositely charged peptides. Custom and porous scaffold images are from (31) and (48), respectively.

**Figure 2.2: Cell adhesive hydrogels.** Formation of growth-plate-like structures in RGD-modified alginate hydrogels with co-transplanted osteoblasts and chondrocytes, showing cartilage (left), transition (middle), and bone and marrow space (right) regions. Magnification x200. From ref (72).

**Figure 2.3: Force-dependent activation of TGF-β.** (Left) TGF-β is sequestered in the ECM within a propeptide (black) containing an RGD sequence, to which cell surface integrins can bind. (Middle) Contraction of the actin cytoskeleton against a stiff (> 10 kPa) substrate can cause the propeptide to undergo a conformational change, resulting in TGF-β release. (Right) On a soft (< 5 kPa) substrate, cytoskeletal contraction results in deformation of the matrix rather than the pro-peptide, and the TGF-β is not relinquished. Modified from ref (91). TGF-β latency and activation is discussed in detail in Chapter 4.

**Figure 2.4: Presentation and release of growth factors from TE scaffolds.** Anticlockwise, from top: growth factors within TE scaffolds may be loaded into polymers whose rate of degradation or diffusive properties can be modulated to tailor release rate, and which may be combined into systems releasing multiple factors with distinct kinetics (99)(100). The exposure of cells to different growth factors with time may therefore imitate developmental pathways and healing responses. An alternative to presenting growth factors in soluble form is to bind them to a surface in either random or specific orientations, with the possible use of a spacer molecule (106)(107)(108). Non-covalent associations with matrix
components, particularly glycosaminoglycans (GAGs), can effect slow release and in some cases may potentiate binding to membrane receptors (114)(115). Cell demanded release is based on the presence of protease-sensitive peptide sequences within the growth factor protein (116)(109).

Figure 2.5: Vascularisation of tissue engineering scaffolds. Centre/bottom: new blood vessels form initially by endothelial cell organisation into tubes, which are later stabilised by smooth muscle cells and pericytes. From left: many attempts to induce this process within TE scaffolds have involved the use of vascular endothelial growth factor (VEGF). A low level of sustained release is demanded as burst release results in the formation of unstable, leaky vessels (120). Subsequent release of platelet-derived growth factor-BB (PDGF-BB) helps to recruit smooth muscle cells, which favours vessel maturation (123). Materials strategies for releasing growth factors are indicated in Figure 3. Gene transfer has been used to generate populations of cells that constitutively express either VEGF, or a stabilised variant of hypoxia-inducible factor 1α (HIF-1α). In the latter case, the protein translocates to the nucleus where it initiates a hypoxic response involving the activation of a host of genes and upregulation of angiogenic growth factors (124). Co-culture of different cell types leads to paracrine and membrane interactions that can enhance angiogenesis. Endothelial cells (EC) and pericytes in co-culture increase the production of tissue inhibitor of metalloprotease (TIMP) -2 (EC) and -3 (pericytes), which inhibits tube regression (119).

Figure 2.6: Synthetic mimics of biological structures. Many characteristics of ECM macromolecules have been reproduced in simpler compounds with biologically inspired structures. (a) Certain protein functions, including integrin binding for cell attachment and protease degradability, can be isolated to short amino-acid sequences. These sequences can be combined with synthetic polymers or incorporated into complex peptides to enable cells to attach to or break down the material, respectively (43)(68)(69)(154)(81)(82). (b) Some glycoproteins involved in bone mineralization, such as bone sialoprotein, possess runs of negatively charged amino acids. Peptides that incorporate these sequences, and synthetic polymers with negatively charged chemical groups, can display improved mineral-nucleating activity (149)(149)(150)(155)(156). (c) Growth factor action has been demonstrated in peptides possessing receptor-binding domains; heparin binding sequences may also be included to aid growth factor sequestration (125)(126). Random peptide libraries have allowed the identification of peptides with affinity to particular receptors, and dimerization of these molecules in some cases can improve receptor binding and physiological response (128). Growth factor action is sometimes potentiated through the actions of glycosaminoglycans (GAGs) such as the binding of heparan sulphate to the FGF-1/FGF receptor 2 complex. This specific interaction can be achieved using short heparin oligosaccharides (137). (d) Furthermore, the protein-binding function of ECM GAGs such as chondroitin sulphate can be mimicked by grouping sulphated oligosaccharides by polymerization, by sulphonating natural carbohydrates such as dextran, or by sulphonating synthetic polymers (111)(133)(134)(135)(136)(157).
Figure 3.1: Alginate structure and gelation. (Left) Alginate monomers showing (1,4)-linkages, and polysaccharide fragment (159). G = α-L-guluronate, M = β-D-mannuronate. (Right) Idealised schematic of calcium binding to alginate in egg-box dimers and intra-cluster associated multimers. Zigzag lines represent G blocks, wavy lines represent M and MG blocks, and circles represent calcium (or other divalent /multivalent) ions. Note that in the dimer structure, the ratio of calcium to G residues (and hence COOH groups) is 0.25, whereas in the multimer it is 0.5.

