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A Multi-Modal Collagen Nucleic Acid Delivery System for the Modulation of Inflammation and the Promotion of Angiogenesis

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

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Network of Excellence for Functional Biomaterials

National University of Ireland, Galway

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Abstract

Tissue engineered organs and implants hold promise for the replacement of damaged and diseased organs. However, the foreign body response (FBR) is a major obstacle that compromises the function of tissue engineered constructs, typically causing them to fail. Two components of the FBR are the inflammatory response and a lack of vascularization. To overcome these, this project has delivered anti-inflammatory and pro-angiogenic nucleic acids. A collagen system was developed to release interleukin-6 (IL-6) siRNA and endothelial nitric oxide synthase (eNOS) pDNA in a staggered manner. Collagen hollow microspheres were fabricated using a template-method and displayed an ability to load and release pDNA, maintaining the capability to transfect 3T3 fibroblasts in vitro, while reducing vector-associated toxicity. Following the identification of an optimal microsphere size, hollow collagen microspheres were assembled into a collagen sphere-in-hydrogel system that displayed a staggered release profile in vitro. This system was assessed in vivo in a subcutaneous rat model. The doses of IL-6 siRNA and eNOS pDNA were first individually optimized for their ability to reduce the volume fraction of inflammatory cells (7 days) and increase the length density of blood vessels (14 days), respectively. The identified optimal doses were combined and the ability of the system to decrease the volume fraction of inflammatory cells and increase the length density of blood vessels was confirmed at both 7 and 14 days. Analysis of the tissue using Raman spectroscopy revealed that in addition to changes in inflammation and angiogenesis, there were also changes in the extracellular matrix (ECM) of the tissue at seven days. While changes in sulfated glycosaminoglycans (sGAG) content of the ECM were not detected, changes in the binding of sGAG of the ECM to growth factors was observed. Two growth factors tested, VEGF_165 and bFGF showed increased binding to sGAG extracted from eNOS pDNA treated samples at seven days, increasing the angiogenic potential of the ECM. Overall, the system shows promise for the treatment of pathologies in which inflammation and angiogenesis are dysregulated.
Chapter One

Literature Review

Sections of this chapter have been previously published in:


1.1 Introduction

The long-term goal of engineered tissues is to replace, repair or enhance the function of a damaged or compromised organ. Through the combination of a suitable material (scaffold), cells and biological signals, engineered tissues can be produced that are capable of replacing native tissue both structurally and functionally.[1] However, a major obstacle with regard to tissue-engineered products is the tendency of the host to reject foreign implants. An immune response to the implanted engineered tissue may be due to the scaffold material, the implantation of foreign cells or any incorporated bioactive agent.[2,3] The initial response involves protein adsorption onto the material surface and complement activation. This is followed by infiltration of inflammatory cells such as neutrophils and macrophages. A robust inflammatory response can result in the formation of a fibrous capsule surrounding the implant, isolating it and compromising its function. However, some materials, particularly natural extracellular matrix (ECM)–based implants, cause only a minimal foreign body response. Despite a minimal response, ECM-based materials can still fail to integrate well with the surrounding tissues, and may be degraded by native enzymes or a fibrous capsule may form around them. Implants are prone to failure due to inadequate vascularization. Without blood supply, diffusion can only support an implanted tissue up to a thickness of 2 mm. Thus, any cells incorporated into a tissue engineered graft are starved of oxygen and nutrients, as there is a build-up of metabolic waste. This compromises the function of the newly implanted tissue by reducing the viability of any delivered cells or cells which have infiltrated the scaffold.

Strategies to overcome these drawbacks are a part of current tissue engineering paradigms.[4] These strategies involve the incorporation of appropriate signals to enhance the functionalisation of tissue-engineered scaffolds.[5] These signals are often anti-inflammatory or pro-angiogenic in nature, in an attempt to directly influence the viability of the implanted scaffold. However, a key consideration is not only the nature of the signal, but
also the form in which the signal needs to be delivered. Early attempts at delivering therapeutic proteins have proven to be somewhat unsuccessful due to the short \textit{in vivo} half-life of these proteins. In order to increase the bioactivity of the proteins, biomaterials were used as reservoirs to prolong their release and protect them from degradation. The bioactive protein is released over time through a combination of diffusion from and degradation of the scaffold. The use of nucleic acid delivery to provide a more prolonged supply of bioactive proteins has also become popular. By delivering the gene encoding a particular protein of interest, a more robust supply of therapeutic may be provided through host cells. In addition, in the last decade, RNA interference (RNAi) techniques have entered mainstream usage as therapeutics, allowing for the blockage of translation of a protein of interest. Thus gene therapy offers versatility that is not possible with protein delivery strategies, such that a particular protein may be either up- or downregulated to manipulate the response of the body to a pathological insult or injury, or the implantation of a engineered tissue. Despite this, gene therapy is not without its disadvantages, with viral vectors being associated with toxicity and integration into the host genome, while non-viral vectors have low transfection efficiencies and only transient gene expression. The next sections will detail gene therapy strategies that have been reported to reduce inflammation and induce angiogenesis.

1.2 Gene therapy

Gene therapy holds promise as a molecular medicine strategy to regulate the pathophysiology of disease.[6] Originally devised as a strategy to correct defective genes in patients born with a genetic defect, gene therapy has more recently been used to provide long-term expression of therapeutic proteins in an attempt to overcome the short half-life and associated difficulties with protein therapy. The delivery of \textit{p}DNA encoding therapeutic proteins to cells \textit{in vivo} allows for a sustained release of therapeutics. In addition, more recent strategies involving RNAi to reduce the expression of proteins implicated in the
pathophysiology of disease states have shown promise both as a tool to study disease states and also as a therapy to reduce the impact of disease and induce healing.

The key to the success of any gene therapy approach is to have an efficient means of delivery. The vectors utilized in gene therapy fall into two main categories: viral and non-viral. While viral systems are much more efficient and generate far greater levels of transgene expression than non-viral systems, concerns exist about their safety owing to the fatality of a number of patients enrolled in clinical trials.[7,8] Additionally, difficulties in scale-up, limits on the size of the nucleic acid that can be delivered, mutagenesis and immunogenicity are a concern.[9] As a result, extensive research efforts have focused on the development of more efficient non-viral vectors that overcome the drawbacks associated with viral systems. However, as well as having low transfection efficiencies, non-viral systems may also induce toxicity. The ideal non-viral vector is capable of efficient delivery of genetic material and has no associated toxicity and is biodegradable.[10–15] No current ‘ideal’ system exists with these characteristics. The main classes of non-viral delivery vectors are cationic polymers and cationic liposomes.[10] The concept behind the use of cationic polymers and lipids is that a cationic particle (cationic polymer/liposome + pDNA/siRNA) will adsorb better onto the anionic cell membrane via electrostatic interaction, and hence will be uptaken more efficiently than naked pDNA/siRNA, which typically yields very low levels of transfection in vivo and in vitro. Additionally, by packaging the nucleic acid, its stability is increased and the nucleic acid is protected from degradation by endogenous nucleases. One difficulty in screening suitable vectors for gene therapy is the lack of a definite correlation between in vitro and in vivo efficiencies, and that the cell lines in which vectors are typically assessed on two dimensional tissue culture plastic in vitro behave dramatically differently than cells in the in vivo environment. In addition, non-dividing cells are notoriously difficult to transfect, and, depending on the cell
population present in the micro-environment, this may also be a consideration and a major obstacle to overcome.

1.2.1  pDNA delivery

The delivery of pDNA to a cell is a complex process in which many barriers and obstacles have to be overcome. Cells are typically not amenable to the uptake and integration of foreign genetic material. Thus, a number of strategies are necessary to deliver the pDNA to the cell and induce expression of the protein of interest. In order for a pDNA strategy to be successful, it is necessary for the foreign pDNA to navigate through a number of hurdles.[16] For a start, the pDNA must gain entry to the cell through the phospholipid bilayer. This in itself is difficult as the negative nucleic acid is repelled by the charge on the cell membrane. As a result a vector is required, typically with a neutral or positive charge. This allows for uptake of the pDNA/vector complex via endocytosis. Non-viral vectors are either polymeric or liposomal systems (systems discussed in section 1.3). However, even when a pDNA enters the cell, with or without the help or a vector, it still has a number of steps to overcome before transfection can occur. The pDNA must avoid degradation by late endosomes or lyosomes. Vectors allow this by escaping the endosome mostly via endosomal buffering and the so-called ‘proton sponge’ effect.[17] This theory states that unprotonated amines absorb protons, leading to increased influx of chloride ions (Cl\(^-\)) and water. This results in swelling and rupture of the membrane. Once it has escaped the endosome, it is necessary for the pDNA to dissociate from the vector and enter the nucleus. Not until it has entered the nucleus can pDNA be transcribed and the protein of interest produced. This process is summarised in figure 1.
Figure 1-1: pDNA delivery and transfection of the cell. A cationic vector is required to enable negatively charged pDNA to cross the cell membrane. Escape from the late endosome/lysosome is achieved via the proton-sponge theory, finally allowing for the pDNA to reach the nucleus and transcription.
1.2.2 RNAi

RNAi is a more recent development in the gene therapy field, and offers further opportunities for gene regulation by allowing for the silencing of mRNA.[18–20] MicroRNA (miRNA) are endogenous, short, non-coding RNAs that are formed following processing of long primary miRNAs (pri-miRNAs) by the enzymes Drosha and Dicer.[18] The miRNA is then loaded into the RNA-induced silencing complex (RISC), and the complementary strand cleaved by Ago2. This allows for binding of the mature miRNA to mRNA through RISC and subsequent translational repression and mRNA. Synthetic small interfering RNAs (siRNAs) also utilise this pathway, and this is summarised in figure 1-2. However, there are some key differences between siRNAs and miRNAs. One key difference is in the complementarity of the bases. While synthetic siRNAs match perfectly with the sequence they target, miRNA needs only to match with nucleotides 2 – 7 (the seed region) for inhibition to occur. As a result, miRNA can alter the expression of a number of genes, and also specific genes may be a target for a number of miRNA. In contrast, synthetic siRNAs are designed with perfect complementarity to the target sequence, and thus have only one target mRNA. In addition, since siRNAs are synthetic they can cause an immune response, depending on the dose and RNA sequence. However, it has been shown that the use of collagen as a vector to deliver synthetic siRNAs in vivo prevented immunostimulatory effects, even when the siRNA contained an interferon (IFN)-inducible sequence.[21] In fact, delivery of a monocyte chemoattractant protein-1 (MCP-1) targeting non-chemically modified siRNA using atellocollagen was shown to reduce ear swelling and MCP-1 expression in a contact hyper-sensitivity inflammatory mouse ear model.

1.3 Non-viral gene therapy vector systems

Gene therapy offers a more versatile and prolonged supply of therapeutic proteins compared with protein delivery. Theoretically, the use of a cell’s own machinery to produce a supply of therapeutic proteins by gene therapy is superior to the supply of a finite amount of said
protein. However, the low levels of transfection exhibited by naked pDNA or RNA, both in vitro and in vivo, necessitates the use of a vector to increase transfection efficiencies. The primary function of non-viral gene vectors is to shield the negative charge on the nucleic acid, and thus allow it to cross the cell membrane. In addition, vectors can facilitate endosomal escape and localisation to the cell nucleus, depending on possible functionalisation strategies. The two main classes of non-viral vectors are cationic polymers and liposomes. Cationic polymers and liposomes will be discussed in the following section in the context of the ideal carrier for pDNA and RNAi, weighing up the associated benefits and drawbacks.

1.3.1 Cationic polymers

Cationic polymers condense and package nucleic acids (either pDNA or siRNA) through an electrostatic interaction, forming particles known as polyplexes.[22,23] By binding with the negatively charged nucleic acid, cationic polymers form condensed, positively charged nanoparticles which are more amenable to cellular uptake. Cationic polymer vectors offer a wide range of advantages over their viral counterparts, including ease of large-scale production, ease of functionalisation and relatively low toxicity and immune responses. A number of properties are considered important and related to transfection efficiency. These include amine type (primary, secondary or tertiary) and content. Amines are necessary to complex nucleic acids, with primary amines shown to be most effective for condensing nucleic acids to form positively-charged particles. Other properties which have been shown to have an effect on transfection include molecular weight, structure, buffering capacity, biodegradability and size of the polyplex particle.[16] Chain length has been shown to have an effect on the uptake and cytotoxicity of cationic polymers, [24] while a comparison has been made between linear and knotted polymers in terms of transfection capability and cellular viability.[25] Thus, a range of polymers have been designed with varying combinations of these parameters, and tested in vitro with the most promising candidates.
The siRNA enters the cell and is uptaken by the RNA-induced silencing complex (RISC). The complementary siRNA strand is cleaved, and the programmed RISC then binds to the mRNA target, with translational repression and subsequent occurrence of mRNA degradation.
tested in preclinical models. However, while in many cases these properties can be incorporated into polymers and a resultant increase in transfection observed, this is often combined with an increase in cytotoxicity. Therefore, the design of polymers with efficient transfection capability but also minimal toxicity is a balancing act.

In addition to the properties of the polymer, efforts have been made to increase efficacy of polymer vectors through functionalisation.[26] By designing the polymer with free reactive groups, functionalisation becomes possible. Through these free groups, cell penetrating-peptides, antibodies, glyco-moieties and nuclear localisation sequences (NLS) may be added to further enhance the efficacy of the vector system by increasing internalisation and trafficking of the nucleic acid to the nucleus. In addition, polyethylene glycol (PEG) moieties have been used to increase the circulation life and to avoid immune detection of vector systems that are delivered systemically via the circulation. For example, TAT-peptide conjugated to PEG-Polyethyleimine (PEI) polyplexes resulted in a 600% increase in transfection compared with PEI polyplexes alone delivered to the lung.[27] A reduction was also observed in the total number of cells, polymorphonuclear monocytes (PMNs) and TNF-α present in the bronchial alveolar lavage fluid (BALF) compared with PEI treated lungs. In a similar study, PEI-PEG conjugates were used to deliver siRNA resulting in knockdown of eGFP in the lungs of eGFP positive mice, with no histological abnormalities detected.[28] PEI-PEG conjugates were also used by Debus et al. to deliver mRNA *in vitro*, and were found to outperform both of the two components at the same N:P ratio (ratio of nitrogen in the polymer to phosphates in the nucleic acid). Stable particles were formed of size less than 200 nm with mRNA, which is important as mRNA is very unstable both *in vitro* and *in vivo*. [29]

Superfect® is a sixth generation, partially degraded polyamidoamine (dPAMAM) which has proven useful for drug and gene delivery applications. In particular, the commercially
available dPAMAM has been used *in vitro* for gene delivery due to its ability to bind negatively charged nucleic acids with its high primary amine content. dPAMAM not only increases cellular uptake of nucleic acids by imparting a positive charge, but also improves endosomal escape via a proton-sponge effect, which can be attributed to the presence of tertiary amines in its core. dPAMAM is most often used for *in vitro* transfection applications, but has also been used for gene delivery in preclinical studies in combination with biomaterial delivery systems.[30–32] Despite this, concerns remain about the toxicity of dPAMAM and PAMAM dendrimers in general.[33] Therefore, means to reduce toxicity without negatively affecting the transfection capability of Superfect® are crucial to its progression as a safe gene delivery agent for applications in the clinic. One approach taken to increase the length of transgene expression is to combine polyplexes with biomaterial scaffolds.[34] This approach has been reported in the literature, and will be elaborated on in section 1.5.

### 1.3.2 Liposomes

Liposomes are spherical vesicles which are formed via the self-assembly of lipid bilayers.[35] Liposomes have been used for decades as drug delivery vehicles capable of delivering hydrophilic or hydrophobic therapeutics *in vivo*. Despite the upsurge in the availability of cationic polymers for gene delivery, liposomes have remained in widespread use due to a number of favourable characteristics such as relative safety, high-loading capacity, ease of large-scale production, versatility and ease of functionalisation.[35] As with cationic polymers, functionalisation is a key aspect to increase uptake and nuclear localisation of nucleic acids, as well as to avoid clearance by immune cells. As with cationic polymers, this can be achieved by functionalisation with various antibody fragments, cell-penetrating peptides, glyco-moieties and NLS, as well as PEG conjugation. Lipoplexes conjugated with an anti-myosin antibody and TAT-peptide displayed preferential accumulation in the ischemic myocardium, demonstrating an ability to target lipoplexes to
specific regions. Addition of a cyclic RGD ligand specific to tumour endothelial cells to a liposomal siRNA delivery system resulted in reduced angiogenesis and tumour volume when used to deliver an siRNA targeting vascular endothelial growth factor (VEGF).[36]

Another key aspect of liposomes is that external stimuli may be used to trigger release of the therapeutic. One such stimuli is ultrasound. Using a combination of microbubbles and PEI, Chen et al. were able to localize pDNA to the heart and then cause destruction of the microbubble by ultrasound, allowing for release of the PEI/pDNA polyplexes and subsequent transfection.[37] This added functionality makes liposomal delivery appealing for therapies which are to be delivered systemically and have a particular target organ or tissue.

Dharmafect® is a commercially available liposomal transfection reagent which has been developed specifically for the intracellular delivery of small RNA molecules such as siRNA and miRNA. It is typically used for in vitro applications, and has a number of different formulations optimized for RNA delivery to specific cell types. Dharmafect® has also been used in vivo, specifically to knockdown expression of SPARC (secreted protein, acidic and rich in cysteine), which has been implicated in fibrosis of the skin and lungs. Delivery of SPARC siRNA with Dharmafect® significantly reduced the expression of collagen I and III in a bleomycin-induced model of fibrosis.[38]

Liposomes are also amenable to local delivery through the use of biomaterials as reservoirs.[39–41] Examples include the attachment of lipoplexes to stents to enhance re-endothelialization following deployment.[42] By providing a continuous supply of lipoplexes, from a stent in this case, the length of gene expression has been reported to increase, and thereby the therapeutic value of the treatment amplified.
1.4 Biomaterials for tissue engineering

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences to produce biologically active tissue replacements to maintain, restore or replace diseased tissue.[1] However, the success of tissue engineering strategies depends on the identification of a suitable biomaterial. Without a suitable biomaterial, tissue engineering applications are unlikely to be therapeutically and clinically efficacious. Hence, the choice of a biomaterial and the form it takes is of vital importance. Synthetic materials have many desirable qualities among which are control over material properties, ease and consistency of synthesis and options for functionalization. However, synthetic materials often suffer from \textit{in vivo} toxicity and poor control over biodegradation. Natural biomaterials may be either ECM-derived or natural biopolymers, such as chitosan or alginate, which are derived from crustaceans and seaweed, respectively. ECM-based scaffolds provide an ideal substrate for tissue engineering applications. Through their inherent bioactivity, molecular recognition and biodegradability, they provide an ideal scaffold which cells recognize, infiltrate and remodel, creating local microenvironments and gradients of chemotactic activity to drive neotissue formation. Examples of both natural and synthetic scaffold materials that have been used for tissue engineering application are discussed in the subsequent sections.

1.4.1 Natural biomaterials

Natural biomaterials encompass a wide range of materials including native ECM constituents (collagen, elastin, fibrin, glycosaminoglycans (GAGs) etc.), decellularised ECM and natural biopolymers such as alginate, chitosan and keratin. Natural ECM-based materials are ideal substrates for cell adhesion, remodeling and degradation, with no risk of toxic degradation products. However, the wide variety of material properties available with synthetic materials is not possible, and functionalization can be more difficult. Furthermore, other aspects to consider for any therapy that is to be translated clinically are ease of
sterilization, batch-to-batch variability and, in the case of allogenic and xenogenic-derived materials, disease transmission and allergic reactions.

Collagen is the most abundant protein of the ECM in the human body and thus is an ideal candidate material for tissue engineering applications. Collagen provides structural support and tensile strength to various tissues of the human body, including skin, ligaments, tendon, and bone. It consists of a right-handed triple helical structure with the repeating amino acid triplet sequence: Glycine-X-Y, where X is often proline and Y is frequently hydroxyproline. The presence of the small amino acid Glycine at every third position on the polypeptide chain allows for close packing of the triple-helix along the central axis.[43] Non-helical, globular regions exist at either end of the collagen molecule, and prevent fibril formation prior to cleavage by appropriate proteinases. The properties that have contributed to its popularity as a scaffold material include its relatively low toxicity, natural degradation by matrix metalloproteinases (MMPs), ease of crosslinking and functionalization as well as the homology of the amino acid sequences that exist between species.

The use of xenogenic collagen in the fields of biomaterials, drug delivery and tissue engineering is widespread.[44–49] Much research has focused on the formulation of collagen into a wide variety of different forms. Preclinical studies have been reported for many forms of collagen and some have been used clinically. Collagen sponges have a wide range of applications varying from skin wound healing[50,51] to bone[30,52–56] and cardiac repair.[32,57] Collagen hydrogels, due to their injectable nature and in situ self-assembly, have also been used for a variety of applications including regeneration of the myocardium,[58,59] intervertebral disc,[60,61] as a dermal substitute,[62] for corneal applications[63] and for regenerating cartilage.[64,65] Aligned forms of collagen have been developed and utilised to act as a bridge for tendon,[66–68] spinal cord[69] and peripheral nerve regeneration.[70] Collagen films have primarily been used for wound healing[71] as
they create a barrier between the wound and the external environment, thus reducing the possibility of infection. Collagen can be functionalised to add additional therapeutic benefit and bioactivity. For example, sponges and hydrogels can be functionalised with therapeutic genes[30,56,72] or proteins,[51,53] or used to deliver stem cells[31,32,59] whilst collagen microspheres not only have high capacity for loading but also offer localised and sustained delivery of bioactive / therapeutic molecules such as genes,[73] proteins[74] or stem cells.[65,75]

Despite the use of collagen in vitro, in pre-clinical models and in the clinic, there are a number of issues that continue to cause concern. One major obstacle to the clinical translation of these therapies is the source of the collagen. As figure 1-3 outlines, the main sources of collagen type I are porcine skin, bovine tendon and human cadavers. Unfortunately, each of these sources has drawbacks associated with its use. The major drawbacks are possible disease transmission, allergic reactions[76], contamination with pathogens as well as antigenicity and immunogenicity associated with the use of collagen telopeptides, although substantial evidence to prove this is lacking.[77] In addition, batch-to-batch variability as well as cultural considerations add to the concern on animal-derived collagen. As a result, much effort has been made in the field of recombinant protein production[78] to utilize expression systems that will allow the formation of recombinant human collagen in a consistent, efficient and safe manner.

Recombinant collagen has been developed in a number of different expression systems, including yeast, silkworm, mammalian cells, transgenic animals and bacterial systems. Recent developments have seen the production of post-translationally hydroxylated collagen in relatively large amounts using a transgenic tobacco plant.[79] This type of collagen is likely to bypass the concerns that exist with animal-derived collagen. Recombinant collagens have been used in bone[55], ocular[80,81] and skin applications. They have been
used mostly as sponges, but also more recently as electrospun fibres and gels, and have been compared favourably with xenogenic collagens with respect to processability and therapeutic efficacy.[82] Despite these promising signs, recombinant technologies have suffered from a number of drawbacks which include high cost, low yield and the lack of co-factors or enzymes in the systems which are critical to the stable formation of bioactive and biofunctional collagens. Because of these disadvantages, conveniently extracted xenogenic collagen has remained the standard for use both in research and clinically.