Figure 3.2 Correlation between affinity for 92% G and 92% M alginates and ionic radii of some metal ions. Note that 92% G alginate is mostly composed of G blocks and the 92% M of mostly M blocks. Affinity data is from ref. (40), and ionic radii are from ref. (185).

Figure 3.3: Experimental concept. Alginate solution is crosslinked into a hydrogel by calcium, strontium, and zinc ions. The ions are gradually released as the gel degrades, leading to sustained biological action on encapsulated or nearby cells.

Figure 3.4: Representative stress-strain plot for alginate gels over first 1 mm (~10%) strain. 3G.Ca gel shown. Modulus = 345 KPa, R² = 0.9999.

Figure 3.5: Modulus (E) of alginate hydrogels crosslinked with calcium and strontium over first 1 mm of compressive deformation (~10% strain). Effect of alginate type P < 0.001; Effect of crosslinking ion P = 0.044. Mean ± SD, n ≥ 5.

Figure 3.6: Weight profiles of alginate gels swollen in PBS. a) Calcium-alginate gels, b) Strontium-alginate gels. Note that time is plotted on a log axis. Mean ± SEM, n = 6.

Figure 3.7: Gel lifespan (day of peak swelling) for alginate gels of different compositions. Effect of alginate type P < 0.001; effect of crosslinking ion P < 0.001; interaction between ion and alginate type P < 0.001. Mean ± SD, n = 6.

Figure 3.8: Cumulative strontium release from alginate gels as measured by ICP. Crosses indicate the day of maximum swelling, i.e. the onset of gel degradation.

Figure 3.9: Toxicity of potential crosslinking solutions. Relative total metabolic activity (alamarBlue® fluorescence) of Saos-2 cells 24 hours after 30 minute exposure to potential crosslinking solutions. Total molarity of crosslinking solutions including ZnCl₂ = 100 mM. Effect of zinc p < 0.001. * 1 mM ZnCl₂ versus no zinc p < 0.001. Mean ± SD, n = 6.
Figure 3.10: LDH activity of Saos-2 cells encapsulated in 2% w/v RGD-alginate gels and grown on TCP, relative to day 0 gels. Results for AlgG gels, AlgM gels, and TCP are displayed separately for clarity. Effect of crosslinking ion \( p < 0.001 \); * strontium-containing gels vs non strontium-containing gels at day 7 \( p < 0.001 \). Effect of crosslinking ion \( p = 0.082 \). Effect of alginate type at all time points \( p < 0.001 \). Mean ± SD, \( n = 6 \).

Figure 3.11: AlamarBlue® reduction by Saos-2 cells, encapsulated in 2% w/v RGD-alginate gels and grown on TCP, relative to day 1 TCP (background subtracted). Results for AlgG gels, AlgM gels, and TCP are displayed separately for clarity. Effect of alginate type at 1 day N.S. \( (p = 0.864) \) and 7 days \( p < 0.001 \). For AlgG gels at day 7, effect of Sr versus no Sr \( p < 0.01 \) and effect of ion \( p < 0.05 \). * Sr versus Ca and Zn gels at day 7 \( p < 0.05 \). For AlgM gels at day 7, effect of Sr versus no Sr N.S. \( (p = 0.526) \) and effect of ion N.S. \( (p = 0.129) \). Mean ± SD, \( n = 6 \).

Figure 3.12: LIVE/DEAD® images of clusters of Saos-2 cells within 2% w/v AlgM RGD-alginate gels after 21 days of culture. Live cells appear green and dead cells red. Arrows indicate isolated dead cells, and arrowheads point to clusters of live cells. Fluorescent microscopy; 40x magnification; scale = 200 µm.

Figure 3.13: Gene expression of Saos-2 cells grown on TCP or in 2% w/v AlgM RGD-alginate gels at day 21, relative to day 0 TCP. COL1A1, BSP and RUNX2 shown, as indicated. Mean ± full range of values, \( n \geq 3 \) from two independent experiments. * Effect of TCP versus gels \( p < 0.001 \) for all genes; effect of crosslinking ion: COL1A1 \( p = 0.91 \), BSP \( p = 0.53 \), RUNX2 \( p = 0.26 \).

Figure 3.14: Alkaline phosphatase (ALP) activity per cell of Saos-2 grown on TCP or in 2% w/v AlgM RGD-alginate gels with different crosslinking ions. The dotted line represents baseline (day 0) activity, determined for Saos-2 in Ca100 gels. Mean ± SD, \( n = 6 \). Effect of gels versus TCP \( p < 0.001 \). * Sr100 vs Sr20 and SrZn \( P < 0.05 \); ** Sr100 vs Ca100 and TCP \( P < 0.001 \); * Zn versus Ca100 \( P < 0.05 \).

Figure 4.1: TGF-β signal transduction. TGF-β binding to TβRII leads to phosphorylation of TβRI (Alk5). Phosphorylated TβRI in turn phosphorylates Smad proteins (Smad2 and Smad3). These are thought to associate in a trimer with Smad4, also known as the co-Smad. Further association with transcription factors (TF) and transport through nuclear pores result in transcriptional regulation (57).

Figure 4.2: TGF-β latent complex and ECM association. Latency associated peptide (LAP) and transforming growth factor beta (TGF-β) comprise the small latent complex (SLC). The large latent complex (LLC) additionally includes latent TGF-β binding proteins (LTBPs). Based on a figure from (91).

Figure 4.3: In vitro concentrations of free and latent TGF-β over time. Concentrations of free and latent TGF-β over 48 hours in DMEM on BSA-coated 6-well plates, as determined by ELISA. \( n = 3 \), mean and range of values shown. * Free versus latent TGF-β concentrations \( p = 0.05 \).
Figure 4.4: Signalling response to different concentrations of free and latent TGF-β by human foreskin fibroblasts (HFF-1). Cells were incubated in the presence of free or latent TGF-β at the indicated concentrations for 1 hour prior to fixing. Confocal microscopy; scale = 200 µm.

Figure 4.5: Signalling response by human foreskin fibroblasts (HFF-1) to 10 ng.mL⁻¹ free and latent TGF-β in the presence of inhibitors of TGF-β activation. - = no inhibitor; RGD = soluble RGD peptide; ALMC = aprotinin, leupeptin, Marimastat, CP471474; TGFBR1 = TGF-β receptor inhibitor. Scale = 200 µm.

Figure 4.6: Signalling response by passage 4 bovine articular chondrocytes to 10 ng.mL⁻¹ free and latent TGF-β in the presence of protease inhibitors. (Top) immunostaining for phosphoSmad3. Scale = 200 µm. (Bottom) Western blot membrane showing phosphoSmad3, and total Smad3 after stripping. Abbreviations as per Figure 4.5, plus PIMC = Roche protease inhibitor cocktail, Marimastat, CP471474.

Figure 4.7: Conjugation chemistry. (Top) Traut’s reagent reacts with primary amines (lysines) to introduce thiols to proteins. (Bottom) Co-reacting a protein (LTGF-β shown) with Traut’s reagent and PEGDA causes the SH-LTGF-β to react with PEGDA, producing a PEGylated protein.