Gelatin has been used in tissue engineering as a scaffold material. It is denatured collagen and consists of polypeptide fragments of various sizes, ranging from 16 to 150 kDA. It has proven popular in the fields of tissue engineering, regenerative medicine and drug delivery owing to its similarity to collagen. That is, its favourable tissue response, ability to promote cellular infiltration and biodegradability. However, due to the destruction of the α-chains and disruption of the helical structure during hydrolysis, it degrades quicker and has less mechanical strength in comparison with collagen. These properties make it less appealing than collagen, but nonetheless numerous studies have shown its worth as a biomaterial scaffold. Forms of gelatin have proven capable as scaffolds for engineering adipose tissue[83] articular cartilage[84,85] and dermal tissue,[86] as well as for drug delivery.[87,88] In an attempt to overcome issues associated with rapid degradation, a number of crosslinkers have been used in conjunction with gelatin, including glutaraldehyde[89] and genipin.[90] In addition to being suitable for processing into scaffolds, gelatin has also been processed into microspheres, further emphasising its usefulness for drug delivery[89,91–93] as well as for cell delivery.[94,95] Gelatin is often used as a coating for medical devices to increase cell adhesion and to improve the tissue response to implants. It is also used in composite scaffolds for these reasons, and as a coating for tissue culture plastic in vitro to increase adherence of certain cell lines.
Despite the popularity of collagen and gelatin as a biomaterial for tissue engineering, a wide range of other natural materials have also been used. Of these, fibrin has been utilised in a wide range of tissue engineering applications, including wound healing and regeneration of the myocardium.[96–98] One aspect that has made fibrin particularly appealing is that fibrin glues are FDA approved and are commercially available e.g. Tissell™ and Evicel®. Fibrin glue delivery to the post-infarcted myocardium has been shown to preserve wall thickness, either with or without the inclusion of skeletal myoblasts,[99] while its angiogenic nature has been previously established.[100] Fibrin has shown an ability to bind and modulate the release of biomolecules.[101,102] Its angiogenic capacity has been increased by functionalization of fibrin with pDNA encoding pleiotrophin. In this case, pleiotrophin pDNA in fibrin resulted in increased angiogenesis compared with either fibrin alone or pleiotrophin pDNA alone.[103] It has been shown that using fibrin as a reservoir system for endothelial nitric oxide synthase (eNOS) can enhance its angiogenic capacity, using both viral[104] and non-viral gene therapy vectors.[39] By incorporating a peptide with a C-terminal heparin binding domain, Sakiyama-Elbert et al. allowed for the controlled release of heparin-binding growth factors, in this case bFGF.[105] This system functionalised with neural growth factors has subsequently been shown to increase neurite outgrowth in vitro[106] and in an in vivo spinal cord model[107] relative to unmodified fibrin, as well as to increase endothelialisation in vitro when VEGF is incorporated. [108] Fibrin has also been used for cartilage regeneration, both as a therapeutic and for model development.[109,110]

Hyaluronan (HA) is a negatively charged GAG. It is one of the main components of the ECM. HA is unique amongst GAGs in that it is not sulphated. HA has been implicated in water homeostasis and as a lubricant and shock absorber in joints such as
Figure 1-3: **Sources of collagen and forms into which extracted collagen can be processed for tissue engineering.** Collagen can be extracted from bovine sources, porcine sources, human cadavers or genetically altered expression systems such as plants. Following extraction it may be processed into various forms for tissue engineering, including spheres, gels, sponges, films and fibres.
the knee and the intervertebral disc. The molecular weight of HA can vary greatly, and this can alter its biological activity. Smaller molecular weight HA chains exhibit pro-inflammatory properties while large molecular weight HA chains are anti-inflammatory in nature. [111,112] Extremely high molecular weight HA has even been implicated in the cancer resistance of the naked mole rat, a small, long-living rodent that is remarkably cancer resistant.[113] Like collagen, due to the fact that it is a major component of the ECM, HA has been used as a tissue engineering scaffold. In particular, it has been widely used to repair cartilage[114–116] and the intervertebral disc[117,118] because of the relatively large presence of HA in these tissues. HA too is often used in conjunction with other materials for these applications, such as collagen type II[60,61] and chitosan.[119,120] HA has been used as a scaffold for adipose tissue engineering,[121] and as a delivery system for mesenchymal stem cells (MSCs) to the infarcted heart bringing associated benefits such as reduced inflammation and fibrosis and increased perfusion[122,123], while HA microspheres have been fabricated for drug delivery.[124]

Decellularized matrices are widely used in tissue engineering and regenerative medicine. The rationale for the use of ECM for a particular tissue is due to the specificity of the matrix for that tissue, and that it contains signals that can help that specific tissue to regenerate. This approach has been used to engineer heart valves, where the scaffolds retain the structure of the native heart valve following decellularization.[125–127] In the case of the heart, decellularized myocardium can be formed into a gel and has been reported to be injected into the infarcted myocardium of a donor.[128–130] Generally, the tissue forming the implant is explanted from animals or cadavers, treated with various detergents and other agents to remove cells and DNA, then implanted into the host. The correct decellularization procedure is vital to the success of any implant, because inadequate treatment can result in an immune response.[131] This can prove catastrophic for the implant and the host. Scaffolds may also be populated with cells from the host directly prior to implantation or
cultured \textit{in vitro} for a set period prior to implantation, as with the heart.\cite{132} However, tissues can also be taken from one site in the body of a donor, decellularized and implanted into a different location in the host. Scaffolds have been produced from cholecyst-derived ECM,\cite{133,134} urinary bladder ECM,\cite{135} small intestine ECM\cite{136} and placental ECM\cite{137} amongst other sources. However, concerns remain with this type of biological scaffold, particularly that of disease transmission from different species, while the use of human-derived scaffolds also poses this risk, as well as still being reliant on donors.

Alginate is an anionic polysaccharide derived from brown algae. It forms a gel in the presence of calcium ions (Ca$^{2+}$). This property, along with its relative \textit{in vitro} cytocompatibility, has led to its use as a hydrogel in the field of tissue engineering.\cite{138} Due to its inert nature, alginate has been used to immobilise islet cells as a therapy for diabetes. In combination with anti-inflammatory drugs, alginate improved the functionality of the transplanted islet cells in a diabetic mouse model.\cite{139} Alginate has also been used in adipose,\cite{140} bone,\cite{141} and cartilage tissue engineering.\cite{142} Despite its extensive use in tissue engineering research, cells do not readily attach to alginate scaffolds, and must be physically entrapped. Unlike native ECM components, alginate does not contain binding sites for cells. But with the more recent paradigm shift towards more interactive scaffolds, this lack of cell adhesion domains has become a negative feature. In order to overcome this, RGD sequences are often engineered into alginate systems to increase cell attachment and infiltration.\cite{143–145} Alginate systems have also been functionalised with both proteins\cite{146} and nucleic acids.\cite{147} A note of caution must also be attached to the use of alginate in that it has been shown that alginate has a similar effect on a macrophage cell line (RAW264.7 cells) as lipopolysaccharide (LPS), such as pro-inflammatory cytokine production and activation of NF-κβ.\cite{148} While \textit{in vitro} results do not always correlate with what is seen \textit{in vivo}, this activation of inflammatory cells must be an important consideration when selecting an appropriate biomaterial for \textit{in vivo} applications.
Chitosan is a linear polysaccharide derived from chitin, which is found in the shells of crustaceans. It is commercially used in bandages due to its clotting ability and its antibacterial properties.[149] Chitosan has been used preclinically for applications related to neural,[150] bone[151] and tissue engineering of the intestines.[152] It has been used to deliver stem cells to the kidneys[153] and also to the ischemic limb[154] and myocardium.[155] In the ischemic myocardium in particular, the radical oxygen species (ROS) scavenging properties of chitosan are useful in increasing the survival and engraftment of implanted stem cells.

A considerable range of natural biomaterials are available for use as biomaterial scaffolds for tissue engineering. However, materials which are naturally present as components of the ECM stand out as useful owing to their biodegradation by native enzymes, and in particular for the tendency of host cells to populate and re-model them. The fact that minimal functional changes are required of the material to assemble them as scaffolds that are amenable to implantation, with the exception of crosslinking which is necessary to prolong lifetime in vivo, makes ECM-based materials attractive from a translational perspective.

1.4.2 Synthetic biomaterials

Synthetic materials offer a considerable range of material properties as well as the ability to tune and modulate properties dependent on requirements. Where they traditionally fall short is in a lack of tissue integration and the generation of toxic degradation products. Despite these drawbacks, the versatility amongst synthetic polymer materials allows for a wide range of utilization in tissue engineering. Additionally, attempts have also been made to develop synthetic materials that mimic the natural ECM by incorporating bioactive ligands necessary to recapitulate developmental processes and drive neotissue formation, as well as MMP cleavable sites to ensure timely in vivo degradation.[156] Therapeutic molecules may also be
easily tethered and encapsulated within synthetic materials, thus acting as reservoirs to prolong the release of therapeutics.

PEG is a widely used synthetic biomaterial due to its relatively inert nature. It forms a hydrogel at body temperature and pH without the need for an initiator and produces no byproducts, emphasizing its inert nature. However, this inert nature is detrimental for cell infiltration. To counter this, a number of features have been engineered into PEG hydrogels to modulate their interaction with cells and tissues. These include ligands for cell attachment, MMP-cleavable domains and binding domains for growth factors. Modified PEG scaffolds have been used in many different applications including in the myocardium,[157,158] tendon[159] and cartilage[160], while it has also been used for gene delivery.[161,162] Reports in the literature indicate that functionalization of a PEG hydrogel with an RGD sequence results in an improved foreign body response (FBR) as there is a reduction in the expression of pro-inflammatory markers.[163]

Polyglycolic acid (PGA) is also widely used in tissue engineering applications, due in part to its biodegradable nature. It is FDA approved for use as resorbable sutures. It has been reported for its use in the engineering of cartilage tissue,[164] along with a number of other applications such as in the myocardium. The use of a PGA scaffold to deliver embryonic stem cells to a mouse infarct model improved blood pressure and ventricular function eight weeks after induction of myocardial infarction (MI).[165] It was notable that implantation of the scaffold alone did not improve survival or ventricular function, suggesting that the scaffold was simply acting as a vehicle to deliver the cells. However, a cells alone injection was not included in the study design, and hence it is difficult to determine whether the combined effect of the scaffold and the cells was greater than the effect that an injection of cells alone. A note of caution with regard to the use of PGA as a material for implants is necessary however, with studies revealing that the degradation products of PGA can cause
inflammation. Glycolide, a metabolite produced during the hydrolysis of PGA, can cause activation of the classical complement inflammatory pathway.[166]

Poly(D,L-lactic-co-glycolic acid) (PLGA) has been used as a controlled release device in vivo due to its biodegradable properties. Scaffolds fabricated from PLGA were assessed in vivo and compared with a porcine small intestinal submucosa (SIS) scaffold.[167] It was found to induce a much greater inflammatory response at 14 days than the SIS scaffold, but a scaffold composed of a mixture of PLGA and SIS elicited an intermediate inflammatory response, indicating potential strategies to minimize the response of PLGA-based scaffolds. In another study, incorporation of SDF-1α into a PLGA scaffold reduced the inflammatory response and increased angiogenesis and stem cell recruitment following implantation in a subcutaneous model.[168] PLGA microspheres may also be fabricated and have proven popular for drug delivery, with both nucleic acids[169] and proteins[170] delivered.

Polycaprolactone (PCL) is a biodegradable polyester which degrades via hydrolysis of its ester linkages. It can be formed into nanofibrous meshes via electrospinning, and has been used as a scaffold in cartilage tissue engineering[171] as well as in nerve[172] and cardiac tissue engineering. To enhance its suitability for cell attachment PCL is often coated with ECM-based materials. In one such study, type II collagen and chondroitin sulfate were grafted onto a porous PCL scaffold.[173] This resulted in increased adhesion and proliferation of seeded chondrocytes, as well as reduced toxicity, and an increase in type II collagen and GAG secretion. PCL is often used in combination with other materials to form composite scaffolds. For example, PCL is deemed to have inadequate mechanical properties for bone tissue engineering, so it is often combined with a stiff material such as β-tricalcium phosphate (β-TCP) to increase the overall mechanical strength of a bone scaffold.[174]

Poly(lactide-co-e-caprolactone) (PLCL) is a biodegradable and elastic synthetic polymer. Because of these properties, it has proven particularly popular in the tissue engineering of
blood vessels.[175] Other applications for which PLCL has been investigated include bone[176] and cartilage.[177] However, in the case of cartilage, PLCL was not deemed suitable. While PLCL promoted the attachment and proliferation of chondrocytes, it also resulted in an increased expression of genes associated with cartilage destruction and ossification in vitro.[177]

A wide variety of synthetic, polymeric biomaterials have been developed and tested for tissue engineering applications. Given the great variety of tissues for which these polymers are required, no consensus exists over which material is best. Each material has advantages and disadvantages associated with its use. As yet, no ideal material is currently available that can adequately promote tissue regeneration, and degrade at the appropriate rate to be replaced by new tissue, while at the same time not producing any toxic degradation products. A further characteristic important in load-bearing applications is how the mechanical properties vary over time as the scaffold degrades. This is particularly important in applications such as bone, heart valve and myocardial tissue engineering, amongst others.

1.4.3 The foreign body response (FBR)

Whether the implanted biomaterial is synthetic or naturally derived, the body will always react to the implantation of a foreign material. This is known as the foreign body response (FBR), and is the host response to the implantation of a foreign object. While the body will react to the implantation of every material, resolution of the response depends on the material properties.[2,178,179]

Immediately following implantation, a biomaterial acquires a layer of host proteins which is associated with the surface chemistry of the material. This occurs before any interaction with host cells, and governs the type of cells that interact and their phenotypes. Material properties and particularly surface chemistry have an effect on protein deposition on the surface. The proteins that typically absorb on the surface include fibrinogen, albumin and
fibronectin. These proteins form a provisional matrix composed mostly of fibrin around the implant. This matrix acts like a thrombus and initiates a wound healing-like inflammatory response. Inflammatory cells such as neutrophils and macrophages are attracted by the build-up of chemokines and other chemoattractants in the provisional matrix. In addition, non-specific attachment of antibodies activates the complement system. The presence of inflammatory cells amplifies the inflammatory signal and increases the accumulation of inflammatory cells at the site of the implant. There is a resultant increase in the expression of pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α).[180] An angiogenic response also occurs to facilitate efficient transport of cells and nutrients towards the implant. This angiogenic response is stimulated by both the fibrin deposition, as fibrin is a potent angiogenic factor, as well as by various angiogenic factors released by inflammatory cells in the vicinity of the implant. These include VEGF, fibroblast growth factor (FGF) and platelet derived growth factor (PDGF). Macrophages in particular have been implicated in the vascularization of implanted tissue engineered scaffolds, with the degree of angiogenesis associated with the phenotype.[181]

The site of implantation is a further consideration with regard to the FBR. The difference in response between a collagen disc implanted subcutaneously and supra-epicardially has been studied.[182] It was shown that there was an increase in pro-inflammatory cytokine expression supra-epicardially compared with subcutaneously, while there was also an increase in the influx of inflammatory cells. Furthermore, it has been shown that while the FBR does not differ so much between strains of a particular species, differences do exist between species. The difference in FBR between rat and mouse following implantation of a crosslinked collagen sponge was revealed, with less capsule formation and slower cellular infiltration in the mouse compared with the rat.[183]
Throughout the FBR, there is a change in the ECM of the surrounding matrix. What begins as a mostly fibrin-based matrix changes over time with various types of collagens, laminins, elastins as well as proteoglycans and GAGs being present. This matrix is continuously remodelled by fibroblasts and inflammatory cells expressing MMPs. A balance exists between the level of MMPs and the factors that suppress them, tissue inhibitors of matrix metalloproteinases (TIMPs). In the case of degradable materials, the response continues until the scaffold has been degraded and is replaced by host tissue. However, in the case of a non-degradable, or slowly degradable materials, a capsule will form around the implant, isolating it from the surrounding tissue. Activated fibroblasts respond to the milieu of inflammatory signals and change the matrix over time. As the inflammatory signals subside, and the expression of transforming growth factor-β (TGF-β) increases, there is a shift in the balance of MMPs:TIMPs, with a tendency towards increased TIMP activity and reduced MMPs. This leads to the formation of a matrix rich in collagen type I and type III, along with fibronectin and proteoglycans, characteristic of fibrosis. Thus, a fibrotic capsule is formed around the implant. This is a major barrier that impedes the function of tissue engineered constructs. Such impedance in function is also seen in implanted devices such as glucose sensors[184] and neuroelectrodes.[185] Thus strategies to minimize the FBR and reduce its impact on implants, as well as increase the integration of implants are of significant importance to tissue engineered constructs and medical devices.

1.5 Multi-modal delivery from biomaterials

Wound healing is driven by various factors that are present at distinct time-points in the regeneration process.[186–188] Following tissue injury and after the formation of a clot, there is an initial inflammatory phase, characterized by the presence of cells such as pro-inflammatory macrophages and neutrophils. Subsequently, there is an increased production of radical oxygen species (ROS) and MMPs.[189] Gradually, this inflammatory response subsides and is replaced by a proliferative phase during which increased angiogenesis and
extracellular matrix (ECM) deposition occurs, and there is a shift in macrophage phenotype from a pro-inflammatory to a more regulatory phenotype. There is also a reduction in capillary formation as the wound enters the remodeling phase, with constant re-organization of the matrix through the activity of fibroblasts, MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs). It is apparent that biological phenomena such as angiogenesis, inflammation and tissue remodeling are complex and are interlinked processes that are controlled in a spatiotemporal manner. That is, the appearance of various factors in the local microenvironment is carefully orchestrated and follows a defined path. Thus, to assume that these processes can be manipulated and re-capitulated by delivery of a single factor is to oversimplify complex biology. In fact, this phenomenon explains the failure of single factor delivery systems in the clinic. For instance, delivery of VEGF alone often leads to the formation of immature leaky blood vessels characteristic of tumours.[190] In addition, these vessels are often prone to regression over time, compromising the perfusion of the tissue. In comparison, systems that deliver multiple growth factors have led to increased maturation of newly formed vessels. [191] It is evident, therefore, that there is a clear need to engineer biomaterials that can control the release of factors over time, in an attempt to control processes such as these. It is conceivable to design materials capable of controlling the release of multiple factors such that there is a differential release of specific factors over time and can mimic the spatiotemporal nature of biological processes.

1.5.1 The need for multi-modal delivery

The use of single factor delivery to treat various pathological and disease states thus far has proven insufficient from a clinical standpoint. This is hardly surprising given the complexity of many disease states. For instance, if one examines the normal wound healing response, there is an initial inflammatory phase, followed by a proliferative phase and finally the remodeling phase. During compromised wound healing, such as with diabetic wounds, there is dysregulation in all of the phases.[192] In addition, with the reduced endothelial nitric
oxide synthase (eNOS) activity and nitric oxide (NO) availability due to the increased production of ROS, there is diminished angiogenesis, and issues with inflammation as well as changes in ECM composition.[193,194] When designing a therapy, one has to carefully consider the target pathological stage, and determine the primary cause of impaired wound healing. In the case of diabetes, this would mean deciding whether to target the chronic inflammatory state, attempt to reduce ROS expression, try to overcome the lack of sufficient angiogenesis or attempt to modulate the changes in ECM composition. However, it is apparent that in order to deal adequately with the pathological state, a multi-faceted approach is necessary. This is true of not only diabetes but also of a number of other conditions such as critical limb ischemia (CLI) and myocardial infarction (MI). But the delivery of multiple factors with antagonistic functions at one time can complicate the process, given that processes such as inflammation and angiogenesis are so intricately and closely linked that they follow a very distinct time-course. Thus not only is the delivery of multiple factors necessary but it must be controlled in a temporal manner, allowing for changes from one phase of the pathological condition to the next. For this reason, biomaterials that can control the timing of delivery of multiple factors relative to each other have therapeutic value.

In addition to their use as therapeutic delivery systems, biomaterials that can modulate the temporal release of multiple factors are of biological significance. Using biomaterials, which can impart control over the temporal release of factors, are useful tools that permit an investigation of cross-talk between multiple factors. For example, scaffolds with these multi-modal release properties can be used to study interactions between various angiogenic factors to determine how mature and stable blood vessels are formed in all four dimensions. By utilizing these materials in a systematic way, the mechanisms behind complex processes may be elucidated, and more relevant and efficacious therapies developed.
1.5.2 Biomaterial strategies to achieve multi-modal delivery

The current paradigm in tissue engineering is the use of biomaterials as reservoirs to extend the release of therapeutics. These strategies involve the use of protein therapeutics, nucleic acids or drugs. However, from a biomaterials standpoint, the parameters of significance are the physico-chemical properties of the therapeutic, and its optimal combination with a biomaterial. Typically, therapeutics may simply be loaded and physically entrapped within scaffolds.

The therapeutic is subsequently released over time through a combination of diffusion and degradation of the scaffold. This has proven moderately successful with both natural and synthetically-derived scaffolds. However, especially for synthetic scaffolds, there is no significant control over the release of the therapeutic given that the degradation is not enzymatically driven. The only control is that of the concentration and physical form of the material. With natural materials, enzymatic degradation will trigger release of loaded therapeutics. However, this property can be engineered into synthetic scaffolds.[195,196] In addition to enzyme-mediated degradation, binding sites for therapeutics may be engineered into material systems.[105,106] Another possibility is the modification of the therapeutic or the use of a variant such that it has a sequence that will bind to the natural scaffold. This has been shown with VEGF and fibrin matrices previously.[197] While these strategies offer control over release of a single-loaded therapeutic, alternative strategies are necessary to control the release of multiple factors in a temporal and controlled manner in an attempt to re-capitulate natural developmental and regenerative processes.

1.5.2.1 Scaffolds and hydrogels

Biomaterials in the form of porous scaffolds and hydrogels are the most common scaffolds used in tissue engineering. Typically therapeutics are physically incorporated into the biomaterial either during or after the fabrication process. Delivery of VEGF and FGF-2 from
a PLGA scaffold (or bridge) has been reported to promote angiogenesis in a rat spinal cord hemi-section model to treat the ischemia associated with the injury.[198] Dual delivery of the growth factors resulted in an increase in endothelial cell infiltration and blood vessel formation. This resulted in a trend towards increased neurite ingrowth into the implanted bridge. Thus, delivery of factors to promote angiogenesis may help to overcome the ischemic environment that negatively affects regeneration. However, in this study the use of single factor delivery was not used as a control, and thus it is impossible to say definitively that multiple factor delivery is more powerful than either single factor delivery, or to interpret the interaction between the factors as a function of release from the biomaterial.

Layered PLG scaffolds containing distinct regions of VEGF and anti-VEGF were constructed to create spatially restricted angiogenic regions.[199] It was observed that increased angiogenesis occurred in the VEGF layers while minimum angiogenesis was observed in the anti-VEGF layers, as the anti-VEGF blocked the activity of the VEGF as it diffused away from the VEGF layer. The layered scaffold was assessed in an ischemic limb model and it was observed that scaffolds containing both the VEGF and anti-VEGF layers produced greater perfusion of the limb than the blank scaffold. The importance of this study is that it spatially restricts the activity of a factor by delivering an antagonist to restrict its zone of activity. The region of action can be increased or decreased by altering the doses of VEGF and anti-VEGF, respectively.