Figure 4.8: PEGylation of model proteins. (Top) attempt 1; (Bottom) attempt 2. Unmodified protein (-) and reactions carried out in sodium phosphate buffer (SPB), SPB with 5 mM EDTA (SPEB), or SPB with 100 mM EDTA (SPEB+).

Figure 4.9: Primary sequence of TGF-β1 propeptide. Grey letters correspond to signal peptide (residues 1-29), blue letters to LAP (residues 30-278; 249 amino acids), and black to TGF-β1 (residues 279-390; 112 amino acids). The eighteen lysine residues (K) are highlighted in red, and cysteines (C) in gold. (240)

Figure 4.10: PEGylation of LTGF-β. Positions of LAP and free TGF-β are indicated. LTGF-β was run either alone or after reacting for 1 hour with PEGDA +/- Traut’s reagent (Traut’s). Batches (a-c) are indicated.

Figure 4.11: Release of latent TGF-β from UV photopolymerised PEGDA hydrogels over time. Acid was added at 24 hours (arrow) and concentrations measured by ELISA. LTGF-β was either unmodified, or reacted with PEGDA for 1 hour +/- Traut’s reagent (Traut’s). LTGF-β from the batch c reaction was used. Effect of reaction conditions p < 0.05 from 0.25 - 24 hours. * LTGF-β versus LTGF-β + PEGDA p < 0.05.

Figure 4.12: Signalling response to LTGF-β and PEG-LTGF-β by HFF-1. Cells were exposed to LTGF-β and PEG-LTGF-β (batch b) at the indicated concentrations for 1 hour. + ACID = acid activation of LTGF-β; + Mn²⁺ = 500 µM MnCl₂. Free equivalent concentrations of LTGF-β are shown, without correction for loss or modification of protein during PEGylation reactions. Confocal microscopy; scale = 200 µm.
Figure 4.13: Signalling response to PEG-LTGF-β by human foreskin fibroblasts (HFF-1) and comparison to unmodified LTGF-β at different concentration corrections. HIGH and LOW refer to concentration corrections detailed in Table 4.4 and explained in the accompanying text. Estimated concentrations (free or free equivalent) were 10 ng.ml⁻¹ for all conditions. (Top) Immunostaining for phosphoSmad3, PEG-LTGF-β batch b used. Confocal microscopy; scale = 200 µm. (Bottom) Western blot membranes – two repeats from the same experiment. (a) and (b) refer to PEG-LTGF-β batches. For consistency, the lane order of the top right membrane was altered in Photoshop. Membranes are also shown after being stripped to remove phosphoSmad3 antibody.

Figure 4.14: Signalling response to PEG-LTGF-β by human foreskin fibroblasts (HFF-1) with acid activation and plasmin digestion. Estimated concentrations (free or free equivalent) were 1 ng.ml⁻¹ for all conditions, and the PEG-LTGF-β (batch b) was corrected using the HIGH concentration estimation (Table 4.4). + acid = acid activation; + P90 = plasmin digestion for 90 minutes; + P180 = plasmin digestion for 180 minutes. (Top) Immunostaining for phosphoSmad3. Confocal microscopy; scale = 200 µm. (Bottom) Western blot membranes – two repeats from the same experiment. Membranes are also shown after being stripped to remove phosphoSmad3 antibody.

Figure 4.15: Viability of bovine chondrocytes cultured for 28 days in PEG-HA gels with and without TGF-β. (Top) Total metabolic activity as indicated by alamarBlue® reduction, reflecting cell proliferation. Effect of gel p < 0.001; effect of ‘free TGF-β’ versus ‘no TGF-β’ and ‘PEG-LTGF-β’ p < 0.001; effect of ‘latent TGF-β’ versus ‘no TGF-β’ p = 0.72. *‘no TGF-β’ versus ‘PEG-LTGF-β’ p < 0.05; ** ‘no TGF-β’ versus ‘PEG-LTGF-β’ p < 0.001. (Bottom) LDH release from cultured hydrogels, reflecting cell lysis. Effect of ‘free TGF-β’ versus ‘no TGF-β’ and ‘PEG-LTGF-β’ p < 0.001. Mean ± SD, n = 5.