An alginate affinity-based system was utilized to deliver insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF) in a sequential manner to the infarcted myocardium.[200] This was found to increase formation of blood vessels and reduce apoptosis in the myocardium, with reduced fibrosis also being apparent. While this study illustrated the potential advantages of a delivery system compared with a saline injection, it is difficult to decipher whether either growth factor delivered via a biomaterial will be
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<td>Poly(propylene fumarate) (PFF) and gelatin microparticles</td>
<td>VEGF and BMP-2</td>
<td>12 μg VEGF and 2 μgBMP-2</td>
<td>Bone formation</td>
<td>n/a</td>
<td>Rat critical-sized</td>
<td>Increased bone volume</td>
<td>66</td>
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<td>bone defect</td>
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<td>PFF and gelatin microparticles</td>
<td>VEGF and BMP-2 [dose study]</td>
<td>VEGF dose of 0, 6 or 12 μg and BMP-2 dose of 0, 6 or 12 μg</td>
<td>Bone formation</td>
<td>Release profile – 90% VEGF released at 5 days, 40% BMP-2 released at 25 days (both in collagenase buffer)</td>
<td>Rat critical-sized</td>
<td>Increased bone volume as BMP-2 dose was reduced, minimal effect of VEGF dose</td>
<td>67</td>
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<td>bone defect</td>
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<tr>
<td>PLGA microspheres and PPF scaffold surrounded by a gelatin hydrogel</td>
<td>VEGF and BMP-2</td>
<td>2 μ VEGF and 9.2 μg BMP-2</td>
<td>Bone formation</td>
<td>Release profile – 90% VEGF, 20% BMP-2 at 7 days</td>
<td>Rat subcutaneous and critical sized bone defect</td>
<td>Increased blood vessel volume and bone volume (subcutaneous model)</td>
<td>68</td>
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<td>Brushite-chitosan</td>
<td>PDGF and VEGF</td>
<td>250 ng PDGF and 350 ng VEGF</td>
<td>Bone formation</td>
<td>Release profile – 80% PDGF, 60% VEGF at 21 days</td>
<td>New Zealand rabbit bone defect model</td>
<td>Increased bone formation</td>
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<td>Chitosan hydrogelpoly(ethylene glycol)-b-poly(L-lactide-co-caprolactone) (PELCL) electrospun membrane</td>
<td>VEGF and PDGF</td>
<td>10 μg/ml</td>
<td>Angiogenesis</td>
<td>Release profile – 90% VEGF, 60% PDGF at 15 days</td>
<td>New Zealand white rabbit vascular graft implantation in the left carotid artery</td>
<td>Endothelial cells attached to lumen and smooth muscle cells attached on outer surface, with no thrombosis observed</td>
<td>70</td>
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<td>Biomaterial system</td>
<td>Therapeutics</td>
<td>Doses used</td>
<td>Target</td>
<td>In vitro characterization</td>
<td>In vivo model</td>
<td>Effect observed</td>
<td>Reference</td>
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<tr>
<td>Collagen/fibronectin hydrogel and alginate microparticles</td>
<td>VEGF and MCP-1</td>
<td>1000 ng/mg VEGF and 50 ng/mg MCP-1</td>
<td>Angiogenesis</td>
<td>Release profile – 100% VEGF, 75% MCP-1 at 2 days</td>
<td>Subcutaneous mouse model</td>
<td>Increased number of blood vessels, blood vessel diameter and blood vessel maturity</td>
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<tr>
<td>PLGA and PLGA/PSA microspheres in a HA/methylcellulose hydrogel</td>
<td>EGF and EPO</td>
<td>Not specified</td>
<td>Brain regeneration</td>
<td>Release profile - 80% EGF, 25% EPO at 10 days</td>
<td>Mouse stroke model</td>
<td>Reduced inflammation, apoptosis and cell death, increased neuronal repair</td>
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<tr>
<td>Alginate gel and PLGA microspheres</td>
<td>HSP27 and VEGF</td>
<td>3 µg TAT HSP-27 and 0.65 µg VEGF</td>
<td>Anti-apoptosis and angiogenesis</td>
<td>100% HSP27, 30% VEGF at 10 days</td>
<td>Mouse ischemic hindlimb model</td>
<td>Reduced apoptosis, increased arteriole and capillary density</td>
<td>75</td>
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<td>Gelatin microspheres</td>
<td>IGF-1 and VEGF</td>
<td>100 µl of 20mg/ml microspheres</td>
<td>Angiogenesis</td>
<td>n/a</td>
<td>Rat myocardial infarction model</td>
<td>Reduced infarct size, increased number of capillaries, reduced inflammation and reduced apoptosis</td>
<td>76</td>
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<tr>
<td>Alginate-albumin particles</td>
<td>FGF-2 and HGF</td>
<td>500ng FGF-2 and 125 ng HGF</td>
<td>Angiogenesis</td>
<td>Proliferation and migration assays to assess dual delivery system</td>
<td>Rat chronic heart failure model</td>
<td>Increased number of blood vessels, reduced collagen density and improved cardiac function</td>
<td>77</td>
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<td>PLGA microparticles</td>
<td>VEGF, HGF and ANG-1 (and endothelial progenitor cells)</td>
<td>2.5 µg of each factor</td>
<td>Angiogenesis</td>
<td>n/a</td>
<td>Mouse ischemic hindlimb model</td>
<td>Enhanced neovascularization and perfusion</td>
<td>78</td>
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<td>Collagen microspheres</td>
<td>bFGF and HGF</td>
<td>5 µg bFGF and 20 µg HGF</td>
<td>Angiogenesis</td>
<td>n/a</td>
<td>Mouse ischemic hindlimb model</td>
<td>Increased capillary density and maturation index of blood vessels</td>
<td>79</td>
</tr>
<tr>
<td>Biomaterial system</td>
<td>Therapeutics</td>
<td>Doses used</td>
<td>Target</td>
<td>In vitro characterization</td>
<td>In vivo model</td>
<td>Effect observed</td>
<td>Reference</td>
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<tr>
<td>Collagen microspheres</td>
<td>bFGF and HGF</td>
<td>5 µg bFGF and 20 µg HGF</td>
<td>Angiogenesis</td>
<td>n/a</td>
<td>Mouse ischemic hindlimb model</td>
<td>Increased capillary density and maturation index of blood vessels</td>
<td>79</td>
</tr>
<tr>
<td>Fibrous PELA scaffold</td>
<td>VEGF and bFGF (pDNA polyplexes)</td>
<td>Not specified</td>
<td>Angiogenesis</td>
<td>Release profile – 40% VEGF, 30% bFGF at 15 days</td>
<td>Rat subcutaneous model</td>
<td>Increased blood vessel density</td>
<td>80</td>
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<tr>
<td>PLG scaffolds and microspheres</td>
<td>VEGF and FGF-2</td>
<td>4 µg VEGF and 2 µg FGF-2</td>
<td>Angiogenesis</td>
<td>Protein encapsulation efficiency and protein remaining after scaffold leaching</td>
<td>Rat spinal cord hemisection model</td>
<td>Increased endothelial cell infiltration</td>
<td>81</td>
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<tr>
<td>Fibrin gel and microspheres</td>
<td>eNOS and RAB18 (pDNA lipoplexes)</td>
<td>10 µg of each pDNA</td>
<td>Angiogenesis and inflammation</td>
<td>[Release profile previously characterized]</td>
<td>Diabetic rabbit ear ulcer model</td>
<td>Reduced inflammation, increased angiogenesis and improved wound closure</td>
<td>82</td>
</tr>
<tr>
<td>PEG hydrogel (as a coating on single walled carbon nanotubes)</td>
<td>DX and VEGF</td>
<td>0.9 µg DX and 45 ng VEGF released over 9 days</td>
<td>Inflammation and angiogenesis</td>
<td>Release profile - .8 µg DX, 35 ng VEGF at 4 days</td>
<td>Chick embryo chorioallantoic membrane (CAM) assay</td>
<td>Reduced inflammatory cell density and increased blood vessel density</td>
<td>84</td>
</tr>
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</table>
Table 1-1 (continued): Multi-modal delivery using biomaterial systems.

<table>
<thead>
<tr>
<th>Biomaterial system</th>
<th>Therapeutics</th>
<th>Doses used</th>
<th>Target</th>
<th>In vitro characterization</th>
<th>In vivo model</th>
<th>Effect observed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-hydroxyethyl-methacrylate, N-vinyl pyrrolidinone, and polyethylene glycol hydrogel (as a coating on a glucose sensor)</td>
<td>DX and VEGF</td>
<td>20 µg DX and 900 ng VEGF</td>
<td>Inflammation and angiogenesis</td>
<td>n/a</td>
<td>Rat subcutaneous implant</td>
<td>The combination of DX and VEGF reduced inflammation but also reduced angiogenesis</td>
<td>85</td>
</tr>
</tbody>
</table>
sufficient as these groups were not included in the study. Similarly, an alginate system was used to enhance the regeneration of ischemic muscle[201] with VEGF and IGF delivered along with myoblasts. It was observed that there was a reduction in the defect area at six weeks. Increased angiogenesis and perfusion were seen, while there was an improvement in the tetanic force generated in the anterior tibialis muscle when both factors were delivered compared with either factor alone or bolus delivery of the factors. The use of a biomaterial system thus ensured a more extended presence of the delivered factors in the local environment.

Alginate-sulfate has been used to sequentially deliver three factors to induce neovascularization.[202] The use of alginate-sulfate allows for the loading of heparin-binding factors, or VEGF, PDGF-BB and TGF-β in this case. An initial burst release of VEGF is followed by a more prolonged and delayed release of PDGF-BB and TGF-β, similar to that which occurs in normal blood vessel formation. When using only alginate, no difference was observed in the release profile of these factors, with release also occurring much quicker for each of the factors. In vivo, this differential release profile proved capable of increasing blood vessel density after one and three months. The timing of the release of multiple factors, and subtle changes in material characteristics can thus have an effect on biological processes.

A poly (ethylene glycol) (PEG)-maleimide gel was formed with RGD sequences and growth factor binding domains, with the ability to control the growth factor release via protease-cleavable linkers.[203] Dual delivery of HGF and VEGF was found to improve function in the rat myocardium following ischemia/reperfusion. However, there was no difference in the release profile of the growth factors as they were released simultaneously. Time-delayed release of one of the factors relative to the other, or incorporation of a separate factor to be differentially released can further improve this system. A brushite-chitosan system was used to control the release of angiogenic factors. VEGF and PDGF were delivered in a bone defect model.[204] The authors compared the in vitro and in vivo release profiles using
radiolabelled growth factors. The same patterns were observed with minimal differences between *in vitro* and *in vivo*. It was found that the combination of growth factors greatly increased the formation of new bone that occurs with either growth factor alone, due to increased blood vessel formation and maturation.

Single-walled carbon nanotubes have been developed for use as biomedical sensors. However, inflammation results from implantation and compromises the function of the sensor. Thus, carbon nanotube sensors have been coated with a PEG hydrogel loaded with dexamethasone (DX) and VEGF. In a chick embryo chorioallantoic membrane (CAM)[205] the release profile was assessed and revealed that the DX release was quicker than the VEGF. Using both DX and VEGF, the therapeutic index, defined as the ratio of vasculature density to inflammatory cell density, was higher than that of the control. However, there was little difference between this group and the VEGF alone group, while the group delivering DX in bolus form followed by sustained release of VEGF had an improved therapeutic index. This may be due to an interaction between the functions of DX and VEGF. Thus, it may be that a more precise control over the release profiles, such that there is a greater difference between DX and VEGF release, would result in an improved outcome. Similarly, a hydrogel formed from 2-hydroxy-ethyl methacrylate, N-vinyl pyrrolidinone, and PEG was loaded with DX and VEGF and used as a coating to reduce the foreign body response to implanted glucose sensors.[206] VEGF increased angiogenesis and inflammation, while the DX had the opposite effect. Thus a system that can tune the release of the two therapeutic components of the system is useful in a scenario in which both inflammation and angiogenesis must be controlled.

1.5.2.2 *Micro- and nanoparticles*

Wang et al developed an anti-cancer strategy using a nanocapsule delivery system. To treat a subcutaneous tumor model in mice, nanocapsules were used to sequentially deliver an agent to disrupt the vasculature, combretstatin A4 (CA4), and an anti-cancer agent, paclitaxel (PTX).[207] Sequential release was achieved by the slow hydrolysis of the ester linkage
between the PTX and the poly (lactic acid) (PLA) polymer, which delayed its release more than that of the CA4. It was found that sequential delivery of these factors using the nanocapsule system resulted in reduction in tumor volume and 100% survival of the treated mice. A similar anti-cancer strategy was developed using liposomes decorated with low-density lipoprotein receptor-related protein receptor (Angiopep-2) and neuropilin-1 receptor (tLyP-1). Yang et al. delivered VEGF siRNA and PTX to subcutaneous xenograft tumor models in mice. It was found that dual delivery resulted in reduced levels of VEGF, increased apoptosis and a reduction in tumor volume. This was observed both when the liposomes were delivered directly to the tumor and when delivered intravenously.[208]

Dual delivery of siRNA targeting IL-10 along with a pDNA vaccine encoding for hepatitis-B surface antigen (gWizHBsAg) using poly (ethylenimine) (PEI)-PLGA microparticles was observed to tune the immune response in a mouse in vivo model.[209] This system however was designed to co-deliver the nucleic acids to the same cells, and thus there was no sequential release. This system can be modified to alter the release profile, allowing for sequential delivery of nucleic acids.

Cittadini et al. used gelatin microspheres to produce an initial burst release of IGF-1 followed by a sustained release of VEGF.[92] It is not clear how this release profile is achieved, although it appears simply due to an increased natural affinity between the gelatin and the VEGF over the IGF-1. Dual-delivery of both IGF-1 and VEGF together had complimentary effects: reducing inflammation, increasing angiogenesis, and reducing the infarct size. Alginate-albumin particles have also been used in a similar way to deliver angiogenic factors to the myocardium in a chronic heart failure model.[210] The interaction between FGF-2 and HGF when they were delivered simultaneously was examined, and its ability to promote angiogenesis. It was observed that delivery of both factors increased not only the number of blood vessels but also the number of mature vessels. This resulted in an improved performance of the heart, in terms of systolic and diastolic function. A system
that modulates the release of one factor relative to another can be used to study the spatiotemporal relationship between the factors, which in turn can lead to improved efficacy.

PLGA microparticles were used to deliver combinations of angiogenic factors to induce angiogenesis in a CLI model.[211] Endothelial progenitors were also delivered to aid in the angiogenic process. PLGA particles formed using a double-emulsion process were loaded with VEGF, HGF or Ang-1, and used in various combinations in an in vivo matrigel plug assay. It was observed that delivery of all three factors was superior in terms of blood vessel formation to dual delivery of VEGF and HGF, which itself was superior to delivery of VEGF alone. In the ischemic limb model, delivery of VEGF, HGF and Ang-1 along with endothelial progenitors increased perfusion of the ischemic limb when injected into the muscle. The PLGA particles were designed to release the growth factors over a period of up to two weeks, with any difference in release likely to be minimal. However, the fact that microparticles were used allows for the possibility of their incorporation into a scaffold or hydrogel to further tailor the release, or to modulate the release of factors relative to one another and thus increase the degree of control over their interaction. Collagen microspheres were loaded with bFGF and HGF and used to induce angiogenesis in a CLI model.[212] In vivo release studies showed that when bFGF and HGF were delivered simultaneously to the limb increased perfusion of the limb resulted than that of either factor alone at a higher dose. Thus the synergism of the two factors results in a greater effect than with either factor alone.

1.5.2.3 Fibrous scaffolds

A cellulose acetate hollow fibre system was used to prolong the release of angiogenic factors and was tested in vivo using a matrigel™ plug assay.[213] It was found that delivery of VEGF followed by delivery of sphingosine 1-phosphate (S1P) resulted in the formation of a mature vasculature compared with either factor alone or both factors simultaneously. While this does prove the necessity for sequential delivery, the delivery system was not ideal. The hollow fibres were implanted and then injected with the angiogenic factor every 24 hours, with VEGF injected for the first three days and S1P subsequently injected for three days.
Thus, while the system is not suitable as a therapeutic delivery system, it has shown its usefulness as a tool for studying the interaction between various factors. This was also observed when the same system was used to study the interaction between bFGF and PDGF in a subcutaneous matrigel™ plug assay.[214] In this case, the switch from bFGF to PDGF was on the third day. It was observed that delivery of bFGF followed by PDGF resulted in greater blood vessel formation and maturation compared with either factor alone, both factors delivered simultaneously or PDGF followed by bFGF.

Electrospun fibres may also be loaded with different factors during the fabrication process, negating the need for continual application of therapeutics following implantation as in previously described studies. This has been shown by Man et al., who fabricated electrospun fibres with poly (vinyl pyrrolidone)/bovine serum albumin as the core fluid and poly (ε-caprolactone) (PCL) solution as the sheath fluid. TGF-β1 was loaded within the core fluid and a bone marrow-derived stem cell (BMSC) affinity peptide (E7) was attached to the PCL shell. Release studies revealed that the TGF-β1 was released gradually over 21 days after an initial burst over the first five days. In vitro studies show that the presence of the E7 peptide increased the attachment and proliferation of the BMSCs, while the gradual release of TGF-β1 promoted cartilage differentiation. Thus, this fibre-based scaffold shows potential to promote the attachment of stem cells and direct their differentiation, depending on the cytokine loaded within the scaffold.[215] Electrospinning can also be used to form differentially loaded membranes. Electrospun membranes were fabricated with VEGF and PDGF loaded in the inner and outer layers, respectively.[216] When implanted in vivo, it was observed that the group delivering VEGF and PDGF allowed endothelial cells to attach to the lumen and smooth muscle cells to form a layer on the outside.

Fibrous scaffolds composed of poly (DL-lactide)–poly (ethylene glycol) (PELA) were loaded with either pVEGF polyplexes, bFGF polyplexes or both, and implanted subcutaneously to observe the angiogenic effect.[217] Polyplexes were formed using the commercially available transfecting agent PEI. It was seen that delivery of both pVEGF and
pbFGF together increased the blood vessel density at two and four weeks. However, there were minimal differences in the release profiles, with any small differences attributed to polyplex size and charge. This shows that release of two complimentary factors is superior to that of one factor; nevertheless the timings of release of each needs optimization.

1.5.2.4 Spheres-in-scaffold/hydrogel composite

The use of biomaterials to deliver multiple therapeutics has been investigated over a number of years.[218] The use of composite scaffolds with microspheres contained within a bulk scaffold (solid or hydrogel) has proven to be a popular method to achieve differential release of factors. Biomaterial systems that are able to mediate the release of VEGF and PDGF so that VEGF is released much quicker than the PDGF have been designed. This was achieved by mixing lyophilized VEGF with PDGF within microspheres, which were then incorporated into the same poly (lactide-co-glycolide) (PLG) scaffold. It was observed that co-delivery of these factors in a temporal manner using a polymeric system resulted in the increased formation of mature and larger blood vessels. This utilization of a biomaterial to mimic the native angiogenic process shows the importance of the temporal delivery of factors, demonstrated by the fact that delivery of single factors with a biomaterial or both factors via bolus injection resulted in a lesser angiogenic response. To further emphasize the crucial temporal control over factors, a similar study utilised a bi-layered scaffold which had VEGF alone in one spatial domain and VEGF/PDGF delivered sequentially in an adjacent region.[219] Following implantation in the ischemic hind limbs of mice, it was observed that in the VEGF alone treated side, small immature blood vessels were formed, but in the region treated with a temporal combination of VEGF and PDGF, there were fewer blood vessels but these vessels were larger and mature, at both two and six weeks. An alginate gel system has been used in the myocardium to deliver these same two factors, VEGF and PDGF, in a sequential manner.[220] Increased vessel density was observed, along with an improvement in myocardial function. The differential release of the two factors, however, was not a result of physical entrapment within microspheres as before, but rather a result of differential
affinity between the different growth factors and the alginate hydrogel. Differential release was not as a result of modification of the material but rather an intrinsic property of the alginate and growth factors. These studies illustrate the ability to utilise the properties of materials for multiple, sequential release. Material properties may also be engineered into substrates and growth factors for single factor release. By engineering increased affinity for a specific factor for which delayed release is appropriate, and non-modification of a factor which is required for an earlier/quicker release, multi-modal systems can also be constructed.

The delivery of more than two factors is also possible through biomaterial scaffolds. Using a PLG scaffold (microspheres and scaffold composite), the temporal presentation of multiple angiogenic factors was investigated. It was found that co-delivery of VEGF and angeopoeitin-2 (ANG-2) followed by PDGF and angeopoeitin-1 (ANG-1) using this system induced the formation of a more stable vasculature characterized by increased alpha-smooth muscle actin (α-SMA) positive vessels.[191] This emphasizes a number of important factors, the first of which is the significance of multiple factor release in biological processes. The second is the critical need of temporal presentation, as in this case the pro-angiogenic factors VEGF and ANG-2 are released first followed by the release of the pro-maturation factors PDGF and ANG-1. This ensures the formation of a stable vasculature, which does not occur with delivery of just VEGF and ANG-2.

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VEGF and ANG-2 are released first followed by the release of the pro-maturation factors PDGF and ANG-1. This ensures the formation of a stable vasculature, which does not occur with delivery of just VEGF and ANG-2.

To induce angiogenesis in the infarcted myocardium, an N-isopropylacrylamide (NIPAAm)-based thermally responsive hydrogel with poly (lactic-co-glycolic acid) (PLGA) microspheres were used to deliver bFGF and IGF-1.[221] However, no added benefit was observed when these growth factors were added to the biomaterial compared with the biomaterial alone as the cytokines lost their bioactivity over time.

The PLGA particles previously described[209] were combined with a dextran hydrogel to create a sequential delivery system. Macrophage inflammatory protein-3α (MIP-3α) was loaded into the dextran hydrogel to be released faster than the PLGA particles and attract dendritic cells. The release of PLGA particles with IL-10 siRNA and a pDNA antigen was enabled as the dextran hydrogel degraded, enhancing the immune response by shifting the T cell response to a Th2 type response.[222] In vitro studies confirmed the ability of the system to attract dendritic cells and reduce the IL-10 expression illustrating the potential efficacy of the system to deliver chemokines and nucleic acids in a single system. The system can be altered to allow for the delivery of alternative combinations of chemokines and genes, enabling use of the system in a range of disease states and pathologies.

A synthetic thermoresponsive hydrogel system was developed by Nelson et al. based on NIPAAm. Through the incorporation of a protein-reactive methacryloxy N-hydroxysuccinimide (MANHS) group, increased protein loading and retention was achieved. By addition of a hydrophilic acrylic acid group, degradation of the hydrogel, and hence protein release, can be increased.[223] A further layer of functionality and control was achieved by adding protein-loaded PLGA microspheres to the gel system. Release of protein from the gel occurred before release from PLGA spheres. This system allows precise control over the timing of release through the incorporation of protein reactive groups as well as
Figure 1-4: Approaches to achieve multi-modal delivery from biomaterial systems. Two typical strategies that have been used to achieve multi-modal release. (A) The use of microspheres in a scaffold to promote differential release of two factors, as the contents of the spheres (drawn in red) are released slower than the contents of the hydrogel (drawn in blue). (B) The use of materials that have a differential affinity for the biomaterial. In this case, the factor drawn in red is released slower than the factor drawn in blue. This may be a natural phenomenon due to the interaction between the biomaterial and the loaded therapeutics, or may be engineered into the material using linker systems.
cleavable sites. However, no biological functionality of a released protein was observed, with bovine serum albumin (BSA) used as a model protein. A similar sphere-in-gel system using chitosan gel and gelatin microspheres was characterized in vitro and used to increase the osteoblastic differentiation of W-20-17 mouse bone marrow cells.[224] This composite chitosan/gelatin microspheres system used to sequentially deliver bone morphogenic protein-2 (BMP-2) and IGF-1 showed an increase in alkaline phosphatase (ALP) activity at five and seven days.

A composite poly (propylene fumarate) (PPF) scaffold with gelatin microparticles was used to deliver VEGF and BMP-2 in a critical-sized bone defect in a rat.[225] Dual delivery improved the bone formation at four weeks, but this effect disappeared at 12 weeks as the response with the combination of VEGF and BMP-2 was similar to the group treated with BMP-2 alone. A more controlled release strategy to optimize the interaction between the angiogenic VEGF and the bone-forming BMP-2 in a temporal manner may yield increased bone formation over BMP-2 alone. In a follow-on study to assess the effect of dose on bone formation, no difference was seen in bone formation following VEGF delivery at 12 weeks.[226] There was no obvious benefit of the delivery of VEGF and BMP-2 over the delivery of BMP-2 alone at 12 weeks. However, the authors speculate that further optimization of loading dose, growth factor ratio and release kinetics can result in improved bone formation. In contrast, another study, using VEGF and BMP-2 in combination, observed an increase in bone formation following dual delivery using PLGA microspheres in a PPF scaffold, surrounded by a gelatin hydrogel.[227] This system was implanted both subcutaneously (ectopic model) and in a critical sized defect model. However, this difference may also be due to the different time point used (eight weeks).

A collagen/fibronectin hydrogel with alginate microparticles embedded within it was used to increase the survival and therapeutic potential of transplanted endothelial cells.[228] VEGF and MCP-1 were delivered in a sequential manner and resulted in an increased number of
blood vessels when compared with delivery of either factor alone. In addition, there was no increased inflammatory response detected at two weeks.

Wang et al. utilized a double-sphere-in-gel system to control the release of two factors, epidermal growth factor (EGF) followed by erythropoietin (EPO), in an attempt to stimulate endogenous stem/progenitor cells in a stroke model.[229] EGF was modified with a 5kDa PEG and encapsulated within PLGA particles while the EPO was encapsulated within biphasic particles consisting of PLGA coated with poly (sebacic acid) (PSA). Both of these types of particles were encapsulated in a hyaluronan methylcellulose hydrogel. The purpose of the hydrogel was two-fold: to retain the particles and growth factors in the local area; and to attenuate inflammation, an intrinsic property of high molecular weight hyaluronic acid.[111,112] It was observed that delivery of EGF and EPO through the composite delivery system improved recovery compared with that of vehicle alone or growth factor delivery via a pump, although the lack of delivery of EGF or EPO alone makes it difficult to determine the importance of dual delivery or the interplay between the two factors. The importance of relevant controls in these studies cannot be underestimated as the effects of each individual component in the system need to be determined, along with the interaction between the factors.

The use of a PLGA sphere/alginate gel system has been developed and tested in a CLI model in mice. It has been used to deliver heat shock protein 27 (HSP27) with a transcriptional activator (TAT) derived from the human immunodeficiency virus (HIV) introduced as a protein transduction domain (PTD) to HSP27 along with VEGF.[230] The hypothesis tested was that the TAT-HSP27 will protect cells in the ischemic environment from apoptosis, while the subsequent release of VEGF induces neovascularization of the ischemic limb. A further layer of complexity was added by the use of either porous or non-porous PLGA spheres. It was found that the combination of TAT-HSP27 and VEGF resulted in a reduction in apoptosis and an increase in arteriole and capillary density, particularly with the porous PLGA spheres. It is worth noting that in the measurement of both apoptosis
and angiogenesis, the cumulative effect of TAT-HSP-27 and VEGF was greater than that of either factor alone, underscoring the positive effect of coupling complementary therapeutic strategies.

A fibrin-based sphere-in-gel system has been reported for the delivery of multiple nucleic acids using lipoplexes.[40] This system has proven efficacious in the delivery of peNOS to diabetic rabbit ear ulcer model. However, increased potency of the therapeutic was observed when the secretory control pRAB18 was added to the system.[231] The delivery of peNOS and subsequent delivery of pRAB18 allowed for increased angiogenesis and reduced inflammation, resulting in improved wound closure at 14 days. Again, delivery of peNOS and pRAB18 proved more efficacious than either of the single genes alone, emphasizing the synergistic nature of these molecules and strategies.

Silk scaffolds have been developed that have dual release, sequential properties. Using either silk nanoparticles[232] or calcium alginate beads,[233] sequential release of factors was achieved. However, in both cases, no therapeutic molecule was added to the system, with model molecules used to assess the release profiles. Also, *in vivo* studies were not performed, making it difficult to comment with any certainty on the potential of the systems.

### 1.6 Inflammation

#### 1.6.1 Overview

The inflammatory response is an essential part of the healing process. This response is required to remove necrotic and apoptotic cells, cleaved and damaged ECM molecules, and to initiate subsequent angiogenesis and tissue repair. [234] However, excessive and chronic inflammation leads to the formation of a hostile environment for regeneration and repair, resulting in further cell death. Excessive inflammation and ECM remodeling leads to the formation of a scar through the upregulation of matrix metalloproteinases (MMPs) and increased deposition of collagen type I and III.[235] This is typically characterized by increased neutrophils infiltration and pro-inflammatory macrophages retention. This
amplifies the pro-inflammatory cytokine response, along with MMP activity and the presence of ROS. It has been shown that improved wound healing occurs following a lesser inflammatory response in wound healing of foetal skin, with a reduced expression of pro-inflammatory cytokines, TGF-β, and overexpression of interleukin-10 (IL-10).[236] A similar effect has been observed in foetal myocardium, with reduced inflammation allowing for complete functional restoration.[237] While scarless healing cannot be completely attributed to the absence of inflammation, it does present evidence that a reduced inflammatory response can result in a more favourable outcome.

Thus, strategies to reduce inflammation can prove to be of benefit to treat conditions in which inflammation causes greater damage than the injury itself, or when inflammation becomes chronic.

1.6.2 Biomaterials to modulate inflammation

Implanted biomaterials can cause an inflammatory response, with the level of this response dependent on the material of choice, along with the site in the body into which it is implanted. This is known as the foreign body response and has been discussed in section 1.4.3. Therefore, the choice of materials is of the upmost importance, with an obvious preference for materials that cause a very minimal response. Typically, anti-inflammatory strategies using biomaterials have involved loading anti-inflammatory therapeutics into biomaterial systems, with therapeutic release in vivo aiding to alleviate the inflammatory response. These anti-inflammatory signals are composed of either anti-inflammatory drugs, proteins or nucleic acids, while the delivery of stem cells has also been shown to result in a reduced inflammatory response. In addition, a number of naturally occurring biomaterials have been shown to have intrinsic anti-inflammatory signals. These include high molecular weight hyaluronan and chitosan, which has ROS-scavenging properties. However, primarily the anti-inflammatory effect is derived from anti-inflammatory therapeutics loaded within the biomaterial.
One of the main reasons why anti-inflammatory therapy has become important in relation to biomaterials is to protect the implant itself and prevent rejection by the body. This can be elucidated with an example of islet cell delivery. Ideally, the islet cells will not be rejected by the body, and can produce insulin efficiently to correct diabetes. However, this is not the case in actuality as the host often rejects these implanted cells and their function is quickly compromised. Attempts to immuno-isolate cells using biomaterials have not proven successful and thus anti-inflammatory signals are necessary to prevent rejection by the host immune system. Encapsulation in a biomaterial alone has not proved efficacious, and thus the incorporation of anti-inflammatory agent is the next logical step. Su et al. encapsulated islet cells in a PEG-based gel and conjugated an inhibitory peptide to the IL-1 receptor (IL-R1).[238] This increased the survival of encapsulated cells in vitro following exposure to IL-1β, IFN-γ and TNF-α, while islet cells were also able to continue glucose-stimulated release of insulin when incubated with β-cell specific T-lymphocytes. A large-scale study was performed to identify a suitable anti-inflammatory drug for local immunosuppression of islet cells.[139] Encapsulation of islet cells in alginate microcapsules, along with the identified anti-inflammatory curcumin, improved survival of the islet cells in vivo, with reduced fibrosis of the capsules and improved glycaemic control in a chemically-induced mouse type 1 diabetes model.

The incorporation of SDF-1α into a PLGA scaffold reduced the inflammatory response when implanted into the subcutaneous space in mice.[168] While SDF-1α is typically associated with increased angiogenesis, it can also exert anti-inflammatory effects through its mobilization and homing effect on stem cells.[239,240] Thus it was shown that incorporation of SDF-1α in PLGA scaffolds reduced the inflammatory tissue response through the increase in autologous stem recruitment to the implant site. Furthermore, a reduction in pro-inflammatory cytokines was detected, with reduced expression of a number of key mediators including IL-1α, IL-6 and TNF-α, while there was an increase in VEGF expression.
Table 1-2: Biomaterials used to modulate the inflammatory response.

<table>
<thead>
<tr>
<th>Biomaterial system</th>
<th>Therapeutic</th>
<th>Dose</th>
<th>In vitro characterization</th>
<th>In vivo model</th>
<th>In vivo outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG hydrogel (10,000 Mw)</td>
<td>IL-1R inhibitory peptide (IL-1RIP)</td>
<td>1% IL-1RIP</td>
<td>Cell viability and insulin secretion from MN6 cells</td>
<td>n/a</td>
<td>n/a</td>
<td>221</td>
</tr>
<tr>
<td>Alginate microcapsules</td>
<td>Curcumin and Dexamethasone</td>
<td>1 mg/ml Curcumin, 2 mg/ml Dexamethasone</td>
<td>n/a</td>
<td>Diabetic mouse subcutaneous and interperitoneal implantation</td>
<td>Increased blood glucose level following treatment with Curcumin.</td>
<td>136</td>
</tr>
<tr>
<td>PLGA (75:25) scaffold</td>
<td>SDF-1α</td>
<td>50 µg SDF-1α</td>
<td>Stem cell migration assay</td>
<td>Mouse subcutaneous implantation</td>
<td>Increased vessel density</td>
<td>164</td>
</tr>
<tr>
<td></td>
<td>IL-10 pDNA polypeptides and MSCs</td>
<td>2 µg IL-10 pDNA</td>
<td>IL-10 secretion Metabolic activity</td>
<td>Rat intramuscular implantation model</td>
<td>Increased stem cell survival Increased ratio of regulatory to inflammatory macrophages</td>
<td>70</td>
</tr>
</tbody>
</table>
Table 1-2 (continued): Biomaterials used to modulate the inflammatory response.

<table>
<thead>
<tr>
<th>Biomaterial system</th>
<th>Therapeutic</th>
<th>Dose</th>
<th>In vitro characterization</th>
<th>In vivo model</th>
<th>In vivo outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen sponge</td>
<td>IL-10 pDNA polyplexes and MSCs</td>
<td>2 µg IL-10 pDNA</td>
<td>n/a</td>
<td>Rat myocardial infarction model</td>
<td>Improved cardiac function and stem cell survival</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Increased ratio of regulatory to inflammatory macrophages</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reduction in apoptosis</td>
<td></td>
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<tr>
<td>Chitosan hydrogel</td>
<td>ADSCs</td>
<td>4x10^6 ADSCs</td>
<td></td>
<td>Rat myocardial infarction model</td>
<td>Increased ICAM-1, VCAM-1 and SDF-1 expression and ADSC retention</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cell adhesion and expression of adhesion genes in response to ROS</td>
<td></td>
<td>Reduction in ROS, apoptosis and infarct size</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Increased wall thickness and blood vessel density, improved cardiac function</td>
<td></td>
</tr>
<tr>
<td>Chitosan hydrogel</td>
<td>ADSCs</td>
<td>2x10^6 ADSCs</td>
<td>n/a</td>
<td>Rat acute renal ischemia-reperfusion model</td>
<td>Reduction in apoptosis and ROS expression</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Increased stem cell retention and renal cell proliferation</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>Reduction in serum levels of creatinine and blood urea nitrogen</td>
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<tr>
<td>Tetraethylene glycol and cyclohexyl methacrylate nanoparticles</td>
<td>IL-1RA</td>
<td>5 µg IL-1RA</td>
<td>Nanoparticle size</td>
<td>Rat intra-articular model</td>
<td>Increased retention of IL-1RA compared with saline delivery</td>
<td>226</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Target specificity</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>NF-κB activity in NIH3T3 fibroblasts</td>
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<tr>
<td>Poly(cyclohexane-1,4-diyacetone dimethylene ketal) (PCADK) microparticles</td>
<td>P38 inhibitor</td>
<td>50 µg P38 inhibitor</td>
<td>Particle size</td>
<td>Rat myocardial infarction model</td>
<td>Reduced P38 activation, superoxide and TNF-α production</td>
<td>227</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Activation of RAW 264.7 macrophages</td>
<td></td>
<td>Reduced fibrotic area and improved cardiac function</td>
<td></td>
</tr>
</tbody>
</table>
Table 1-2 (continued): Biomaterials used to modulate the inflammatory response.

<table>
<thead>
<tr>
<th>Biomaterial system</th>
<th>Therapeutic</th>
<th>Dose</th>
<th>In vitro characterization</th>
<th>In vivo model</th>
<th>In vivo outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(cyclohexane-1,4-diyaceton dimethylene ketal) (PCADK) microparticles</td>
<td>Superoxide dismutase (SOD)</td>
<td>80U SOD</td>
<td>Particle size Superoxide scavenging</td>
<td>Rat myocardial infarction model</td>
<td>Reduced superoxide production and apoptosis Improved cardiac function</td>
<td>228</td>
</tr>
</tbody>
</table>
A collagen scaffold with MSCs was functionalized with IL-10 polyplexes in an effort to reduce inflammation in an intramuscular model. It was observed that IL-10 polyplex treatment reduced inflammation and promoted increased MSC survival. When this system was implanted in an MI rodent model, reduced myocardial inflammation was induced along with functional recovery of the heart, in terms of improved ejection fraction. Furthermore, a change in macrophage phenotype was detected. Macrophages were seen to change from a classically activated, pro-inflammatory phenotype in control groups to an alternatively activated, anti-inflammatory treatment following treatment with IL-10 polyplexes and MSCs.

Chitosan is a polysaccharide, derived from crustaceans. It has been used for many biomedical applications. Specifically, it is commercially available as a bandage due to its clotting ability and its anti-bacterial properties. The addition of adipose-derived mesenchymal stem cells (ADSCs) to a chitosan hydrogel was seen to improve their survival in the infarcted myocardium. In vitro studies demonstrated the ROS scavenging properties of chitosan and its degradation products. MI was induced by permanent ligation of the coronary artery, followed by injection of the chitosan/ADSCs system. Treatment with the chitosan/ADSCs system resulted in a reduction in ROS, as observed by a reduction in dihydroethidium (DHE) staining. An improvement was observed at four weeks in ejection fraction and fractional shortening, while there was a reduction in apoptosis seen at one week. Additionally, there was a reduction in infarct size as well as an increase in wall thickness and vessel density in the infarct site. This study demonstrates the anti-ROS properties of chitosan, as it hypothesized that chitosan enhances stem cell retention and survival in the myocardium (which was confirmed by in vivo bioluminescence imaging) partly due to its ability to scavenge ROS. This system was also utilized to treat ischemic injury in the kidney. A similar effect was observed, with a reduction in ROS expression, increased cell retention and increased number of blood vessels.
Nanoparticles have been used to deliver anti-inflammatory therapies. Whitmire et al. fabricated a new block co-polymer that assembles into sub-micron particles and contains a moiety for tethering proteins.[245] To this moiety, IL-1RA, the naturally occurring antagonist to the IL-1R, was conjugated. These particles were injected into the intra-articular joint space, where it was shown that they significantly enhanced the retention time of IL-1RA. Polymer particles fabricated from poly(cyclohexane-1,4-diylacetone dimethylene ketal) (PCADK) were loaded with a p38 inhibitor to modulate the post-infarction inflammatory response in the myocardium.[246] In an intramuscular model the particles themselves were found to be non-inflammatory, while in an MI model, the particles significantly reduced superoxide and TNF-α production. This resulted in a reduction in fibrotic area as well as improved cardiac function. A similar study utilized the same particles to deliver superoxide dismutase (SOD) to the infarcted heart, which reduced superoxide expression and apoptosis.[247] A subsequent improvement in cardiac function was also observed.

1.7 Angiogenesis

1.7.1 Overview

Angiogenesis is a key requirement in the formation of complex tissues and organs. When a tissue reaches a critical thickness, blood vessels are required to provide oxygen and nutrients to cells, as well as to remove wastes and byproducts of metabolism. Additionally, blood vessels provide a mechanism for immune cells to monitor, quickly react and extravasate into damaged tissue.

Angiogenesis is defined as the sprouting of blood vessels from pre-existing blood vessels.[248] However, it is not a simple process, and requires a very defined and structured sequence of events to occur in order to produce regular, mature blood vessels.[249] Pathological angiogenesis, as typically seen in tumours, results in the formation of leaky, heterogenous vessels with tortuous anatomy.[250] These structural and functional abnormalities result in the formation of regions of ischemia. Therefore, it is important to
understand the mechanism of angiogenesis, such that the strategy used to induce angiogenesis does not result in abnormal vessel formation.\[251\] Full understanding of the process of angiogenesis is important when choosing the appropriate factor to induce functional and mature angiogenesis. However, despite a greater understanding of the molecular mechanisms of angiogenesis, therapies capable of inducing the formation of stable, functional vasculature in ischemic tissues have not yet materialized. However, this increased mechanistic understanding has led, in turn, to an improved understanding of pathological angiogenesis, which has been associated with chronic inflammatory diseases and cancer. Indeed, major strides have been made to block pathological angiogenesis, with therapies in the clinic to treat macular degeneration as well as a range of cancers. A therapy capable of revascularizing ischemic tissue would be a major step forward in the treatment of conditions such as MI and CLI. However; as yet no such treatment has been forthcoming.

During normoxia, hypoxia-inducible factor-1 alpha (HIF-1α) is hydroxylated by the enzyme prolyl hydroxylase (PHD) which results in its degradation by proteases.\[252\] However, during hypoxia, PHD is rendered inactive, and as a result HIF-1α is not degraded and initiates an angiogenic response to compensate and increase the supply of oxygen to the tissue. The release of pro-angiogenic signals such as VEGF, FGF and ANG-2 first results in the detachment of pericytes and the loosening of endothelial cells of the local vasculature.\[249,250,253\] A provisional ECM is formed in which endothelial cells migrate, with a tip cell leading the way flanked by stalk cells which proliferate to lengthen the newly formed sprout. The vessel matures in response to signals such as PDGF, ANG-1 and TGF-β, which stimulate pericyte coverage and endothelial cell quiescence. This mechanism is depicted in figure 4. The stages of increased permeability and tip cell selection, stalk elongation and subsequent maturation and pericyte attachment are shown.

1.7.2 Biomaterials to promote angiogenesis

Tissue engineering strategies have long sought to create vascularized scaffolds capable of implantation to create viable tissue constructs. With the use of the appropriate signals and
Figure 1-5: Mechanism of angiogenesis – (A) Initial pericyte detachment, increased vascular permeability and tip cell formation (shown in green), followed by (B) lumen formation, elongation of the stalk and endothelial cell proliferation along with sprouting of adjacent vessels. Pericytes are also recruited to the newly formed vessels, which (C) mature and form a stable vessel in response to pro-maturation factors.
cell types, in vitro pre-conditioning of cell-seeded biomaterials has enabled the formation of a vasculature which may then be implanted in vivo. However, the appropriate conditions to form a mature vasculature capable of amalgamating with host vasculature are not yet clear from the literature as opinions differ. Differences emerge with regard to the most appropriate cell types, signals, biomaterials and, crucially, the spatiotemporal manner of combining these key elements. However, another strategy exists which seeks to deliver angiogenic factors to ischemic tissues with biomaterials-based systems. Rather than implant vascularized scaffolds to form tissue, the hope is that by delivering the appropriate angiogenic cues from within a prolonged release system, the scaffold and the local tissue will become vascularized as the therapeutic is released over time. The use of biomaterials as delivery systems to promote angiogenesis may help to create functional, mature vasculature, rather than the immature, transient vessels which are often the result of bolus delivery strategies which neglect the temporal nature of blood vessel formation, development and stability.

A range of materials have been used to deliver angiogenic factors, both natural and synthetic. However, ECM-based biomaterials have proved to be particularly popular and efficacious, in particular those which have an intrinsic pro-angogenic effect. These materials include fibrin, elastin and collagen. A collagen scaffold has been shown to increase angiogenesis in the myocardium following cryoinjury.[254] The addition of VEGF_{165} to collagen scaffolds has proven to be a particularly popular combination for many applications including orthopedics,[30] subcutaneous[255] and in the myocardium.[256] In all cases addition of VEGF_{165}, whether as a protein or in the form of an encoding pDNA, resulted in an increased level of blood vessels compared with the collagen scaffold alone. Fibrin gels have been used to promote angiogenesis in a range of tissue types, with the addition of various angiogenic factors such as eNOS,[104] pleotrophin[103] and VEGF_{121},[108] Progenitor cells have also been added to scaffolds to promote neovascularization. These include bone marrow mononuclear cells added to fibrin gels[257] and bone marrow-derived...
MSCs[258] and circulating angiogenic progenitor cells added to collagen scaffolds.[259] Addition of adipose-derived stromal vascular fraction cells to a collagen scaffold increased healing and angiogenesis in a diabetic porcine wound model.[260]

Non-ECM based biomaterials have also been used to induce angiogenesis. The delivery of VEGF$_{165}$ pDNA via an alginate hydrogel induced neovascularization in a CLI model with an increase observed in blood vessel density.[261] Delivery of bFGF via a chitosan hydrogel induced increased angiogenesis in a rabbit MI model at four weeks. [262] Combinations of ECM-based and other natural biomaterials have also been utilized. For example, chitosan/collagen hydrogels have been loaded with recombinant human granulocyte macrophage-colony stimulating factor (GMCSF) to increase angiogenesis,[263] while a chitosan-based pDNA complexing agent was added to a collagen scaffold to deliver VEGF to induce angiogenesis.[255]

Synthetic materials require the addition of an angiogenic factor more so than some natural biopolymers due to the fact that the molecules on their own can rarely induce angiogenesis. Therefore, functionalization with an angiogenic agent is key. The addition of lentivirus encoding VEGF$_{165}$ to a porous PEG hydrogel increased blood vessel formation following implantation.[264] PEG hydrogels are relatively inert materials without cell attachment sites, and thus peptide sequences (RGD etc.) have been incorporated to promote cellular attachment, along with MMP-cleavable regions to increase degradation. Cell attachment sequences are of particular importance if the hydrogel itself is to be infiltrated by host cells and vascularized. Incorporation of both RGD and MMP-cleavable sequences along with loading of VEGF$_{165}$ and HGF increased angiogenesis and stem cell recruitment following MI, as well as reducing fibrosis.[203] Poly (ethylene glycol) diacrylate (PEGDA) hydrogels were similarly engineered to contain cell adhesion and MMP-degradable sequences, and loaded with soluble VEGF$_{121}$.[265] When this material was implanted in both subcutaneous and CLI models, it was observed to increase vascularization. PEG hydrogels with integrin binding sites and protease sensitive linkers were loaded with VEGF$_{165}$ (both soluble and
Table 1-3: Examples of biomaterials used to induce increased angiogenesis.

<table>
<thead>
<tr>
<th>Biomaterial system</th>
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<th>Dose</th>
<th>In vitro characterization</th>
<th>In vivo model</th>
<th>In vivo outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen sponge</td>
<td>n/a</td>
<td>n/a</td>
<td>Adhesion and differentiation of cardiovascular cells</td>
<td>Cardiac cryoinjury model</td>
<td>Increased arteriole and capillary density</td>
<td>232</td>
</tr>
<tr>
<td>Collagen/calcium phosphate sponge</td>
<td>VEGF pDNA</td>
<td>0.35 µg VEGF pDNA/mm³</td>
<td>n/a</td>
<td>Mouse subcutaneous and intra-femoral models</td>
<td>No significant difference observed in blood vessel density</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Release studies – 80% released after 96 hrs HEK293 VEGF secretion and proliferation</td>
<td>Subcutaneous mouse model</td>
<td>Increased bone volume observed in intra-femoral model</td>
<td>233</td>
</tr>
<tr>
<td>Collagen sponge</td>
<td>VEGF pDNA</td>
<td>356 µg of naked pDNA VEGF or 70 or 320 µg of complexed pDNA VEGF</td>
<td>Release studies – 80% released after 96 hrs HEK293 VEGF secretion and proliferation</td>
<td>Subcutaneous mouse model</td>
<td>Increased blood vessel number per mm³ at 14 and 21 days</td>
<td>233</td>
</tr>
<tr>
<td>Collagen sponge</td>
<td>VEGF</td>
<td>20 ng (Low) or 100 ng (High)</td>
<td>Release studies – 80% released after 96 hrs HEK293 VEGF secretion and proliferation</td>
<td>Rat right ventricle free wall resection</td>
<td>Increased cell mobilization and proliferation (High dose VEGF)</td>
<td>234</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VEGF bioactivity - Endothelial cell and MSCs proliferation and lactate production measured at 3 days</td>
<td></td>
<td>Increased vessel density at 7 (High) and 28 days (High and Low)</td>
<td>234</td>
</tr>
<tr>
<td>Fibrin hydrogel</td>
<td>eNOS</td>
<td>3x10⁷ pfu Adenovirus eNOS</td>
<td>n/a</td>
<td>Alloxan-induced rabbit ear ulcer model</td>
<td>Increased epithelialization at 14 days</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Increased surface area of blood vessels at 7 days</td>
<td>102</td>
</tr>
<tr>
<td>Fibrin hydrogel</td>
<td>Pleiotrophin pDNA</td>
<td>250 µg pleiotrophin pDNA</td>
<td>n/a</td>
<td>Rat myocardial infarction model</td>
<td>Increased arteriole density at 5 weeks</td>
<td>101</td>
</tr>
</tbody>
</table>
Table 1-3 (continued): Examples of biomaterials used to induce increased angiogenesis.

<table>
<thead>
<tr>
<th>Biomaterial system</th>
<th>Therapeutic</th>
<th>Dose</th>
<th>In vitro characterization</th>
<th>In vivo model</th>
<th>In vivo outcome</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Fibrin hydrogel    | VEGF        | 1-10 µg | Binding of VEGF to fibrin matrices  
|                    |             |      | Effect on endothelial cell growth | n/a           | n/a           | 106       |
| Fibrin hydrogel    | Bone marrow mononuclear cells (BMMCs) | 2x10^7 cells | n/a | Rat myocardial infarction model | Increased microvessel density | 235       |
| Collagen sponge    | Bone marrow-derived mesenchymal stem cells (BMSCs) | 1x10^7 cells | Cytotoxicity of biomaterials and BMSCs proliferation | Rabbit ischemic hind limb model | Increased oxygen saturation ratio, mature blood vessels | 236       |
| Collagen sponge    | Circulating angiogenic cells exposed to osteopontin | 5x10^6 cells | Cell metabolic activity | Alloxan-induced rabbit ear ulcer model | Improved wound closure 
|                    |             |      |                           | Increased surface density of blood vessels |          | 237       |
| Collagen sponge    | Stromal vascular fraction cells | 1x10^5 cells | Cell attachment to scaffold | Porcine diabetic wounds on back | Increased wound healing at 7, 14 and 21 days 
|                    |             |      |                           | Increased blood vessel density 
|                    |             |      |                           | Increased VEGF and bFGF expression |          | 238       |
| Alginate hydrogel  | VEGF pDNA   | 25 µg VEGF pDNA | Release studies – complete release at 20 days | Mouse hindlimb ischemia model | Increased blood perfusion 
|                    |             |      | Alginate mediated gene delivery | | Increased vessel density | 239       |
Table 1-3 (continued): Examples of biomaterials used to induce increased angiogenesis.

<table>
<thead>
<tr>
<th>Biomaterial system</th>
<th>Therapeutic</th>
<th>Dose</th>
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<th>In vivo model</th>
<th>In vivo outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan hydrogel</td>
<td>FGF-2</td>
<td>7.5 µg FGF-2</td>
<td>n/a</td>
<td>Rabbit myocardial infarction model</td>
<td>Reduced infarct area</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Improved cardiac function</td>
<td></td>
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<td></td>
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<td></td>
<td>Reduced fibrosis in border zone</td>
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<td></td>
<td></td>
<td></td>
<td>Increased capillaries in non-infarcted area</td>
<td></td>
</tr>
<tr>
<td>Heparinized collagen/chitosan sponge</td>
<td>Granulocyte-</td>
<td>30 µg</td>
<td>Release studies – Sustained release over 11 days (followed by degradation by collagenase)</td>
<td>Rat subcutaneous model</td>
<td>Increased infiltration of CD68 positive cells</td>
<td>241</td>
</tr>
<tr>
<td></td>
<td>macrophage</td>
<td></td>
<td>Swelling, degradation and mechanical properties</td>
<td></td>
<td>Increased number of blood vessels</td>
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<tr>
<td></td>
<td>colony-stimulating factor (GM-CSF)</td>
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</tr>
<tr>
<td>PEG hydrogel (Mw 20,000) with gelatin</td>
<td>Lentivirus</td>
<td>3x10^7 pfu</td>
<td>Cell viability and infiltration</td>
<td>Mouse subcutaneous model</td>
<td>Increased number of transduced cells</td>
<td>242</td>
</tr>
<tr>
<td>microspheres</td>
<td>encoding VEGF</td>
<td></td>
<td>Virus activity</td>
<td></td>
<td>Increased lectin staining (angiogenesis)</td>
<td></td>
</tr>
<tr>
<td>PEG hydrogel (Mw 20,000) with incorporated</td>
<td>VEGF and HGF</td>
<td>1 µg each factor</td>
<td>Release profile – 100% release of each factor following treatment with high concentration collagenase, 65% with lower dose, 40% in PBS at 4 days</td>
<td>Rat myocardial infarction model</td>
<td>Increased number of blood vessels, reduced fibrosis and improved myocardial function</td>
<td>193</td>
</tr>
<tr>
<td>RGD, growth factor binding and protease</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>cleavable sequences</td>
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<tr>
<td>PEG hydrogel (Mw 20,000) with incorporated</td>
<td>VEGF</td>
<td>80 µg/mL A-PEG-VEGF</td>
<td>Degradation and NIH3T3 cell spreading</td>
<td>Mouse subcutaneous and hindlimb ischemia model</td>
<td>Increased vascular volume (subcutaneous)</td>
<td>243</td>
</tr>
<tr>
<td>RGD, growth factor binding and protease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Increased perfusion (hindlimb)</td>
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<tr>
<td>cleavable sequences</td>
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</table>
Table 1-3 (continued): Examples of biomaterials used to induce increased angiogenesis.