Figure 4.16: Collagen staining of PEG-HA hydrogels with encapsulated chondrocytes cultured for up to 28 days. (Top) Picrosirius red staining of wax-embedded sections fixed on day 7 of culture. Brightfield microscopy; 10x magnification; scale = 200 µm. (Bottom) Confocal images of hydrogels immunostained with collagen II (green) plus DAPI (blue). 20x magnification; scale = 100 µm.

Figure 4.17: Eosin staining of PEG-HA hydrogels with encapsulated chondrocytes cultured for 21 and 28 days. Brightfield microscopy; 4x and 10x magnification as indicated; scale = 500 µm for 4x magnification, and 200 µm for 10x magnification.

Figure 4.18: DAPI staining of PEG-HA hydrogels with encapsulated chondrocytes cultured for up to 28 days. Fluorescent microscopy; 4x magnification; scale = 500 µm.

Figure 4.19: Macroscopic appearance of PEG-HA hydrogels after 28 days of culture. Some residual colour is left over from the alamarBlue® assay. Membrane diameter = 12 mm.
Figure 4.20: Viability of bovine chondrocytes in PEG-HA gels with and without TGF-β. (Top) Total metabolic activity (alamarBlue® assay) of bovine chondrocytes encapsulated in PEG-HA hydrogels over a 5 week culture period. Effect of gel $p < 0.001$ (all intergroup comparisons $p < 0.001$). * 'PEG-LTG-β' versus 'no TGF-β' $p < 0.01$; ** 'PEG-LTG-β' versus 'no TGF-β' $p < 0.001$. (Bottom) Total LDH activity of chondrocytes cultured in hydrogels for 34 days, reflecting number of live cells. * $p < 0.001$ versus 'no TGF-β', $p < 0.01$ versus 'PEG-LTG-β'. Mean ± SD, $n = 8$ (alamarBlue®), $n = 7$ (LDH).

Figure 4.21: Collagen II staining of PEG-HA hydrogels with encapsulated chondrocytes cultured for up to 34 days. Collagen II = green, DAPI = blue; confocal microscopy; 20x magnification; scale = 100 µm.

Figure 4.22: Collagen I staining of PEG-HA hydrogels with encapsulated chondrocytes cultured for up to 34 days. Collagen I = green, DAPI = blue; confocal microscopy; 20x magnification; scale = 100 µm.

Figure 4.23: Collagen VI staining of PEG-HA hydrogels with encapsulated chondrocytes cultured for up to 34 days. Collagen VI = green, DAPI = blue; 20x magnification; scale = 100 µm.

Figure 4.24: Macroscopic appearance of PEG-HA hydrogels after 34 days of culture. Base (membrane) diameter = 6 mm.

Figure 4.25: Histology of PEG-HA hydrogels with encapsulated chondrocytes cultured for 34 days (wax-embedded sections). Stains are as labelled; brightfield microscopy; 4x and 10x labels indicate magnification; 'zoom' shows close-ups of 4x images; scale = 500 µm for 4x magnification, 200 µm for 10x magnification, and 125 µm for zooms of 4x images.

Figure 4.26: Total protein in PEG-HA hydrogels after 34 days of culture. * $p < 0.01$ versus 'no TGF-β'. Mean ± SD, $n = 7$.

Figure 4.27: LIVE/DEAD® staining of bovine chondrocytes within PEG-HA hydrogels after 34 days of culture. Live cells appear green and dead cells red. Confocal microscopy; scale = 100 µm.

Figure A.I: LDH standard curve results for Saos-2 homogenised in alginate solution and gels.