<table>
<thead>
<tr>
<th>Biomaterial system</th>
<th>Therapeutic</th>
<th>Dose</th>
<th>In vitro characterization</th>
<th>In vivo model</th>
<th>In vivo outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG hydrogel (Mw 3,400) with incorporated RGD and protease cleavable sequences</td>
<td>VEGF</td>
<td>512 ng VEGF per gel and 1.9 ng PEG-VEGF per gel (soluble and non-soluble)</td>
<td>Degradation, Endothelial cell tubule formation and length</td>
<td>Mouse cornea implantation model</td>
<td>Increased functional, perfused blood vessels</td>
<td>244</td>
</tr>
</tbody>
</table>
immobilized). Endothelial cells cultured in these matrices organized themselves into networks of capillary-like structures, while they were infiltrated with host vasculature following implantation in mouse corneas.[266]

As has been previously mentioned, Mooney et al. have reported on the development of multi-modal delivery systems, mostly to deliver angiogenic factors in a spatiotemporal manner. This approach has involved using a PLGA scaffold with PLGA microspheres embedded within, and has been used in an attempt to recapitulate natural processes involved in angiogenesis. A number of these studies have shown the importance of spatiotemporal delivery and presentation of angiogenic factors, and the resulting improvement.[191,218,219]

1.8 Inflammation–angiogenesis: link and balance

Inflammation and angiogenesis are two key processes which are disregulated in numerous disease states as well as being important in the integration (or rejection) of biomaterial and tissue engineered scaffolds. In response to injury, or implantation of a foreign material, an initial inflammatory response occurs which is required to remove any necrotic cells or damaged ECM. This subsides and leads to the formation of granulation tissue rich in blood vessels, and ECM deposition and remodeling. This can lead to a scar in the case of defective wound healing, or a fibrotic capsule in the case of implantation of a material. Many examples of compromised wound healing are characterized by excessive inflammation, inadequate angiogenesis or both. For example, in diabetic wounds, macrophages are known to be locked in a pro-inflammatory state,[189] while angiogenesis is also compromised due to a reduction in NO levels. Reduced NO is a consequence of the increase in ROS, which reacts with the NO to produce another free radical, peroxynitrite.[267] This NO depletion impairs wound healing[194] through a reduction in angiogenesis.[193] Similarly, in the myocardium following MI, the presence of ROS reduces the effect of NO, and hence there is a reduction in vasodilation and angiogenesis necessary to compensate for the injury. The inflammatory response associated with MI is also detrimental to functional healing, as the
ECM is degraded and replaced by a new matrix rich in collagen type I. Therefore it is clear that modulation of these two processes is essential for an improved outcome in a number of disease states, as well as to increase the integration of biomaterial implants. However, given that these two processes are interlinked, it is important to modify these processes in such a way that they do not inappropriately affect the other i.e. complete nullification of inflammation would reduce a number of key angiogenic signals, while uncontrolled angiogenesis can lead to the formation of immature blood vessels and an influx of inflammatory cells. Therefore it is vital to strike the appropriate balance in reducing inflammation, but not to such an extent that angiogenesis is compromised, and vice-versa. Strategies to manipulate and optimize the balance between inflammation and angiogenesis could prove to be key to treating a range of disease states, as well as improving the integration of biomaterials following implantation.

1.8.1 Interlinked processes

Inflammation and angiogenesis are two dynamic processes which interact with one another in a spatial and temporal manner. For example, a number of angiogenic growth factors and cytokines are secreted by inflammatory cells during wound healing.[268] This is emphasized by both macrophages and endothelial cells. Pro-inflammatory macrophages secrete many cytokines and growth factors. These soluble factors promote inflammation and also induce angiogenesis through the promotion of vessel permeability and endothelial cell activation, survival and proliferation. However, these vessels are often disorganized and immature. Regulatory macrophage phenotypes can promote the stabilization and maturation of these blood vessels. Nevertheless, during impaired healing this maturation of blood vessels does not occur and, as a result, the vessels regress. This is the case following MI, when this regression of blood vessels results in the formation of an avascular scar. Even so, the promotion of angiogenesis can lead to an influx of inflammatory cells, especially when the blood vessels are immature and leaky. Endothelial cells, which form the vasculature, are also a key component of the inflammatory response. Following an injury, the expression of
adhesion molecules on the surface of the local endothelium is one of the first responses of the immune system.[269,270] The expression of molecules such as ICAM-1, VCAM-1 and PECAM results in the influx of inflammatory cells such as monocytes and neutrophils to the injured tissue. Thus, this newly formed vasculature with leaky, immature vessels will result in increased infiltration of inflammatory cells.

In the area of cancer research, the interplay between inflammation and angiogenesis has been studied extensively. This has been driven by the pursuit of anti-tumour therapies such as angiogenic blockers which target pro-angiogenic growth factors including VEGF. In addition to anti-angiogenic approaches, strategies to modify the inflammatory response in the tumour microenvironment have become prevalent. A link has been made between tumour progression and the increase in the number of regulatory macrophages in the tumour microenvironment.[271,272] These macrophages suppress inflammation in the tumour microenvironment and promote angiogenesis, possibly due to hypoxia in the tumour. This aids the growth of the tumour through an increased blood supply. A number of studies have attempted to reduce the immunosuppressive environment present in the tumour either through a reduction of the expression of anti-inflammatory cytokines[209] or the targeted death of regulatory macrophages through the delivery of pro-apoptotic peptides.[273] In actual fact, strategies to treat cancer are often the opposite of those treating many pathological conditions. In cancer the strategy is to increase inflammation and reduce angiogenesis in the tumour environment, thus creating an inflammatory environment and starving the tumour of its blood supply. However, in many other disease states such as diabetic wound healing, CLI and MI, the strategy has traditionally been to reduce inflammation, promote angiogenesis, or indeed both, to improve healing. Thus, strategies to treat cancer may seem quite unconventional and counter-intuitive. Nonetheless, approaches used to treat cancer may prove to be important to aid drug discovery through a basic mechanistic understanding of the interplay between inflammation and angiogenesis.
1.8.2 Effect of inflammation and angiogenesis on ECM/GAG composition

The ECM was once considered an almost inert matrix that provided mechanical support and a scaffold in which cells grew. However, it is now well-established that the ECM is a dynamic matrix capable of regulating a range of cellular functions including cell interaction, communication and proliferation.[274] By altering its composition, the affinity of the ECM for binding and releasing growth factors can be altered, as well as its mechanical strength. Through these changes, the ECM can react to stimuli and direct cell fate.[275] Thus the importance of the ECM and specifically its changing composition during pathological states is firmly established, both in terms of its effect on inflammation[276] and on angiogenesis.[277]

The formation of a fibrotic scar composed of fibrillar collagens which results from injury is a clear indication that there is a change in ECM composition following disease. However, as well as this ‘end-stage’ change which is apparent during fibrosis, dynamic changes in ECM composition occur over time which are more subtle in nature. The ECM composition can change due to a number of stimuli, and potentiate the response of cells in the microenvironment.[274,277–283] The ECM dynamically regulates tissue regeneration by transmitting mechanical forces, exposing cryptic adhesion sites and releasing sequestered growth factors as a result of protease activity. Changes in matricellular protein expression in the context of cardiac injury and remodeling have been studied.[284] Matricellular proteins are typically upregulated during development, injury and remodeling.[285,286] They have a role in a number of inflammatory, angiogenic and fibrotic pathways, and potentiate their effect through an association with cytokines, growth factors and other effector molecules. Hence, they play a key role in inflammation, angiogenesis and subsequent remodeling following ischemic injury.[287] Thrombospondins, tenascins, SPARC (secreted protein, acidic and rich in cysteine), osteopontin and periostin are just some of the matricellular proteins that have been shown to have altered expression in the myocardium following MI. Furthermore, changes in myocardial glycosaminoglycan (GAG) content may play a crucial
role, with GAGs and associated proteoglycans implicated in the initiation and modulation of inflammation. [288] Already, increased expression of the enzyme xylosyltransferase-I (XT-1) has been identified and linked with increased GAG content in patients with dilated cardiomyopathy. [289] In addition, differential GAG content, sulfation and growth factor activity has been identified in aged myocardium compared with that of younger hearts. [290] GAGs are implicated in both age-related and disease-associated ECM remodeling, and are likely to play a key role in orchestrating both inflammation and angiogenesis in a range of pathological states. Studies which monitor the dynamic changes in protein levels and GAG expression and sulfation over time, both in terms of content and activity, can lead to new insights into the endogenous mechanisms of activation, regulation and resolution of inflammation as well as control over angiogenesis. This can allow a more mechanistic understanding of the interplay between the two phenomena, leading to improved outcomes for patients and implanted devices. This may involve utilizing biomaterial scaffolds with specific GAGs or GAG-mimetics incorporated, or the use of gene therapy to either increase or reduce the expression of GAG-synthesizing enzymes. By altering the synthesis of GAGs which are up- and downregulated during the healing process, the balance may be tipped towards reduced inflammation and increased angiogenesis, thus improving outcome.

GAG-mimetics have been used in a range of wound healing applications and have proved useful at improving healing through a combination of reduced inflammation and increased angiogenesis. A heparan sulfate mimetic, OTR4120, has been shown to improve healing in both diabetic [291] and burn wounds. [292] It is hypothesized that this effect is through increased regulation of collagen I and III expression, fibroblast proliferation as well as binding to specific growth factors, chemokines and cytokines, such as members of the FGF and VEGF families. Hence it is likely that changes in the composition or activity of GAGs in the matrix can alter the capacity of the tissue for regeneration by modifying the levels of inflammation and angiogenesis following injury. In fact, GAGs have previously been added
to collagen scaffolds to improve the tissue response, specifically in terms of foreign body response and increased angiogenesis, following implantation.[293]

1.9 Project Rationale

The previous sections have discussed biomaterials used in tissue engineering and gene therapy as a means of delivering therapeutics. The foreign body response to biomaterials has been discussed as well as strategies that can be used to modulate the response. These include therapeutics such as gene therapy or protein delivery to alter inflammation and angiogenesis. The crosstalk between inflammation and angiogenesis has been discussed, and how this alters the ECM, or more precisely, how the ECM may co-ordinate this interaction. This project involves development of a composite collagen microsphere/hydrogel system is developed. This system is characterized both in vitro and in vivo as a non-viral gene delivery reservoir. A pro-angiogenic gene, in this case eNOS, is used to promote angiogenesis, while an siRNA is utilized to reduce inflammation through the reduction in IL-6 expression. By combining the collagen microsphere/hydrogel system with the eNOS and IL-6, spatiotemporal delivery of the therapeutics is achieved.

1.9.1 Rationale for using collagen microspheres

Microspheres fabricated from a variety of materials have been used as vehicles to deliver therapeutics and also as reservoirs to prolong therapeutic release.[294] By encapsulating a therapeutic within a microsphere, thereby extending its release, the efficacy of the therapeutic is increased. Microspheres have been used to encapsulate both genes and proteins, with a particular benefit seen with extending the half-life of therapeutic proteins.[246] In addition, by delaying the release of a therapeutic that may have associated toxicity, the dose to be delivered may be increased without an increase in toxicity.[295,296] However, as with any material implanted into the body, microspheres elicit a host response. By using collagen to fabricate microspheres, this host response will be minimized. The form of collagen used in this study, atellocollagen, has telopeptides removed by pepsin treatment,
hence it has reduced immunogenicity compared with unmodified collagen. Collagen, the primary structural protein of the ECM is biodegradable and can be functionalized.

1.9.2 Rationale for using a crosslinked collagen hydrogel

Collagen, as mentioned, is biodegradable, bioactive and a major component of the ECM, promoting cell attachment and proliferation. It is worth noting that many collagen-based products have been approved for use in humans by the FDA, an endorsement of its suitability. These include recently approved products such as CollaGUARD™ and Excellagen™ for wound healing applications. Hence, the next step in the development of collagen-based therapeutics is to incorporate increased functionality through the addition of appropriate and complementary therapeutics. The hydrogel is crosslinked with 4S-PEG via its free amine groups to increase its stability and minimise degradation.[61,297,298]

1.9.3 Rationale for use of a collagen hydrogel/microsphere composite system

Collagen hydrogels and microparticles have been used to deliver a range of therapeutics from cells and proteins to genes.[61,74,297,298] However, by combining a collagen hydrogel and collagen microspheres, a system that can produce a bi-modal release of therapeutics can be fabricated. This approach has previously been used with PLG[218], alginate[220] and many other materials. Release from the collagen hydrogel is quicker than release from the collagen microspheres that are embedded within the hydrogel. Thus, by loading different therapeutics within each phase of the system, i.e. one therapeutic directly into the hydrogel and another therapeutic within the microspheres, it becomes possible to deliver multiple therapeutics in a staggered manner.

1.9.4 Rationale for use of IL-6 siRNA

Il-6 has been identified as a key molecule in the inflammatory response.[299] It is responsible for the influx of inflammatory cells and macrophage activation following injury.[300] IL-6 promotes the expression of ICAM-1 in the ischemic myocardium,
**Figure 1-6: Interleukin-6 functions** - A schematic representing the immune-related functions of interleukin-6 (IL-6). IL-6 is involved in the infiltration and activation of macrophages and monocytes, the activation of adhesion molecules on endothelium, the activation of fibroblast and promotion of matrix deposition, the accumulation of neutrophils, the increase in tissue proteases and the suppression of regulatory T-cells (T\textsubscript{REGS}).
promoting the interaction of neutrophils with cardiomyocytes.[301] It has also been shown that IL-6 is involved in myocyte hypertrophy and the proliferation of fibroblasts, possibly contributing to scarring. In addition, foetal skin wounds display scarring healing and a reduced expression level of IL-6.[302] Administration of IL-6 to foetal skin wounds resulted in scarring, with increased deposition of collagen in parallel bands.[303] The immune-related functions of IL-6 have been summarized in figure 1-6. The use of RNAi is a powerful tool to reduce the expression of molecules implicated in pathogenesis.[304] Since reducing the inflammatory response is a key objective of this project, delivery of IL-6 siRNA is explored as a mechanism to achieve this.

1.9.5 Rationale for use of Superfect® complexed eNOS pDNA

Three isoforms of NOS have been identified, iNOS, nNOS and eNOS. iNOS is transcriptionally regulated by inflammatory cytokines, is calcium independent and produces a high level of NO. It produces more NO than the other forms but it has more of a tendency to uncouple and preferentially produce a superoxide \( \text{O}_2^- \) rather than NO. Superoxide itself is quite toxic, and can combine with NO and form peroxynitrite \( \text{NO}_3^- \), which can be damaging to cells and tissue. nNOS is involved in the transmission of neuronal signals. eNOS has been shown to be pro-angiogenic in nature.[305–307] The mechanism of blood vessel formation by eNOS and NO is shown in figure 1-7. eNOS is constitutively expressed in endothelial cells, is calcium dependent and produces relatively low levels of NO. Both viral and non-viral gene therapy with eNOS has previously been shown to be capable of inducing blood vessel formation in compromised wound models.[40,104] Due to its short half-life, gene therapy approaches with eNOS have seen widespread use.[308] eNOS functions by catalyzing the reaction of L-arginine to L-citruline and NO. NO induces angiogenesis and vasculogenesis through the upregulation of pro-angiogenic factors such as VEGF\(_{165}\), as well as through the enhancement of endothelial cell proliferation, survival and migration as well as EPC mobilization. In addition to its role in angiogenesis, NO also has a plethora of other functions. For example, it regulates vessel tone and has an effect on
Figure 1-7: eNOS-related formation of blood vessels – eNOS catalyses the reaction of L-arginine to L-citruline, with nitric oxide (NO) also formed. NO has a plethora of functions, including those shown on smooth muscle cells (SMC), endothelial cells (EC) and endothelial progenitor cells (EPC). These functions promote arteriogenesis, angiogenesis and vasculogenesis, resulting in vascular remodeling and neovascularization.
inflammation by reducing the interaction between leukocytes and the endothelium in the vasculature.[309]

In order to deliver the eNOS gene, eNOS pDNA must be complexed with a transfecting agent, since transfection using uncomplexed pDNA often result in minimal transgene expression. A transfecting agent will condense the pDNA imparting on it a positive charge, allowing it to gain entry to the cell cytoplasm and increase transgene expression compared with uncomplexed pDNA. Superfect® is an activated, partially degraded polyamidoamine (PAMAM) dendrimer with a defined spherical architecture and terminal charged amino groups which allows it to compact pDNA. It is commercially available and has been used in vitro to efficiently transfect a wide range of cell types with minimal cytotoxicity. In addition, Superfect® has been used previously in conjunction with collagen biomaterials for non-viral gene delivery in vitro[72] and in vivo.[31,32] Considering its history in in vivo applications, as well as its reduced in vitro cytotoxicity when compared with other commercial reagents such as PEI, Superfect® was considered as a suitable transfection reagent for the purpose of this study.

1.10 Objectives and hypotheses

The overall hypothesis of this thesis is that a biomaterial composed of hollow collagen microspheres embedded within a crosslinked collagen hydrogel can allow for the release of IL-6 siRNA and eNOS pDNA, which will downregulate the inflammatory response and upregulate angiogenesis in a temporal manner, thus modulating the inflammatory-angiogenic response and altering the GAG composition of the ECM.

The in vitro studies involved the fabrication of hollow collagen microspheres and their characterization as a gene delivery reservoir of complexed pDNA. Embedding the appropriately sized nucleic acid-loaded microspheres within a crosslinked collagen hydrogel and loading another nucleic acid within the hydrogel created a collagen-based release system for the delivery of multiple nucleic acids. The size of sphere to be used was assessed via
uptake studies with differentiated and activated THP-1 cells. Characterization of the dual release system involved assessing the release of the loaded nucleic acids, as well as the degradation, cell viability and biomaterial-mediated transfection.

The in vivo studies were key to assess the potential of the collagen microsphere/hydrogel system to modulate inflammation and angiogenesis, since these two phenomenon involve so many cell types and interactions which cannot be modelled in vitro. The system was tested in a subcutaneous Lewis rat model. Assessment of the effect on inflammatory response, angiogenesis and changes in GAGs present in the ECM at 7 and 14 days was performed.

1.10.1 Phase 1 - Development of a nucleic acid reservoir

1.10.1.1 Objectives

1. Fabricate collagen hollow spheres using the template method, and assess the charge and morphology of the microspheres using zeta sizer and electron microscopy.

2. Characterize the loading and release over time in vitro of pDNA polyplexes spectrophotometrically using Cy3™ labeled pDNA.

3. Assess the ability of polyplexes released from spheres to transfect 3T3 fibroblasts in vitro using the luciferase reporter system.

4. Assess the ability of collagen hollow spheres to modulate the cytotoxic effect of commercially available transfection reagents on 3T3 fibroblasts in vitro.

1.10.2 Hypotheses

1. By the manipulation of electrostatic charges, hollow collagen microspheres can be fabricated using the template method.

2. Hollow collagen microspheres can act as a nucleic reservoir by loading polplexes and releasing them over time.

3. Polplexes released from collagen hollow microspheres following loading remain bioactive and can transfect 3T3 fibroblasts in vitro.
4. By modulating the release of polyplexes using collagen hollow spheres, the toxicity associated with commercially available transfection reagents on 3T3 fibroblasts in vitro can be modified.

1.10.3 Phase 2 – In vitro development and optimization of a dual release collagen nucleic acid reservoir system

1.10.3.1 Objectives

1. Identify an appropriate size of microsphere which is not internalized by activated macrophages so that hollow collagen microspheres can act as reservoirs external to cells and release their therapeutic gene to a large number of cells rather than be uptaken by macrophages.

2. Characterize the release profile of siRNA and pDNA polyplexes spectrophotometrically from the collagen microsphere/hydrogel system in vitro using Cy3™ labeled siRNA and pDNA.

3. Assess the ability of released siRNA and pDNA complexes to transfect primary fibroblasts in vitro and characterize the subsequent knockdown of IL-6 and upregulation of eNOS at 3, 7 and 14 days.

1.10.3.2 Hypothesis

1. An optimal size of hollow collagen microsphere exists that avoids uptake by activated macrophages, and thus is suitable for use as a reservoir of nucleic acids.

2. When embedded within a crosslinked collagen hydrogel, this optimally sized hollow collagen microsphere can form a gene delivery reservoir system which differentially releases two separate doses of nucleic acids.

3. Nucleic acids released over 14 days from this dual release collagen hydrogel/microsphere system are still bioactive and are capable of transfecting primary fibroblasts in vitro over 14 days.
1.10.4 Phase 3 – In vivo evaluation of system efficacy at modulating inflammation and promoting angiogenesis in a spatiotemporal manner

1.10.4.1 Objectives

1. Optimise the dose of IL-6 siRNA delivered through a collagen hydrogel/sphere system to reduce the volume fraction of inflammatory cells at seven days.

2. Optimise the dose of eNOS pDNA delivered through a collagen hydrogel/sphere system to increase the surface and length density of blood vessels at 14 days.

3. Assess the effect of IL-6 siRNA and eNOS pDNA delivery from collagen hydrogel/sphere system on volume fraction of inflammatory cells and surface and length density of blood vessels at 7 and 14 days.

4. Assess the changes in total GAG composition and relative quantities of heparan sulfate (HS) and chondroitin sulfate (CS) in the ECM, as well as the relative affinity of the extracted GAGs for VEGF\textsubscript{165} and bFGF at 7 and 14 days and relate these changes to the inflammatory (volume fraction of inflammatory cells) and angiogenic parameters (surface and length density of blood vessels).

1.10.4.2 Hypothesis

1. Delivery of IL-6 siRNA via a collagen microsphere/hydrogel system will reduce the volume fraction of inflammatory cells at seven days in a dose-dependent manner relative to delivery of a non-targeting control (NTC) siRNA via the same collagen microsphere/hydrogel system.

2. Delivery of eNOS pDNA via a collagen microsphere/hydrogel system will result in an increase in the surface and length density of blood vessels in a dose-dependent manner at 14 days, relative to an unloaded collagen microsphere/hydrogel system.

3. Co-delivery of the optimal IL-6 siRNA and eNOS pDNA doses using a collagen microsphere/hydrogel system in an in vivo subcutaneous rat model will result in a reduction in the volume fraction of inflammatory cells and an increase in the length and surface density of blood vessels.
4. Changes in the volume fraction of inflammatory cells and surface and length density of blood vessels will result in subsequent changes in the GAG composition of the ECM, specifically related to changes in the relative amounts of HS and CS, as well as the relative affinity of these extracted GAGS for growth factors such as VEGF$_{165}$ and bFGF.
1.11 References


Literature Review


Literature Review


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Chapter Two

Development of an extracellular matrix based nucleic acid reservoir

The main findings of this chapter have been previously published in:

2.1 Introduction

Gene therapy, through the up- or downregulation of dysregulated genes, has been suggested as a therapy for various disease states and disorders. However, research in this field is still far from clinical acceptance due to adverse effects and concerns of delivery systems used, both viral (safety)[1] and non-viral (efficacy and toxicity).[2,3] Recently, there has been a shift towards the development of non-viral, polymeric-based gene carriers. These are generally cationic polymers that form complexes (polyplexes)[4] with negatively charged nucleic acids.[5] These complexes enable protection of the nucleic acid but also facilitate its cellular uptake and intracellular trafficking towards the nucleus.[6] Moreover, the use of polyplexes can help overcome some of the limitations that are typical of viral-vectors such the size of nucleic acid that can be packaged, as well as limitations of immunogenicity, mutagenesis, reproducibility and scale-up.[1,7] Nevertheless, the use of polyplexes for clinical applications is hampered by their toxicity and a lack of stability in vivo, which can lead to a low efficiency of transfection.[2]

Cytotoxicity of polyplexes has been studied extensively, and it is generally known that these systems can potentially destabilize cell membranes, induce autophagy, apoptosis and necrosis.[2,8,9] Polymer molecular weight, charge of polyplexes, polyplex size and their ability to be cleared by the organism have all been associated with their cytotoxicity.[2,8] To address these issues, many modifications to the formulations of these polymeric systems have been attempted, such as the addition of poly (ethylene glycol) (PEG) groups[6], change in the molecular weight of the polymers, and the addition of a biodegradable backbone to the polymer.[10] However, despite these advances, toxicity still remains a major obstacle yet to be fully overcome. In the current work, another approach is explored to address cytotoxicity and stability of polyplexes by optimizing the method of delivery rather than modifying the polyplexes. It is hypothesized, that the toxicity of polyplexes can be masked by controlling the delivery rate using a reservoir system that is of uniform and controlled size composed of an extracellular matrix (ECM) based protein, type I collagen. A reservoir will extend the
duration of polyplex release (lengthening the duration of transgene expression), and decrease the associated toxicity. From current literature, it is known that the use of natural polymers for gene delivery, in both particle and matrix form, is advantageous due to their inherent non-toxic and biodegradable nature. In fact, matrices composed of natural polymers have been previously used and shown to prolong the transgene expression both in vitro and in vivo. It is also hypothesised that the modulation of the polyplexes release may decrease their toxicity and also prolong their ability to transfect cells by increasing their stability by protection from the extracellular environment. To ensure a uniform release, it is essential to have control over both the size and the architecture of the reservoir system.

Furthermore, the composition of a delivery system is another important aspect to consider. A reservoir system composed of type I atellocollagen (collagen type 1 digested with pepsin to eliminate telopeptides containing antigenic epitopes) will have several advantages compared to a system composed of other polymers as it will be recognised as an ECM constituent and will impart site specificity to the delivered gene carrier as collagen can be recognized by cells via sequence motifs such as Arg-Gly-Asp (RGD). Due to its suitability as a biomaterial for a delivery system, various methodologies have been attempted to develop type I collagen-based reservoir systems such as the emulsion method, spray-drying and microphase separation. In addition, collagen has been used to coat particles, and it has been shown that cells adhere to and grow more readily on collagen coated microspheres than on uncoated microspheres.

Although promising outcomes are reported from the use of spheres created using the previously mentioned techniques, drawbacks including difficulties with reproducibility and control over size remain to be resolved. Recently, it has been demonstrated that it is possible to fabricate hollow spheres of a controlled size and in a reproducible manner with natural polymers using a template method. However, to date there have been no reports of hollow reservoir systems that are made from fibrous proteins. Control over size and homogeneity are two important criteria for clinical translation. A uniform microsphere based
Extracellular matrix based reservoir delivery system with a prolonged release profile will further increase the efficiency and length of transgene expression, while limiting any toxic effects of the loaded polyplex cargo.

The hypothesis tested in this study was that collagen hollow spheres can be fabricated using the template method, and can act as a reservoir for gene delivery reservoir by loading and releasing polyplexes, maintaining their ability to transfect cells and modulating polyplex toxicity in vitro.

Thus, the objectives of the study are:

1. To fabricate collagen hollow spheres using the template method.
2. Assess the charge and morphology of the microspheres using zeta sizer and electron microscopy.
3. Characterize the loading and release over time in vitro of pDNA polyplexes spectrophotometrically using Cy3™ labeled pDNA.
4. Assess the ability of polyplexes released from spheres to transfect 3T3 fibroblasts in vitro using the luciferase reporter assay.
5. Assess the ability of spheres to reduce the effect of polyplexes on the metabolic activity of 3T3 fibroblasts using the alamarBlue® assay.

2.2 Materials and methods

2.2.1 Extraction of collagen

Type 1 atellocollagen was isolated as previously described.[25] Briefly, bovine tendons were blended, washed in buffer (3.7mM Na₂HPO₄, 0.35Mm KH₂PO₄, 51Mm NaCl) and suspended in 0.5M acetic acid. The resulting solution was then pepsin treated (1 g pepsin: 100 g tendon) and filtered to remove insoluble collagen telopeptides. The soluble collagen was then purified by repeated salt precipitation (0.9M NaCl) and centrifugation, followed by dialysis against 0.01M acetic acid. (procedure described in detail in appendix A)
2.2.2 Fabrication of collagen microspheres

Hollow collagen microspheres were fabricated using the template method as previously described in the literature for other natural polymers.[22–24] Commercially available polystyrene beads (Gentaur, Chicago, Illinois) of defined sizes (100nm, 1μm and 10μm), were sulfonated to impart a strong negative charge. Following sulfonation, beads were re-suspended in 0.5M acetic acid. 5 mg/ml of collagen solution was added to the beads at a weight ratio of 4:1 (beads: collagen). The resultant mixture was then stirred for four hours at room temperature. Crosslinking of the collagen coating was performed using pentaerythritol poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-PEG), with the active ester groups reacting with the free amino groups of collagen at a ratio of 1:4 (4S-PEG:Free amino groups in the collagen). The mixture was agitated for two hours at room temperature. To produce a hollow sphere, the polystyrene core was dissolved by washing the coated beads with tetrahydrofuran (THF). The suspension of microspheres was diluted at a ratio of 1:1 with THF and agitated for one hour. The washing step was repeated twice to ensure complete removal of polystyrene. Hollow spheres were washed twice with ethanol and twice with water to ensure removal of any remaining THF. Spheres of three sizes were fabricated: 100 nm, 1 μm and 10 μm.

2.2.3 Zeta analysis

To characterize the charge of microspheres zeta potential was analysed using a zeta sizer (Malvern, Nano-ZS90). The charge on 100 nm, 1 μm and 10 μm was analysed in PBS.

2.2.4 Surface analysis of spheres

Amplitude modulation atomic force microscopy (AFM) was utilized to analyse the surface of the 10 μm microspheres using a commercial system (NanoWizard®-II, JPK Instruments, Germany). A 100 μL drop of aqueous solution containing hollow collagen-coated spheres was pipetted onto a glass microscope slide and air-dried. After the slide was placed on the AFM sample stage, an inverted optical microscope (Eclipse Ti-E, Nikon, Japan) was used to
locate isolated or clusters of spheres. The region of interest was positioned directly under the AFM tip prior to imaging in air. Silicon cantilevers (PPP-NCH, Nanosensors™, Germany) with nominal spring constants and resonance frequencies of 42 N m\(^{-1}\) and 314 kHz, respectively, and typical tip radii of ~7 nm were used. The images were recorded with a resolution of 512 x 512 pixels and a 1 Hz scan rate. Images were processed using WSxM software (Nanotec).[26]

2.2.5 Analysis of polystyrene removal

To verify complete removal of the polystyrene template following THF treatment an infrared spectrum analysis of the spheres was performed. Particular attention was paid to the characteristic peaks of polystyrene. Three samples were examined: uncoated polystyrene beads, collagen coated polystyrene beads and hollow collagen spheres (washed with THF). Samples were examined following drying using a Fourier transform infrared spectrometer (FTIR – Varian 660-IR).

2.2.6 Labelling of plasmid

_Gaussia princeps luciferase_ plasmids (pCMV-GLuc; New England Biosciences, Ipswich, USA) were propagated and isolated using standard techniques. These pDNA was then fluorescently labelled with the Cy5 dye using a Cy5 labeling kit (Mirus, Madison, USA), as per manufacturers instructions. Briefly, the dye was incubated with the pDNA in the provided buffers for one hour. Following the incubation, the pDNA was eluted through a microspin column to remove any unbound dye. The recovered labelled plasmid was then stored in a light-protected environment at -20°C until use.

2.2.7 Formation of polyplexes

Polyplexes were prepared by incubating the labelled plasmid with a partially degraded poly (amido amine) (dPAMAM) dendrimer (Superfect™, Qiagen) (SF). The weight ratio of the partially degraded dendrimer to the pCMV-GLuc used was 9:1. As a control,
polyethyleimine (PEI, Branched, MW=25,000) complexes were formed at a weight ratio of 1:1 (PEI:pDNA).

2.2.8 Loading of microsphere reservoirs with polyplexes

Loading was quantified using a method for detecting complexed pDNA previously validated and standardized.[27] 100 μg of collagen microspheres were re-suspended in 500 μl of phosphate buffered saline (PBS). To the re-suspended microspheres, 2 μg of Cy5 labelled complexed pDNA was added. This mixture of microspheres and labelled complexes was agitated on a mechanical shaker for four hours at room temperature. The microspheres were spun down and the supernatant removed. This supernatant was then measured spectroscopically in a black well plate (excitation=649 nm, emission=670 nm) in a Varioskan™ Flash plate reader (Thermo Scientific, Ireland). The supernatant was compared with a standard curve to determine the amount of polyplexes remaining which enabled quantification of loading efficiency, as above.

2.2.9 Release study

The release profile of Cy5 labelled polyplexes was characterized in PBS at 37°C. Loaded microspheres were spun down; the supernatant was collected and replaced at various timepoints. The collected supernatant was frozen until it was assayed for polyplex content using a plate reader as for loading efficiency.

2.2.10 Transfection

Polyplex-loaded collagen microspheres of three sizes (100 nm, 1 μm and 10 μm) were incubated with 3T3 fibroblasts in complete media (10% serum) to assess the ability of the microspheres to release polyplexes that transfect cells in vitro. Briefly, 10,000 cells were seeded into a 96 well plate and incubated with 50 μg microspheres loaded with 2 μg of complexed pDNA for 48 hours. In addition, polyplexes alone formed using 1 μg pDNA and SF and PEI, (at weight-to-weight ratios previously specified), were used as positive controls. At 48 hours, 50 μl of the supernatant was removed and assayed using a gaussian luciferase
Extracellular matrix based reservoir assay kit to quantify the expression of the excreted luciferase protein. Furthermore, to microscopically determine the ability of microspheres to release bioactive polyplexes and transfect 3T3 fibroblasts in vitro, protein expression was also assessed by delivering a SF complexed green fluorescent protein plasmid (pCMV-GFP) loaded within microspheres and visualizing the expression using an inverted fluorescent microscope after 48 hours.

2.2.11 Transfection following release of polyplexes over six days

Microspheres were loaded with 2 µg of polyplexes. Loaded polyplexes were then incubated in serum-free cell culture media for six days. Following this, the released polyplexes in media were added to 3T3 fibroblasts. This media was replaced after four hours with complete media (10% serum), and the levels of transfection determined after 48 hours.

2.2.12 Cell viability

The influence of the spheres on the metabolic activity of 3T3 fibroblasts was quantified using the alamarBlue® cell metabolic activity assay. 50 µg of microspheres of three sizes (100nm, 1µm and 10µm) were incubated with cells for 48 hours and the effect on metabolic activity was measured and compared with the control (cells grown on tissue culture plastic alone).

2.2.13 Cell proliferation

The effect of each size of collagen microspheres on the proliferation of 3T3 fibroblasts over 48, 120 and 168 hours was assessed. Following incubation with microspheres for the three time periods specified, media was removed and cells washed with HBSS. The cells were then subjected to three cycles of freeze-thaw at -80°C in DNAse free water, and analysed for DNA content using the PicoGreen® reagent, as per the manufacturers’ instructions. Each size was compared with the control, cells grown on tissue culture plastic alone.
2.2.14 Cellular interaction with spheres

FITC-labelled collagen microspheres were incubated with 3T3 fibroblasts in an eight well-chamber slide for 24 hours. The media was then removed, and the cells and spheres washed with Hanks Balanced Salt Solution (HBSS). Cells were then fixed with 4% Paraformaldehyde, permeabilised with 1% Triton-X, and stained with Rhodamine-Phalloidin and DAPI before being mounted and examined under an inverted confocal microscope.

2.2.15 Toxicity of polyplexes

In order to assess the potential of the microspheres to buffer the cytotoxicity associated with large doses of transfecting agents, the effect of polyplexes formed using two commonly used transfecting agents, dPAMAM and PEI (at 9:1 and 1:1 weight-to-weight ratios, as previously specified) was assessed both loaded within microspheres (1 μm) and directly in the cell culture media of 3T3 fibroblasts. A 5 μg dose of pDNA was used in both cases, and compared with the control (cells alone) using an alamarBlue® assay at 48 hours.

2.3 Results and discussion

2.3.1 Collagen hollow sphere fabrication and structural characterisation

The first objective was to obtain a reproducible structure with defined parameters with regard to shape and size. The template method (figure 2-1) facilitated the formation of collagen spheres with a hollow core and controlled size and shape through a manipulation of the charge interaction between the polystyrene bead and the positively charged collagen molecule. The coating process is performed in acidic conditions so that the positively charged collagen[28] forms a coating around the negatively charged polystyrene beads. Following the formation of a coating around the polystyrene bead, the collagen is crosslinked to stabilize the coating. Finally, removing the polystyrene by washing with THF forms a hollow shell, (figure 2-2). Considering that small particles and molecules may diffuse inside the lumen of the spheres, their hollowness is an advantage in terms of loading because it almost doubles the surface area available to interact with the cargo relative to
The spheres obtained have the same shape and size of the polystyrene beads used as a template, as can be seen from SEM images (see appendices). The uniformity and consistency of size is an important consideration when proposing microspheres as a therapeutic delivery vehicle. Microsphere behavior (uptake, inflammatory reaction etc.) is dependent not only on the material components, but also the size. Thus, an ability to accurately control size is of vital importance.

Dissolution of the template was achieved by washing in THF and confirmed by FTIR analysis (see figure 2-3). Complete removal of polystyrene is essential as any remaining polystyrene alters the charge on the particles, affecting loading of positively-charged polyplexes. In addition, polystyrene is not biodegradable and will elicit an inflammatory response if implanted *in vivo*. The surface topography of the sphere, measured by AFM, is shown in figure 2-4 (A). While the surface structure is not readily apparent, the curvature of the sphere is visible. The surface roughness of the sphere was determined to be 1.5 nm by selecting a 1.4 x 1.4 µm² area and performing a second order flattening to subtract out the effect of the curvature of the sphere. The amplitude and phase AFM images (figure 2-4 (B) and (C)) have also been flattened to reveal the surface structure. AFM analysis of the structure of the surface of the sphere showed evidence of a nanofibrous coating, thus indicating the presence of fibrous protein on the surface of the spheres. The surface of the spheres exhibited topographical features associated with the matrix assembly process used in the fabrication of the microspheres.

2.3.2 Characteristics of loading and release

Characterisation of the loading and release properties of the microspheres is a critical step as it directly relates to functionalization of the microspheres. The intended application for these microspheres is as a reservoir system that is capable of releasing polyplexes over time thus extending the lifetime of transgene expression. The first step was to assess the loading efficiency. Each of the three sizes of spheres displayed an ability to efficiently load polyplexes at an efficiency of about 85-90% regardless of the sphere size tested (for 2 µg per
100 μg hollow collagen spheres). Hence, the spheres have a high loading capacity, with an ability to efficiently load about 20 μg of complexed pDNA per 1mg of microspheres. The mechanism of loading is related to the charge of the microspheres. As can be seen from the zeta analysis, all three sizes of spheres measured were negatively charged when measured in PBS, with the two smaller sizes (100 nm and 1 μm) around -7.5 mV and the 10 μm significantly more negative at about -17 mV (see figure 2-5). Hence, the high loading efficiency is due to the fact that spheres and polyplexes are incubated in PBS. Positively charged polyplexes interact electrostatically with the negatively charged microspheres. This electrostatic interaction explains the high loading efficiency of the collagen microspheres. In addition, the phosphates of PBS create a temporary cloud of negative charges around the collagen molecules.[29]

Together, these properties allow for the random diffusion of polyplexes inside the microspheres. Moreover, type I collagen is a molecule rich in aspartic acid,[30] a negatively charged amino acid, which further enhances the loading capacity of the microspheres. This interaction is of importance as it determines the loading ability of the system. The amount of nucleic acid loaded is vital for the success of any gene delivery reservoir. Considering that many in vivo studies frequently deliver as little as 2 μg[13,31] of complexed pDNA and with the fabricated collagen spheres it is now possible to deliver 20 μg of complexed DNA per mg of spheres, this system now enables a high dosing regimen.

Each of the three sizes of microspheres showed an ability to delay the release of polyplexes, and therefore this system was suitable to extend the release of polyplexes and transgene expression over time. Each size showed a similar release pattern of polyplexes over 144 hours (figure 2-8 (B)). Overall, the spheres showed an ability to delay the release of polyplex cargo over the first 96 hours, varying from 40-60%. The polyplex release curves demonstrate a prolonged release of the loaded cargo over 144 hours in vitro, regardless of the size used (figure 2-8 (B)). While it is likely that the release profile will be different in the
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extracellular space in vivo, due to interactions with various proteins of the ECM and degrading enzymes, the microspheres hold promise in prolonging the release of polyplexes.

2.3.3 Cell viability and interactions with microspheres

Considering the ultimate goal is to utilize these hollow collagen microspheres as a reservoir system for delivery of polyplexes, it is crucial that these spheres were not toxic to cells. Spheres of all sizes did not exhibit toxicity (see figure 2-6 (A)) when compared with the control (cells alone grown on tissue culture plastic). As a reservoir for polyplexes, spheres are expected to be in contact with cells for a considerable period of time in vivo and therefore it is reassuring that no cytotoxic effects were seen when assessed with the alamarBlue® assay. Similarly, no effect was seen on cell proliferation when the PicoGreen® assay was performed. As figure 2-7 shows, there was no significant effect on the proliferation of 3T3 fibroblasts (over any of the timepoints) following incubation with any size of collagen microsphere tested when compared with the control. These results confirmed the conclusions derived from the alamarBlue® assay.

This was further validated by imaging 3T3 fibroblasts incubated with FITC-labeled microspheres for 48 hours (see figure 2-6 (B)). The cells showed no changes in morphology when compared with a control - further evidence that the collagen microspheres do not adversely affect viability, and hence are suitable as reservoirs in contact with cells.

Microspheres of all sizes showed a similar viability, with no significant difference as compared with the positive control. No effect on the morphology of 3T3 fibroblasts following incubation with microspheres was seen. SEM images (figure 2-6 (C)) showed the cells extending cellular processes toward the microsphere.

2.3.4 Protein expression and transfection-associated toxicity

Measurement of luciferase activity was used to assess the ability of the spheres to release bioactive polyplexes and transfect 3T3 fibroblasts in vitro. A fibroblast cell line was used as fibroblasts are the predominant cell type present in tissue, and thus are a good cell type to
transfect. There was a correlation between size of spheres and transfection ability. Out of the three sizes, the 1 µm and 10 µm spheres showed the highest ability to transfet in vitro and a similar level of transfection to the SF polyplex alone and PEI controls. However, cells incubated with 100 nm microspheres showed significantly less transfection than the polyplex alone control, and even less than the plasmid alone control (see figure 2-9 (A)). Fluorescent images showed GFP expression in 3T3 fibroblasts (see figure 2-9 (B)) following incubation with microspheres that further illustrates the ability of the microspheres to release bioactive polyplexes. However, the relatively low number of cells transfected and expressing GFP is apparent, indicating the relative low transfection efficiency in non-viral systems compared with their viral counterparts. Polyplexes released over six days by microspheres showed an ability to transfet 3T3 cells in vitro. As figure 2-10 shows, there is a detectable level of transfection from the 1 µm and 10 µm spheres. While it is less than that detected from freshly made up polyplexes, it is still significantly greater than pDNA alone transfection. This confirms the usefulness of the microspheres to act as a reservoir of bioactive polyplexes and extend their release over time.

With no difference seen in the release profiles of polyplexes, different levels of transfection can be linked to the size of the spheres. Uptake of the smaller spheres results in lower transfection levels as polyplexes are released within individual cells, resulting in toxicity, rather than staying outside cells and releasing the loaded polyplexes to many cells. However, following their release over six days, polyplexes from 100 nm spheres showed no detectable levels of transfection. Therefore it appears that 100 nm collagen spheres interact with polyplexes in such a way that polyplex functionality is reduced and released polyplexes are incapable of transfecting cells. It is also likely that due to the smaller size the 100 nm collagen spheres do not protect the polyplexes from degradation in the way that the larger spheres seem to be able to. The ability of the 1 µm and 10 µm microspheres to transfet was visually confirmed by using the GFP reporter plasmid, while for the 100 nm spheres no protein expression was evident.
One of the hypotheses behind this study was that by modulating the rate of delivery of polyplexes, the cytotoxic effect of the transfecting agents can be reduced. This is especially crucial when delivering polyplexes to cells which have a limited ability to regenerate following injury as seen in cardiomyocytes in the myocardium[32] or nucleus pulposus cells in the intervertebral disc.[33] This was examined by using two known cytotoxic transfecting agents in high doses and comparing with the same transfecting agents but loaded within microspheres. High doses of the commercially available transfecting agents, dPAMAM and PEI, had a negative effect on the viability of 3T3 fibroblasts as measured using the alamarBlue® assay, with metabolic activity decreasing to 40% and 50% of control, respectively. However, when the same dose was loaded within 1μm microspheres, there was no negative effect on the metabolic activity when compared with the control (see Figure 2-9 (C)). These results illustrate that the microspheres can deliver a large dose of transfecting agent to cells without compromising cellular viability. There is a significant effect on cell metabolic activity using the transfecting agents on their own. However, this effect is buffered by the utilization of polyplex-loaded microspheres and the metabolic activity is maintained as similar to the control. This shows that by altering the release pattern using an ECM-based reservoir system, the cytotoxic effects of known transfecting agents in vitro can be shielded. It is likely that this ability will be displayed in vivo, with the microspheres capable of delivering a high dose of polyplexes to a target tissue without causing toxicity in the local microenvironment.
Figure 2-1: Fabrication of hollow collagen microspheres using the template method. The process involves the sulfonation of a commercially available polystyrene bead of defined size (100 nm, 1 μm and 10 μm in this case), coating of these beads in a collagen solution, crosslinking of the coating and removal of the polystyrene bead core. (THF: Tetrahydrofuran).
Figure 2-2: **Shell-like structure of collagen microsphere:** TEM image revealing the hollow, shell-like nature of 1 μm collagen microspheres. Following the formation of a collagen coating on the surface of the polystyrene template, crosslinking of the collagen coating and treatment with THF, the hollowness of the microsphere can be seen clearly. (THF: Tetrahydrofuran)
Figure 2-3: **Confirmation of removal of polystyrene core:** FTIR spectrum showing the removal of the polystyrene core following treatment with THF. The characteristic peaks of polystyrene (dotted lines) are removed from the sample following THF treatment. (THF: Tetrahydrofuran)
Figure 2-4: **Surface characterization of microspheres by atomic force microscopy:** AFM image of the (A) surface topography of a hollow collagen sphere. Amplitude modulation AFM (B) amplitude and (C) phase images of the same sphere, displaying the fibrous structure present on the surface of the spheres. (B, C) Images have been 2nd-order flattened to subtract out the curvature of the sphere and reveal the surface structure.
Figure 2-5: Collagen microspheres negatively charged in PBS: Zeta analysis of collagen microspheres in PBS revealed that all three sizes were seen to be negatively charged, with the largest sized microspheres, 10 µm, being more negatively charged than either the 100 nm or 1 µm microspheres. Data represents mean ± SD (n=6). Analysis performed using one-way ANOVA, p<0.05. * denotes statistical significance 100 nm and 1 µm microspheres.
Figure 2-6: **Cell-microsphere interactions:** (A) Percentage viability compared with cell alone control as measured by the alamarBlue® assay, (B) fluorescent images of FITC-labelled hollow collagen microspheres incubated with 3T3 Fibroblasts for 96 hours (Red – Rhodamine-Phalloidin, Blue – DAPI, Green - FITC) showing no negative effects on the morphology of the cells. (C) SEM image of the interaction between 3T3 Fibroblasts and collagen microspheres. Data represents mean ± SD (n=3). Analysis performed using one-way ANOVA, p<0.05. * denotes statistical significance versus control.