Figure A.II: UV absorbance spectra of (left) pure RNA and (right) a contaminated preparation. The pure sample has a 260/280 ratio of 2.02 and a 260/230 ratio of 2.06, and the contaminated sample has 260/280 and 260/230 ratios of 2.02 and 0.27 respectively. Note the absorbance maxima at 260 nm (vertical lines). Screenshots are from NanoDrop software.
Figure A.III: Primer validation for BSP. (Top) Raw (cycling) fluorescence for serial dilutions of cDNA, showing intervals of ~3.3 Cts between 10x dilutions. (Middle) Melt curve analysis of samples, showing a single peak (amplicon). (Bottom) Agarose gel electrophoresis of reaction products, showing a single specific band (amplicon). NTC = no template control (water), No RT = no reverse transcriptase control; numbers indicate ten fold dilutions of cDNA.

Figure A.IV: Viability and numbers of Saos-2 in alginate gels at days 1 and 5 of culture, as determined by quantification of LIVE/DEAD® stained cells. 2% and 3% w/v unmodified AlgG gels. (Top) Percentage live cells within gels. Effect of day 1 versus day 5 p < 0.001. (Bottom) Total number of cells per image. Mean ± SD, n = 6. 5 random fields were analysed per gel.

Figure A.V: Viability and numbers of Saos-2 in alginate gels at days 1 and 5 of culture, as determined by quantification of LIVE/DEAD® stained cells. 2% w/v AlgG + NOVATECH-RGD gels. (Top) Percentage live cells within gels. Effect of day 1 versus day 5 p < 0.001. (Bottom) Total number of cells per image. Five random fields were analysed per gel. Mean ± SD, n = 6.
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Table 3.1: Gel formulations used for materials characterisation, including gel codes referred to in the text.

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Table 4.1: Physical and molecular factors involved in latent TGF-β activation. Also shown (in grey) are integrins known to bind, but not necessarily activate, latent TGF-β. Compiled from information in (91), (220) and (230).

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Table 4.4: Relative apparent concentrations of LTGF-β after 1 hour reacting with PEGDA +/- Traut’s reagent. LTGF-β was reacted either with PEGDA or with PEGDA and Traut’s reagent for 1 hour, and the concentrations before and afterwards were read by ELISA (‘Relative amount = concentration after reaction/concentration before reaction’). The inverse of these ratios defines a correction factor for each condition, used to calculate concentrations (denoted HIGH and LOW as indicated) for use in selected experiments in the next section. N/A – batch c was not used in subsequent cell work.

Table A.I: List of common contaminating compounds in RNA preparations and their characteristic absorbances. Guanidine salts are common in RNA isolation buffers, for example guanidine thiocyanate (absorbance at ~260 nm) is a component of the RNeasy buffers RLT and RW1. (255)
Table A.II: Summary of troubleshooting steps for RNA extraction from alginate and PEG hydrogels. (a – above) Troubleshooting steps in gel homogenisation. (b – page 148) Troubleshooting steps in removing gel debris and other contaminants. (c – page 149) Extractions using Trizol reagent. Brief methods and reasons for taking each step are given. Bold typeface highlights the notable points for each attempt. Alg = ungelld alginate; Ca-Alg = Ca100 gel; Sr-Alg = Sr100 gel; PEG700 = photopolymerised PEGDA Mw = 700; PEG4K = photopolymerised PEGDA Mw = 4000. RNA concentration, and 260/280 and 260/230 nm ratios are stated, and an overall judgement on the success of the extraction is indicated in the far right column: ✔ = method has significant potential (typical yield > 20 ng.ml⁻¹, 260/280 ratio ~ 2, 260/230 ratio >> 1); x = RNA does not meet the required standard; ~ = RNA does not meet the required standard, but some samples or parameters show promise. Sources: manufacturer’s information (Trizol, RNeasy) and reference (255).

Table A.III: Standard curve results for qPCR primers. Working range and dilution indicate the Ct and dilution ranges over which the indicated efficiency and R² values were generated.
It amazes me to consider how much I have experienced since I started my PhD in October 2007. Perhaps the biggest surprise was the group itself, which has surpassed all of my expectations. I have Molly Stevens to thank for building this fantastic team and for allowing me to become part of it. Molly has always permitted me the freedom to shape my projects in the ways I have wanted, and I appreciate her willingness in general to hear my opinions. Her confidence in me has been most reassuring and of great practical value. I wish her and her group all the very best for the future and predict great things to come.

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