[Note: Figure 3 (C) was produced in collaboration with Dr. Gianluca Fontana.]
Figure 2-7: Microspheres do not impact cell proliferation: pDNA content was measured using the PicoGreen™ assay. It can be seen that there is no significant effect on the proliferation rate of 3T3 fibroblasts over 48, 120 and 168 hours when compared to the control (cells grown on tissue culture plastic alone without any microspheres). Data represents mean ± SD (n=3). Analysis performed using one-way ANOVA, p<0.05.
Figure 2-8: **Characterisation of polyplex reservoir:** (A) Loading efficiency of polyplexes within three sizes of hollow collagen spheres shows no significant difference, while release profile in PBS over six days, (B), shows an ability to delay the release of the polyplexes over time. Data represents mean ± SD (n=3). Analysis performed using one-way ANOVA, p<0.05.
Figure 2-9: **Transfection and polyplex toxicity**: (A) Gaussia Luciferase assay to assess the ability of the microspheres to release bioactive polyplexes capable of transfecting cells *in vitro*. In this case, 1 µm and 10 µm spheres released polyplexes that transfected 3T3 fibroblasts with a similar ability to polyplexes alone. (B) 3T3 fibroblasts expressing GFP following treatment with polyplex-loaded spheres (Green – GFP, Blue - DAPI). (C) Cell metabolic activity is dramatically reduced following incubation with cationic polymers. However, this effect is removed following loading of the polymers with collagen microspheres (1 µm). Data represents mean ± SD (n=3). (PEI: Polyethyleimine, SF: dPAMAM, Superfect™, Qiagen). Analysis performed using one-way ANOVA, p<0.05.
**Figure 2-10: Confirmation of long-term bioactivity of polyplex following release from microspheres:** Transfection of 3T3 Fibroblasts after 48 hours following incubation with polyplexes release from spheres over 168 hours. While not as high as freshly made up polyplexes, the level of transfection detected is higher than that by pDNA alone. Data represents mean ± SD (n=3). (PEI: Polyethyleimine, SF: dPAMAM, Superfect™, Qiagen). Analysis performed using one-way ANOVA, p<0.05.
2.4 Conclusions

The results of this study demonstrate that hollow collagen microspheres can be fabricated using a consistent and efficient process. These microspheres displayed an ability to efficiently load a large amount of polyplexes and release them over a prolonged period of time. No cytotoxic effects of collagen microspheres were detected on 3T3 fibroblasts, while polyplex-loaded microspheres efficiently transfected 3T3 fibroblasts similar to as seen with polyplex alone control, and in addition, reduced the cytotoxic effect of large doses of polyplex when compared with a bolus dose. Hence, hollow collagen microspheres show potential for use as a reservoir system for the extended release of polyplexes, modulation of associated toxicity and extended transfection and gene expression.
2.5 References


Extracellular matrix based reservoir


Chapter Three

Development of an extracellular matrix based dual gene delivery reservoir

Sections of this chapter have been submitted for publication in

3.1 Introduction

The advent of improved molecular biology and diagnostic tools, in terms of protein and RNA expression profiles, has allowed for an increased understanding of disease pathways and pathologies. As a result, specific pathways have been identified that are crucial in the progression or resolution of particular disease states. Gene therapy is a promising therapeutic strategy by which these pathways can be up- or downregulated.[1] In this way, disease pathologies may be corrected and directed towards a more satisfactory outcome. Gene delivery typically involves the use of a vector, either viral or non-viral in nature. Safety issues, amongst others, have lessened enthusiasm for viral vectors, and thus non-viral delivery systems have been investigated. However, issues including lack of efficacy, high toxicity and short-term gene expression have dogged non-viral gene delivery strategies.[2]

The use of biomaterials to deliver therapeutic genes is an integral part of current research in regenerative medicine.[3] Biomaterials loaded with nucleic acids, or ‘gene-loaded biomaterials’, have shown much promise as a means to deliver bioactive nucleic acids. By the local delivery of gene-loaded biomaterials, many of the associated drawbacks of gene therapy may be overcome. That is, retention of the therapeutic gene in a biomaterial and its gradual release over time to the local microenvironment can increase gene expression. In addition, there is a reduction in the toxicity associated with the vector delivery system due to its delayed release when compared with bolus delivery.[4] Biomaterials, specifically collagen, have been used to achieve a sustained release of functional, bioactive nucleic acids both in vitro[5] and in vivo.[6,7] Despite the improved delivery of gene therapeutics achieved via biomaterial systems, issues remain with regards to the treatment of complex disease pathologies. In these cases, the delivery of single factors is insufficient to promote complete resolution of pathology and tissue repair. Thus, there has been a move towards strategies that allow for the delivery of multiple therapeutics that will prove more efficacious.[8] However, an important consideration is the relative timing of the delivery of
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each factor. Biomaterial systems offer an opportunity to control the delivery of therapeutics relative to one another, termed ‘multi-modal’ delivery. This may be achieved by a number of means. One approach seen often in the literature to achieve multi-modal delivery of therapeutics involves the use of reservoir systems embedded within a scaffold (sponge, fibre or hydrogel).[9,10] By differential loading of one factor within the scaffold and another in the spheres (which are then embedded within the scaffold) a staggered release pattern may be achieved.

Collagen type I is the main constituent of the extracellular matrix (ECM) and is a biomaterial suitable for both in vitro and in vivo applications due to the ability of cells to infiltrate, re-model and degrade it without the formation of toxic degradation products.[11] Biomaterial scaffolds fabricated from collagen type I have previously been functionalised with a range of therapeutics, such as proteins and nucleic acids.[12–14] Collagen has also been processed into microspheres and used to deliver growth factors and cells.[15–17] In addition, collagen microspheres can be used as a reservoir of pDNA, as has been described in chapter two and elsewhere.[18,19] By combining a collagen hydrogel system and a microsphere reservoir system, a multi-modal biomaterial system can be assembled with two separate release profiles. This assembled platform will enable the delivery of multiple therapeutics from a biodegradable, ECM-based system.

An important aspect when using microsphere-based delivery systems is the selection of appropriate sized spheres. When used as a reservoir, it is imperative that microspheres avoid phagocytosis by cells, particularly macrophages. Studies by Mitragotri et al. have shown the influence of size on the uptake of polystyrene particles by macrophages.[20,21] The surface features of macrophages, particularly membrane ruffles, appear to play a vital role in the ability of macrophages to distinguish between specific sizes of particles. This size effect has significant ramifications for particulate drug delivery systems such as microspheres. In addition, the uptake of spheres effects are likely to be material as well as size-dependent.
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Therefore it is important to identify a size of collagen microspheres that is not readily uptaken by macrophages.

A collagen ‘sphere-in-hydrogel’ system may be used to deliver different nucleic acids to modulate complex pathologies. For example, inflammation and angiogenesis are two pathways often dysregulated during pathological states such as diabetic wound healing, ischemia and during a chronic foreign body response (FBR). The inflammatory cascade results in an increase in tissue macrophages and neutrophils in the local microenvironment, as well as an upregulation in the production of a range of pro-inflammatory cytokines. These pro-inflammatory cytokines include tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6). IL-6 is a key pro-inflammatory cytokine involved in the inflammatory cascade and infiltration of inflammatory cells.[22,23] IL-6 in particular plays a role in the recruitment of monocytes and neutrophils, and the differentiation of monocytes into macrophages.[24] IL-6 signalling may be either classical (through the membrane bound IL-6 receptor) or via trans-signalling. Trans-signalling involves the soluble IL-6 receptor (sIL-6R). Signalling via the sIL-6R has been shown to mediate the pro-inflammatory functions of IL-6, including inhibition of T-cell apoptosis and differentiation of regulatory T-cells.[25–27] Compromised angiogenesis is detrimental from the point of view of nutrient delivery and the removal of waste products of metabolism from tissue. Approaches to increase angiogenesis include the delivery of factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). Endothelial nitric oxide synthase (eNOS) is a pro-angiogenic enzyme that acts as a catalyst in the reaction of L-arginine to L-citruline, resulting in the formation of nitric oxide (NO).[28] NO promotes relaxation of blood vessels and the mobilization of endothelial progenitor cells (EPCs), an important step in the formation of new blood vessels.[29,30] In addition, NO has been shown to reduce IL-6 expression at high doses, in an NF-κB dependent pathway.[31] In contrast, at lower concentrations, NO has a stimulatory effect on NF-κB, exerting a pro-inflammatory effect.
Thus, the hypothesis of this phase of the study is that an appropriately sized collagen microsphere can be identified that will not be uptaken by activated macrophages. By assembling this size of collagen microsphere within a collagen hydrogel, a multi-modal dual-delivery nucleic acid system will be formulated. This system may be used for the delivery of an IL-6 targeting siRNA, and subsequently an eNOS encoding pDNA, which allows for the modulation of an inflammatory gene and a pro-angiogenic gene in a temporal manner.

Therefore, the objectives of this study were:

1. To identify a size of collagen hollow microsphere that is not uptaken by activated macrophages and, hence, is suitable to act as a reservoir system.
2. To assemble the identified suitable size of collagen hollow microsphere within a 4S-PEG crosslinked collagen hydrogel to fabricate a multi-modal release system.
3. To confirm the ability of IL-6 siRNA and eNOS pDNA loaded multi-modal collagen systems to act as reservoirs for gene therapy \textit{in vitro} by assessing the mRNA expression of IL-6 and eNOS in primary cardiac fibroblasts after 3, 7 and 14 days of \textit{in vitro} culture.

\section*{3.2 Materials and methods}

\subsection*{3.2.1 Extraction of collagen}

Type 1 atellocollagen was isolated as previously described\cite{32}. Briefly, bovine tendons were blended, washed in buffer (3.7mM Na$_2$HPO$_4$, 0.35Mm KH$_2$PO$_4$, 51Mm NaCl) and suspended in 0.5M acetic acid. The resulting solution was then pepsin treated (1 g pepsin: 100 g tendon) and filtered to remove insoluble collagen telopeptides. The soluble collagen was then purified by repeated salt precipitation (0.9M NaCl) and centrifugation, followed by dialysis against 0.01M acetic acid. (procedure described in detail in appendix A)
3.2.2 Fabrication of collagen microspheres

Hollow collagen microspheres were fabricated using the template method as previously described in chapter two.[18,19,33–35] Briefly, commercially available polystyrene beads (Gentaur, Chicago, Illinois) of defined sizes (100nm, 1 μm and 10 μm in this case), were sulfonated to impart a strong negative charge. Following sulfonation, beads were resuspended in 0.5M acetic acid. 5mg/ml of collagen solution was added to the beads at a weight ratio of 4:1 (beads: collagen). The resultant mixture was then stirred for four hours at room temperature. Crosslinking of collagen coating was performed using pentaerythritol poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-PEG), with the active ester groups reacting with the free amino groups of collagen at a ratio of 1:4 (4S-PEG:free amino groups in the collagen). The mixture was agitated for two hours at room temperature. To produce a hollow sphere, the polystyrene core was dissolved by washing the coated beads with tetrahydrofuran (THF). The suspension of microspheres was diluted at a ratio of 1:1 with THF and agitated for one hour. The washing step was repeated twice to ensure complete removal of polystyrene. Hollow spheres were washed twice with ethanol and twice with water to ensure removal of any remaining THF. Spheres of sizes of 100 nm, 1 μm and 10 μm were assessed.

3.2.3 Macrophage differentiation and activation

The human monocytic cell line THP-1 was obtained from the American Type Culture Centre (ATCC). The cells were maintained in RPMI 1640 (GIBCO- BRL) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a 5% CO₂ humidified incubator. The mature macrophage-like state was induced by treating THP-1 cells for 24 h with phorbol 12-myristate 13 acetate (PMA) at 100 ng/ml diluted in serum-free medium. Cells were seeded at a density of 100,000 cells per well in a 48-well plate. The following day, plastic-adherent cells were washed twice with cold, sterile Dulbecco’s PBS and incubated with fresh RPMI-1640 lacking PMA but containing 1% FBS,
penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were then activated with 100 ng/ml lipopolysaccharide in RPMI-1640 medium containing 1% FBS.

3.2.4 Primary cardiac fibroblast isolation

Primary rat cardiac fibroblasts were isolated from neonatal pups as previously described[36]. Briefly, neonatal rat ventricle myocytes were isolated from the cardiac ventricles of three to five days old Sprague-Dawley pups. Hearts were removed from the thoracic cavity and placed in a tube containing cold HEPES solution (20 mM HEPES, pH 7.4). Ventricles were separated from cardiac tissue using scissors and minced into several pieces. Subsequently cardiomyocytes and fibroblasts were detached from the extracellular matrix by repeated incubation in collagenase, supplemented with 2 mg/ml trypsin and 20 μg/ml DNase. Cells were collected by centrifugation and tissue clumps were removed by filtration. Subsequently, cells were pre-plated in cell culture dishes in 50 ml DMEM/F12 with 5% fetal calf serum (FCS) for 45 minutes. During this period, most non-cardiomyocyte cells (mainly fibroblasts) attached to the dish, whereas cardiomyocytes remained in solution. Fibroblasts were subsequently cultured in DMEM/F12 medium containing 10% FCS. (see appendix P)

3.2.5 Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial basal medium (EBM-2) supplemented with the endothelial growth medium (EGM-2) bullet kit. Cells were cultured in T75 flasks at 37°C in a 5% CO₂ humidified incubator. The cells were harvested and sub-cultured when >80% confluency was observed. All experiments were performed on cells between passage three and six.

3.2.6 FITC-labelling of collagen microspheres

Collagen microspheres were labeled with fluorescein isothiocyanate (FITC) as described in appendix O. Briefly, microspheres were suspended in PBS and an equal volume of 2 mg/ml FITC in dimethyl sulfoxide (DMSO) was added such that the final FITC volume was 1 mg/ml. The reaction was allowed to occur at room temperature for four hours, after which a
minimum of five washing steps is required until the supernatant is clear. Labelled-spheres were stored in the dark until required.

3.2.7 **Collagen hollow sphere uptake by activated THP-1 and primary fibroblasts**

THP-1 cells were differentiated and activated as described above. First, 300 μg of FITC-labelled microspheres of three sizes was added to 250,000 cells and incubated for 24 h. The cells were then rinsed with Hanks’ balanced salt solution (HBSS) three times to remove any spheres that were not internalized. A cell scraper was used to detach the cells from the surface, which were then fixed with 4% PFA and examined by flow cytometry (BD FACS Canto 6) for internalization. 2x10⁴ cells were analyzed in each sample, and the data processed using FlowJo™ software. This was repeated for primary fibroblasts. The three sizes of microspheres examined were 100 nm, 1 μm and 10 μm. A log scale difference in sizes was chosen in order to see clear differences in size-dependent uptake.

3.2.8 **Dual-release collagen scaffold fabrication**

A dual release system was fabricated by combining hollow collagen microspheres with a collagen hydrogel. Polyplex-loaded microspheres were mixed and re-suspended in a neutralized collagen/4S-PEG crosslinker solution containing a second dose of nucleic acid, prior to gelation. See figure 3-1 for a graphical illustration of the process.

3.2.9 **Cell viability and proliferation**

The effect of the dual-release collagen scaffold on the metabolic activity of primary cardiac fibroblasts and HUVECs was quantified using the alamarBlue® cell metabolic activity assay. 200μg of 1μm microspheres were incorporated into 250 μl of collagen gelling solution and plated on the bottom of the wells of 24 well plates. This was allowed to gel for 30 minutes before 5x10⁴ cells were added. After 48 hours the effect on metabolic activity was measured and compared with the control (cells grown on tissue culture plastic alone). Scaffolds were then degraded with proteinase K at 56°C overnight. The resulting supernatant was analysed.
for DNA content using the PicoGreen® assay as per the manufacturers instructions, and, compared with the control.

3.2.10  *Dual-release collagen scaffold degradation*

The degradation of the collagen scaffold was characterized using a modified Coomassie Brilliant Blue™ (CBB) assay. CBB is anionic and has high affinity for proteins. Thus, the enzymatic degradation by collagenase is correlated with the release of Coomassie-bound collagen. The collagen hydrogels were fabricated (with and without the incorporation of 1 μm collagen microspheres) and stained in 0.1% CBB solution, followed by de-staining and washing with H2O. Collagenase was added in Tris-HCl buffer at a concentration of 10 U/mg collagen. The samples were incubated at 37°C and the supernatant collected and replaced at various timepoints. When fully degraded, the collected supernatant was analysed spectrophotometrically at a wavelength of 595 nm. Data was normalized to the spectrophotometric measurements of completely degraded hydrogels. Non-crosslinked and glutaraldehyde-crosslinked hydrogels were used as controls.

3.2.11  *In vitro IL-6 siRNA silencing studies*

Primary rat fibroblasts were seeded in a 24-well plate at a density of 1x10^5 cells per well. After 24 hours, 0.25 μg of IL-6 siRNA was added in 500 μl DMEM/F12 media, either using a commercially available liposomal transfection reagent, Dharmafect™. IL-6 siRNA was also delivered in naked, unprotected form. A non-targeting siRNA was used as a negative control. After four hours, the media was replaced. After a further 48 hours, the cells were processed for RT-PCR analysis.

3.2.12  *Agarose gel electrophoresis*

Electrophoresis was performed to assess polyplex formation between the eNOS pDNA and a transfection reagent composed of a partially degraded polyamidoamine (dPAMAM), known commercially as Superfect™. This transfection reagent was chosen owing to its favourable transfection capability as compared with a range of non-viral transfection agents.
An extracellular matrix based dual gene delivery reservoir

Polyethylenimine (PEI, MW=25,000), a branched polycation, was used as a positive control and polyplexes formed at a weight-to-weight ratio of 1:1 with eNOS pDNA. Briefly, polyplexes were formed and mixed with a proportional volume of loading dye. Samples were pipetted into the wells of a 0.7% agarose gel (with Syber®Safe dye incorporated) and a voltage of 100 V applied. When the gel was finished running, it was visualized under UV light to observe the movement of the complexes towards the negative terminal.

3.2.13 In vitro eNOS up-regulation

Human umbilical vein endothelial cells (HUVEC) were seeded in a 24-well plate at a density of 1x10⁵ cells per well. After 24 hours, 1 μg of eNOS polyplexes was added in 500 μl of endothelial basal media with supplements. The eNOS pDNA was delivered using the dPAMAM transfecting agent, using three different weight-to-weight ratios. PEI was used as a positive control and polyplexes formed at a weight-to-weight ratio of 1:1 with eNOS pDNA. After four hours of incubation, the media was replaced. After a further 48 hours, media was removed and assessed for nitrate content using the Griess assay and the cells were processed for RT-PCR analysis.

3.2.14 Nitrate content

Nitrate content was determined by using the Griess assay and comparing samples with a sodium nitrate standard curve. Briefly, powdered Griess reagent was re-suspended with distilled water and 50 μl added to samples. Following 5-10 minutes of incubation in the dark, absorbance was read at 540 nm. By comparing with the standard curve of sodium nitrate, the nitrate concentration can be determined. (See appendix V).

3.2.15 Labelling of plasmid

Gaussia princeps luciferase plasmids (pCMV-GLuc; New England Biosciences, Ipswich, USA) were propagated and isolated using standard techniques, as described elsewhere[5]. These plasmids were then fluorescently labelled with the Cy5 dye using a Cy5 labeling kit (Mirus, Madison, USA). Briefly, the dye was incubated with the plasmid in the provided
buffers for one hour. Following the incubation, the plasmid was eluted through a microspin column to remove any unbound dye. The recovered labelled plasmid was then stored in a light-protected environment at -20°C until use.

3.2.16 Loading of microsphere reservoirs with polyplexes

eNOS pDNA complexed with the optimized weight-to-weight ratio of dPAMAM was loaded within 1 μm microspheres, as described in chapter 2. Briefly, 100 μg of 1 μm collagen microspheres was resuspended in 500 μl of phosphate buffered saline (PBS). To this, 2 μg of complexed pDNA was added. This mixture of microspheres and labelled complexes was agitated on a mechanical shaker for four hours at room temperature. The microspheres were spun down and the supernatant removed.

3.2.17 Release study

The release profile of Cy3 labelled siRNA and pDNA was characterized in PBS at 37°C. Polyplex-loaded microspheres were mixed and re-suspended in a neutralized collagen/4S-PEG crosslinker solution containing an siRNA, prior to gelation. The combination of loaded-microspheres and 250 μl of siRNA-containing gel-forming solution was pipetted into 24-well plate, and allowed to gel. After 15 minutes, 250 μl of PBS was added to the gels. The supernatant was replaced at each timepoint. The collected supernatant was frozen until it was assayed for polyplex content by measuring fluorescence.

3.2.18 Scaffold-mediated transfection in vitro

The transfection capability of the dual-release collagen scaffold was assessed in vitro using primary fibroblasts extracted from the myocardium of neonatal rat pups. 1x10^6 cells were plated in the wells of six well plates. After 24 hours, 250 μl of collagen gelling solution with microspheres within it were added to the cells and allowed to gel for 30 minutes. The hydrogel portion of the scaffold was loaded with 1 μg of IL-6 siRNA, while the microspheres within the hydrogel were loaded with 2 μg of eNOS pDNA. A non-loaded collagen hydrogel/microsphere scaffold was used as a control. 1 ml of DMEM/F12 media
An extracellular matrix based dual gene delivery reservoir containing FBS was added following gelation. The media was changed every three days. Samples were processed for RNA analysis at 3, 7 and 14 days.

### 3.2.19 Extraction of RNA

RNA extraction was performed on samples at 3, 7 and 14 days. One mL of TRI Reagent® (Applera Ireland, Dublin, Ireland) was added to each construct and incubated for five minutes at room temperature. Scaffolds were mechanically disrupted using a sterile pipette tip. Phase separation was performed by adding chloroform (Sigma-Aldrich), and total RNA was purified using an RNeasy™ kit (Qiagen), according to the supplier’s recommended procedure (Appendix Y).

### 3.2.20 Real-time Reverse Transcription Polymerase Chain Reaction

RNA quantity and purity were determined spectrophotometrically at 260 and 280 nm using an ultraviolet spectrophotometer (NanoDrop™ ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription (RT) was performed using the ImProm-II™ RT system according to the manufacturer’s protocol (Promega, Southampton, United Kingdom, Appendix T). Gene transcription was examined using real-time RT polymerase chain reaction (PCR). Reactions were performed and monitored using an ABI 7000® sequence detection system (Applied Biosystems, Foster City, CA) using TaqMan® Real-time Gene Expression Mastermix (TaqMan, Applied Biosystems) and specific primers which are detailed in Table 3.1. The primers were designed and their specificity checked using primer-BLAST (available freely from www.ncbi.com) and their efficiency determined by RT-PCR on ten-fold serial dilutions of template cDNA. Gene transcription was normalized to transcription of the housekeeping gene; glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression for each target gene (Appendix AA).
3.3 Results and discussion

3.3.1 Selection of an optimal microsphere size

Flow cytometry was used to assess the uptake of hollow collagen microspheres. Activated and differentiated THP-1 cells had a greater uptake of 100 nm microspheres compared with either of the 1 μm or the 10 μm microspheres (27.9% for 100 nm microspheres compared with 6.1% and 3.1% for 1 μm or 10 μm microspheres, respectively, as seen in figure 3-2 (A)). A similar trend was observed with primary fibroblasts, with much greater uptake of 100 nm microspheres over that of either 1 μm or 10 μm microspheres (25.2 % for 100 nm microspheres compared with 8.6 and 5.9% for 1 μm or 10 μm microspheres, respectively, as seen in figure 3-2 (B)). Thus from these studies the 100 nm microspheres are not suitable to act as a reservoir system since they will be readily internalized by cells such as activated macrophages and may be cleared from the target tissue in which they are implanted. The ultimate aim of this microsphere-based reservoir system is to avoid uptake and remain external to cells, thus maintaining an ability to deliver their loaded cargo to multiple cells rather than being uptaken and release the loaded cargo to single cells.

In addition, although microspheres have been shown to reduce the toxicity associated with polymeric gene vectors, the high loading capacity of microspheres can cause toxicity if a large number of loaded microspheres were uptaken into a single cell and released their cargo of highly charged polymeric vectors. Microsphere uptake is dependent on shape and size, as many studies have reported, using polystyrene particles.[20,21] The results presented in this chapter agree in this respect, with a difference observed in uptake with regards to smaller particles versus that of a larger size and an intermediate. Therefore it seems that the most appropriate size to use is the 1 μm or 10 μm microspheres, rather than the 100 nm microspheres. However, no statistical difference was observed in the uptake of 1 μm microspheres compared with that of 10 μm microspheres.

In terms of loading of pDNA per mg of microspheres, no difference was observed between 1 μm and 10 μm collagen microspheres (see Chapter 2). However, given the difference in
size, 1 mg of 1 μm collagen microspheres would contain more microspheres than 1 mg of 10 μm collagen microspheres. In addition, the distribution of microspheres, and thus of loaded pDNA, will be more uniform throughout the hydrogel using the more abundant 1 μm collagen microspheres than the 10 μm collagen microspheres. Figure 3-3 indicates that 10 μm microspheres may form aggregates during their attempted dispersion throughout a hydrogel. Also, each individual 10 μm collagen microsphere will also contain more pDNA than a 1 μm collagen microsphere. This will imply a more uniform release profile of pDNA from the assembled sphere/hydrogel system. Thus, from an engineering design perspective, the 1 μm collagen microspheres are a more appropriate choice. In addition, it has been suggested that macrophages can become ‘frustrated’ when they cannot internalize a particle due to its large size, and that this can cause an increased inflammatory response as the macrophages releases various reactive oxygen species (ROS) and hydrolytic enzymes in an attempt to destroy the particle it cannot engulf.[37,38] This phenomenon has been observed with carbon nanotubes of length greater than 5 μm.[39] Thus, the 1 μm collagen microspheres are a suitable choice to assemble into a sphere/hydrogel delivery system.

3.3.2 Scaffold characterization

Collagen is an ECM protein that has been used extensively to create scaffolds in the fields of biomaterials, tissue engineering and regenerative medicine.[40–43] The use of collagen owes largely to its relatively low toxicity, natural biodegradation by enzymes and bioactive nature.[11] Cells will readily populate and remodel a collagen scaffold. Despite this, an established drawback of collagen scaffolds is their rapid biodegradation in vivo. In an effort to overcome this speedy degradation, as well as to improve mechanical properties and reduce swelling, crosslinking of collagen is commonplace. However, crosslinking can alter the cell response to a collagen matrix, as has been shown both in vitro and in vivo in a recent study.[44] Ideally, crosslinking of a collagen matrix will extend the lifetime of the construct by retarding degradation, but at the same time have no associated toxicity or negative impact on cells that interact with it. Glutaraldehyde (GTA) is a crosslinking agent that has been used
routinely to preserve implants, particularly heart valve replacements that are typically decellularized and GTA-fixed prior to implantation. However, GTA has toxic effects on cells and is not an ideal crosslinking agent for implantable biomaterials. Collagen hydrogels crosslinked in situ with 4S-PEG have previously been utilised for the delivery of stem cells *in vitro* [45] and *in vivo* [46]. In addition, a collagen hydrogel crosslinked with 4S-PEG was previously used to deliver miRNA-29B in a full thickness murine skin model [6]. In all cases, the 4S-PEG reacts with the free amines in the collagen at an optimal 1:4 ratio. This optimal ratio has been utilized in this study. However, 1 μm collagen microspheres have also been added to the hydrogel solution prior to gelation to add additional functional capability to the system. The effect of 4S-PEG crosslinked collagen hydrogel, with and without the addition of 1 μm collagen microspheres, on the metabolic activity and proliferation of primary fibroblasts and HUVECs was assessed to ensure no toxicity resulted from incorporation of microspheres. There was no negative impact on metabolic activity or proliferation by either the crosslinked hydrogel alone or when 1 μm collagen microspheres were incorporated within it on either cell type, as measured by the alamarBlue® or PicoGreen® assays, respectively (figure 3-4 and 3-5). Thus the 4S-PEG crosslinked-hydrogel combined with 1 μm collagen microspheres was deemed non-cytotoxic when tested with primary fibroblasts or HUVECs.

The primary function of crosslinking is to increase the lifetime and delay the biodegradation of collagen-based biomaterials by restricting access of collagen-degrading enzymes to specific cleavage sites. In this regard, the degradation of the collagen hydrogel was assessed, using a modified CBB assay. Collagen hydrogels were challenged with collagenase (10 U per mg collagen) and the degradation monitored over 144 hours. The 4S-PEG crosslinked hydrogel was analysed both with and without the incorporation of 1 μm microspheres to observe if this affected enzymatically-driven degradation. A GTA crosslinked hydrogel and a non-crosslinked hydrogel were used as positive and negative controls, respectively. After 24 hours there was a significant increase in degradation of the non-crosslinked scaffold
compared with all other groups (figure 3-6 (A)). No difference was detected between the GTA crosslinked collagen hydrogel, the 4S-PEG crosslinked collagen hydrogel or the 4S-PEG crosslinked collagen hydrogel with 1 μm collagen microspheres incorporated within it at 24 hours. When the degradation was stretched over 144 hours, it was observed that all but the GTA crosslinked hydrogels were fully degraded at 96 hours (figure 3-6 (B)). The GTA crosslinked hydrogel was fully degraded after 144 hours. Thus, it appears that while no significant difference was detected between 4S-PEG crosslinked hydrogels and GTA crosslinked hydrogels at 24 hours, this difference becomes apparent from 48 hours. No negative impact was observed on the degradation of a 4S-PEG crosslinked collagen hydrogel with or without the incorporation of 1 μm collagen microspheres. Thus, the addition of 1 μm collagen microspheres to the 4S-PEG crosslinked collagen hydrogel did not impact on its stability when challenged with collagenase.

3.3.3 IL-6 silencing and eNOS upregulation

To confirm the ability of the designed siRNA to specifically silence IL-6 expression in vitro, RT-PCR was performed on IL-6 siRNA treated and non-targeting control siRNA (NTC) treated primary fibroblasts. A non-targeting control is necessary to rule out non-specific silencing due to the presence of double-stranded RNA. As expected, the non-targeting control (NTC) siRNA had no significant effect on silencing IL-6 mRNA expression, while naked IL-6 siRNA (N) and IL-6 siRNA delivered via the liposomal transfection reagent Dharmafect™ (DF) showed a significant reduction in IL-6 mRNA expression (figure 3-7 (A)). An important aspect with gene delivery vectors, in this case the liposomal-based Dharmafect™ reagent, is the possibility of cell death or toxicity due to their use. However, in this case, no toxicity was observed after 48 hours (figure 3-7 (B)). This may be attributed to the set-up of the in vitro knockout experiment, in which the siRNA is added to the monolayer for four hours, after which the media is replaced with fresh media. Thus, toxicity is minimized. In comparison with an in vivo setting in which the siRNA and transfecting agent are not removed after a defined period of time, this is not a realistic situation.
However, the purpose of this experiment was to confirm the siRNA does target and knockdown IL-6 mRNA. To ensure an accurate result minimum cell metabolic activity of 80% is required. In this case cell metabolic activity was unaffected and, thus, the result is valid.

Transfection of pDNA using polymeric systems requires optimization of the weight ratio of pDNA to the polymer in question. The appropriate ratio is required to form a positively charged particle that will be uptaken into the cell and delivered to the nucleus for translation. However, it is often a balancing act to increase the transfection but to minimise the polymer-associated toxicity. In this study, three ratios of dPAMAM were used to deliver the eNOS gene to HUVECs. It is important to maximize transfection and at the same time, minimize vector-associated toxicity. PEI, a commonly used transfection agent for pDNA, was used as a control despite its known toxic effect, while naked, uncomplexed pDNA was used to show the need for a complexing agent to deliver pDNA. Agarose gel electrophoresis was used to show the complexation of eNOS pDNA by either dPAMAM (3:1, 6:1 and 9:1 polymer-to-pDNA weight ratio) or PEI (1:1 polymer-to-pDNA weight ratio). It can be seen in figure 3-8 that the naked pDNA migrates down towards the cathode, while there is no evidence of this in any of the other lanes in the gel. In fact, only in the 3:1 dPAMAM lane can a signal be seen. This has been observed previously with both dPAMAM and PEI, and is explained by the fact that these transfecting agents bind the pDNA so tightly that there is little if any signal from the Sybr®Safe nucleic acid stain.[47] Thus, at the 3:1 ratio, the complex has a net positive charge but the pDNA is not so tightly compacted that the stain is unable to interact with the pDNA. However, at the higher dPAMAM ratios, and using PEI, the stain is unable to intercalate with the pDNA and produce a signal. This has been observed with the PicoGreen® dye, which is also an intercalating stain.[47]

To assess the ability of these complexes to deliver the eNOS gene, RT-PCR analysis and the Griess assay was used. RT-PCR analysis indicated an increase in eNOS mRNA expression following treatment with the eNOS pDNA (figure 3-9(A)). As expected, naked eNOS pDNA
did not induce an increase in eNOS mRNA expression compared with the control. Using dPAMAM, there was an increase in eNOS mRNA expression at the 6:1 and 9:1 weight ratios (44.7 and 77.5 fold increase, respectively), with only a minimal increase at the 3:1 ratio (4.2 fold increase). Using PEI, there was an increase in eNOS mRNA, but less so than using the 6:1 and 9:1 dPAMAM ratios (30.3 fold increase). In addition, the use of PEI resulted in a reduction in metabolic activity that was not observed with dPAMAM. Despite this, there was a trend towards a reduction in cell metabolic activity at the higher dPAMAM ratios (figure 3-9(B)). Thus, no higher ratios of dPAMAM-to-pDNA were explored.

Functionally, delivery of the eNOS gene increases the production of NO as the eNOS enzyme catalyses the reaction of L-arginine to citruline with NO produced as a product. However, NO has a very short half-life (just a few seconds) and is quickly converted to either nitrites (NO⁻₂) or nitrates (NO⁻³).[48,49] The Griess assay detects only nitrates, which is a limitation of its use. However, it still gives an indication of the relative eNOS activity. The nitrate levels followed a somewhat similar trend to the eNOS mRNA levels previously discussed. The use of naked eNOS pDNA resulted in no difference in nitrate levels versus the control (both 0.6 μM), while the use of a transfection agent resulted in a significant increase (figure 3-10). The 3:1 ratio of dPAMAM resulted in a greater increase relative to that which had been observed in the mRNA analysis (5.1 μM). The 6:1 and 9:1 dPAMAM ratios resulted in greater levels of nitrate produced than the 3:1 ratio (8.1 μM and 11.6 μM, respectively), while eNOS pDNA delivered using PEI resulted in a similar level of nitrate as the 3:1 dPAMAM ratio (5.3 μM). As with the mRNA analysis, the 9:1 dPAMAM ratio is the best performing group. Coupled with the fact that the measurement of metabolic activity suggests only minimal associated toxicity, the 9:1 dPAMAM ratio is the optimal ratio for the delivery of eNOS pDNA.

Following confirmation of the knockdown of IL-6 using siRNA and the optimization of the delivery of eNOS pDNA using dPAMAM, these two nucleic acids were added to the composite collagen hydrogel/microsphere system and the release profile and system-
mediated transfection assessed using primary fibroblasts. The release profile was characterized using labeled siRNA and pDNA. Previous studies have shown that when using complexed pDNA and siRNA, methods using intercalating dyes are not suitable.[50] However, using labeled nucleic acids produces standard curves with suitable correlation coefficients and thus the data may be reliably extrapolated. The nucleic acid-loaded scaffold was produced as has been previously mentioned. Briefly, the microspheres were loaded with 2 μg of eNOS pDNA complexed with dPAMAM (optimal 9:1 ratio). These loaded microspheres were then re-suspended within a collagen gelling solution along with the 4S-PEG crosslinker and 1 μg of IL-6 siRNA, either complexed with Dharmafect™ or in naked, uncomplexed form. Prior to gelation, the solution was added to a 24 well-plate and allowed to gel. When gelation had occurred, 250 μl of 1XPBS was added. This supernatant was replaced daily for the first seven days, and then every two days up to day 15. A clear difference can be seen in the release profile of the IL-6 siRNA versus that of the eNOS pDNA (figure 3-11). This is due to loading different components within different phases of the system (siRNA in hydrogel versus pDNA in microspheres). This is evident from the fact that the release of pDNA from within the hydrogel portion of the system follows a similar trend to that of siRNA, as can be seen in figure 3-12 where release of dPAMAM complexed eNOS pDNA from within different phases of the system (hydrogel and spheres) is directly compared. It can be seen that the release profile is dependent upon which phase within the system the eNOS pDNA is loaded.

In addition to the difference between the release from within each phase of the system, a small difference is observed in the release profile of naked siRNA versus Dharmafect™ complexed siRNA, with the Dharmafect™ complexed siRNA being released slightly quicker than the naked siRNA. This may be attributed to the interaction between the nucleic acid and the scaffold itself. The naked siRNA has an overall negative charge, while the Dharmafect™ complexed siRNA has an overall positive charge. The scaffold itself, following crosslinking of the amine groups, has an overall negative charge due to the presence of free carboxyl
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(COOH-) groups. Hence, a delay in the release of uncomplexed versus complexed siRNA can be explained. A further point is that at 15 days the scaffold has not fully degraded and only 60% of the siRNA is released. This may be due to direct interaction with remaining free amine groups in the case of the naked siRNA, or the carboxyl groups in the case of the complexed siRNA. However, this is may be a minor issue if the scaffolds has already degraded by then, as one expects the case to be in an in vivo setting with the presence of various collagen-degrading enzymes.

A key property of any nucleic acid delivery system is the ability of the released nucleic acids to remain bioactive and transfect cells, whether that be in vitro or in vivo. The first step is always to assess scaffold-mediated transfection in vitro. Scaffolds were fabricated with IL-6 siRNA and eNOS pDNA incorporated, as previously described and added to primary fibroblasts. The mRNA levels of IL-6 and eNOS were assessed at 3, 7 and 14 days (figure 3-13). It was observed that there was a minimal reduction in IL-6 mRNA expression at both 3 and 7 days, with no significant difference observed (figure 3-13 (A)). In fact, there was an increase in IL-6 mRNA expression using Dharmafect™ complexed siRNA at 3 days (121.3 %). However, this was reduced at 7 days to a level similar to that of the control (78.7 %), and at 14 days using either Dharmafect™ complexed IL-6 siRNA or naked IL-6 siRNA (both delivered via a collagen hydrogel/microsphere scaffold) there was a significant reduction in IL-6 mRNA expression (42.9 and 48.5 %, respectively). Again, the effect of degradative enzymes such as matrix metalloproteinases (MMPs) will alter the release profile and subsequently the IL-6 expression pattern. Thus the release pattern is expected to be quicker in the in vivo situation due to accelerated degradation, and this will lead to a greater release of IL-6 siRNA, and thus a more effective IL-6 knockdown.

The expression of eNOS mRNA was also followed over 14 days following incubation of fibroblasts with the composite hydrogel/microsphere system. An increase was observed at three days in both scaffolds delivering eNOS pDNA compared with scaffolds without eNOS pDNA (figure 3-13 (B)). At seven days there was much increased eNOS expression relative
to day three, showing the effect of the gradual release of eNOS pDNA over time. Importantly, this level of expression was maintained at 14 days. No effect was observed on eNOS mRNA levels whether IL-6 siRNA was co-delivered using the Dharmafect™ transfecting agent or as a naked nucleic acid. This validates the effectiveness of the system at inducing and maintaining eNOS expression over 14 days through the gradual release of eNOS pDNA.

3.4 Conclusions

The 1 μm sized hollow collagen microspheres were identified and selected for minimal uptake by cells, particularly activated macrophages. The incorporation of microspheres within a collagen hydrogel was found to have no effect on either cell vability, cell proliferation or the degradation profile. The release profile of siRNA and pDNA from a composite collagen hydrogel/microsphere system was assessed. It was observed that two distinct release phases could be achieved by differential loading within either the hydrogel or microsphere portion of the system, with the cargo loaded directly into the hydrogel being released quicker than that loaded within the microspheres, as expected. Furthermore, confirmation of the bioactivity of nucleic acids following release from this collagen system was achieved by monitoring the expression of IL-6 and eNOS mRNA in primary fibroblasts over 14 days. It was observed that IL-6 knockdown was gradually achieved over 14 days, while eNOS upregulation occurred at 3 days but was markedly increased at 7 days and maintained up to 14 days. Delivery of siRNA was at a similar level with or without the use of a complexing agent (Dharmafect™), indicating that the collagen scaffold itself aids as a delivery vehicle, both protecting and modulating the release of two different nucleic acids.
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Figure 3-1: Assembly of multi-modal collagen system. Polyplex-loaded collagen microspheres are re-suspended in a neutralised collagen solution, loaded with siRNA, prior to gelation. The pre-gelling solution may be taken into a pipette or syringe and delivered as required. The polyplexes in the spheres (red) are released slower than the siRNA in the gel (blue), as indicated in the diagram.
Figure 3-2: **Uptake of collagen microspheres after 24 hours.** FACS analysis of the uptake of different sizes of collagen microspheres following 24 hours of incubation with (A) differentiated and activated THP-1 monocytes and (B) primary fibroblasts extracted from neonatal rat pups. Data represents mean ± SD (n=3). Analysis performed using one-way ANOVA, p<0.05. * denotes statistical significance versus other groups.
Figure 3-3: Distribution of 10 μm FITC-labelled collagen microspheres within a collagen hydrogel. Figure (A) shows a aggregate of microspheres, while figures (B) and (C) show more evenly distributed and isolated microspheres. Scale bar indicates 25 μm.
Figure 3-4: Effect of collagen sphere-in-hydrogel system on HUVEC. (A) Cell metabolic activity and (B) proliferation after 48 hours incubation, as measured by alamarBlue® and Picogreen® assays, respectively. Data represents mean ± SD (n=3). Analysis performed using one-way ANOVA, p<0.05.
Figure 3-5: Effect of collagen sphere-in-hydrogel system on primary fibroblasts. (A) Cell metabolic activity and (B) proliferation after 48 hours, as measured by alamarBlue® and PicoGreen® assays, respectively. Data represents mean ± SD (n=3). Analysis performed using one-way ANOVA, p<0.05.
Figure 3-5: **Degradation profile of collagen sphere-in-hydrogel system.** (A) Weight loss after 24 hours treatment with collagenase (10 U/mg collagen) shows no statistical difference between GTA crosslinked hydrogel and 4S-PEG crosslinked sphere-in-hydrogel system. (B) Hydrogel degradation profile over seven days treatment with collagenase (10 U/mg collagen), with little difference observed between 4S-PEG crosslinked hydrogel with and without 1 μm collagen microspheres incorporated within it. Data represents mean ± SD (n=3). Analysis performed using one-way ANOVA, p<0.05. * denotes statistical significance versus control non-crosslinked group.
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Table 3-1: **Primer sequences.** Primer sequences used in this study for gene expression analysis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6_Forward</td>
<td>ATACCACCCCAACAACAGACC</td>
</tr>
<tr>
<td>IL-6_Reverse</td>
<td>GCACAACTCTTTTTCATTTC</td>
</tr>
<tr>
<td>eNOS_Forward</td>
<td>CTGAGAGACCAGCAGAGATACCAC</td>
</tr>
<tr>
<td>eNOS_Reverse</td>
<td>CTGAAGCTCTGGGTCCTGAT</td>
</tr>
<tr>
<td>GAPDH_Forward</td>
<td>GTCAGCCGCATCTTTTGTGC</td>
</tr>
<tr>
<td>GAPDH_Reverse</td>
<td>GCGCCAATACGACCAAATC</td>
</tr>
</tbody>
</table>
Figure 3-6: Interleukin-6 (IL-6) knockdown and viability of primary fibroblasts after 48 hours. (A) mRNA expression of IL-6 is down following treatment with either naked IL-6 siRNA or Dharmafect® (DF) complexed IL-6 siRNA. No effect was seen with a non-targeting control (NTC) siRNA. (B) No effect was seen on metabolic activity following any of the treatments, as measured by the alamarblue® assay. Data represents mean ± SD (n=3). Analysis performed using one-way ANOVA, p<0.05. * denotes statistical significance versus control and NTC siRNA groups. # denotes statistical significance versus naked (N) IL-6 siRNA group.
Figure 3-7: eNOS pDNA complexation with dPAMAM. Agarose gel electrophoresis showing the movement of various eNOS pDNA with degraded polyamidoamine (dPAMAM - SF) at three ratios (3:1, 6:1 and 9:1), polyethyleneimine (PEI) at 1:1 ratio and uncomplexed plasmid (N) on an agarose gel.
**Figure 3-8: eNOS mRNA expression and viability in HUVECs.** (A) eNOS mRNA expression at 48 hours following treatment with eNOS pDNA complexed with degraded polyamidoamine (dPAMAM - SF) at 3:1, 6:1 and 9:1 (SF weight: pDNA weight), polyethyleimine (PEI) at 1:1 and a naked eNOS pDNA control (pDNA). (B) Cell metabolic activity, as measured by the alamarBlue® assay, was not significantly downregulated in any of the SF treated groups, but was in the PEI treated group compared with the control. Data represents mean ± SD (n=3). Analysis performed using one-way ANOVA, p<0.05. * denotes statistical significance versus control. # denotes statistical significance versus PEI 1:1 group.
Figure 3-9: **Nitrate concentration following eNOS pDNA transfection.** Nitrate concentration in HUVEC media, measured by the Griess assay, was significantly increased in groups treated with degraded polyamidoamine (dPAMAM) complexed eNOS pDNA at ratios of 6:1 and 9:1 (weight dPAMAM: weight eNOS pDNA). Data represents mean ± SD (n=3). Analysis performed using one-way ANOVA, p<0.05. * denotes statistical significance versus control. # denotes statistical significance versus PEI 1:1 group.
Figure 3-10: Multi-modal collagen gene reservoir. The release profile of siRNA (complexed with Dharmafect® or in an uncomplexed form) is much quicker than the release profile of pDNA, which can be attributed to the design of the system with siRNA within the hydrogel and pDNA within the microspheres, as depicted in the schematic. Data represents mean ± SD (n=3).
Figure 3-11: Release profile of eNOS pDNA from different phases (microspheres and hydrogel) of scaffold. To confirm that the release is as a result of differential loading (hydrogel v microspheres) rather than simply a difference between pDNA and siRNA release, which was seen in figure 3-10. It can be seen that the release profile from the hydrogel is much faster than that from the microspheres, despite the fact that the same molecule (eNOS pDNA) is being released. Data represents mean ± SD (n=3).
Figure 3-12: **Delivery of bioactive nucleic acids from a multi-modal collagen system.**

(A) The relative expression of interleukin-6 (IL-6) mRNA following incubation with IL-6 siRNA and endothelial nitric oxide synthase (eNOS pDNA) loaded collagen delivery systems. Data represents mean ± SD (n=3). Analysis performed using one-way ANOVA, p<0.05. * denotes statistical significance versus control at individual timepoint.
3.5 References


An extracellular matrix based dual gene delivery reservoir


An extracellular matrix based dual gene delivery reservoir


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Chapter Four

In vivo assessment of collagen microsphere-in-hydrogel system ability to modulate tissue response and promote angiogenesis

The main findings of this chapter have been submitted for publication:

4.1 Introduction

Biomaterials are a key component of tissue engineering and regenerative medicine strategies.[1–3] Biomaterials are designed to aid in the repair and regeneration of tissues by directing the attachment, proliferation and differentiation of cells.[3,4] In this respect, extracellular matrix (ECM)-based biomaterials have attracted interest, particularly those derived from collagen.[5] The primary ECM component of many tissues including skin, tendon and myocardium is collagen. Collagen is conserved between multicellular organisms, and as a biomaterial when isolated has a close resemblance to the natural ECM. In addition, collagen is amenable to functionalization with bioactive agents.[6]

Despite its usefulness and suitability as a biomaterial for use in vivo, collagen-based biomaterials are subject to a foreign body reaction (FBR) following implantation.[7–9] The FBR is the host’s immune response to the implantation of a foreign material. The FBR is comprised of an initial inflammatory reaction characterized by the presence of neutrophils and macrophages stimulating the formation of an immature vasculature.[10] This emphasizes the link between the inflammatory response and angiogenesis. These two key processes, inflammation and angiogenesis, are intimately related and show a temporal overlap.[11,12] That is, a reduction in inflammation can lead to reduced blood vessel formation and vice-versa. Thus, an approach that appreciates the complex nature of the relationship between inflammation and angiogenesis is essential. An approach that can appropriately modify the balance between inflammation and angiogenesis in the FBR will be useful for implantable biomaterials and tissue engineered constructs alike.

Attempts to modulate the FBR have involved the delivery of a single factor, typically an anti-inflammatory agent or a pro-angiogenic agent. However, as stated above, modification of either process can compromise the other. This was illustrated when delivery of an interleukin-10 (IL-10) encoding pDNA via a hexamethylenediisocyanate-crosslinked collagen type I disc resulted in a reduction of not only inflammation but also angiogenesis over 21 days.[13] Conversely, delivery of two angiogenic growth factors, basic fibroblast
growth factor (bFGF) and vascular endothelial growth factor (VEGF), enhanced the FBR to an implanted vascular graft, with evidence of giant cells and graft encapsulation. Further evidence exists that growth factors such as FGF and VEGF, typically associated with the promotion of endothelial cell proliferation and angiogenesis, are also regulators of inflammatory cell infiltration.[14] Thus, it becomes clear that it is necessary to modulate both inflammation and angiogenesis when considering the FBR, but also that a temporal aspect must be considered. That is, that reduction of the inflammatory response should be followed by an increase in the formation of blood vessels.

Multi-modal biomaterial delivery systems can facilitate the delivery of therapeutics in a temporally controlled manner.[15] A collagen hydrogel system with collagen microspheres embedded can allow for the temporal delivery of nucleic acids, as has been shown in vitro using IL-10 pDNA and eNOS pDNA.[16] Using a composite collagen microsphere-in-hydrogel system and differentially loaded nucleic acids, either within hollow collagen microspheres or directly into the hydrogel, the release of two separate doses of nucleic acids may be achieved. In this case, siRNA targeting interleukin-6 (IL-6) is released from the hydrogel, while the release of eNOS pDNA is followed from the embedded microspheres. It is hypothesized that through the delivery of IL-6 siRNA, the inflammatory response will be reduced, while eNOS pDNA can promote the formation of blood vessels. A subcutaneous rat model was used to test the efficacy of nucleic acid delivery to modify the balance between inflammation and angiogenesis during the FBR.

The ECM is a complex network of proteins and glycosaminoglycans (GAGs) that provides both structural and biochemical support to cells.[17] It is well accepted in the field that the local microenvironment, and specifically the ECM, plays a major role in the regulation of cell behavior and function.[18] This is particularly apparent in cancer research where the ECM is dynamically involved in tumour progression, which is closely related to immune evasion and increased angiogenesis in the microenvironment.[19,20] Thus, a means to examine changes in the ECM following modification of the FBR will prove useful. With that
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In mind, Raman spectroscopy was identified as a means to understand the changes in the ECM following modification of the FBR. Raman spectroscopy is a tool that is gaining increased prominence in biomedical research as a means to detect changes in the ECM. It has been used to distinguish between cancerous and healthy skin models in vitro,[21] as well as detecting changes in ECM constituents.[22] Raman spectroscopy generates a molecular fingerprint of the tissue, which can be used to detect the presence of specific ECM components and their relative abundancies. Thus, by comparing between samples, changes in the ECM can be uncovered.

More recently, GAGs and sulfated GAGS (sGAGs), in particular, have been identified as key components of ECM that can dictate immune responses and angiogenesis through interactions with cytokines and growth factors.[23–25] Specifically, changes in sulfated glycosaminoglycans (sGAGs) have been explored as a means by which the ECM controls the angiogenic potential of tissues.[26] Thus, modification of the immune component of FBR to an implanted biomaterial, as well as an increase in angiogenesis will result in changes in the local ECM microenvironment. This can manifest itself as change in content of sGAGs or the amount of growth factor binding activity of sGAGs. By modifying the binding capacity of sGAGs for specific growth factors and cytokines, the sGAG in ECM can modulate the activity of these specific growth factors and cytokines. Through these changes, the ECM, and specifically the sGAGs, can dictate the response of the tissue by alteration of the microenvironment. In this study, we have assessed changes in the binding capacity of extracted sGAG for two key factors in angiogenesis, VEGF_{165} and bFGF. By analysing the binding of extracted sGAG for VEGF_{165} and bFGF, we observe how the ECM changes in response to delivery of eNOS pDNA and IL-6 siRNA by altering its capacity to potentiate growth factor activity.

Thus, the hypothesis of this chapter is that delivery of the optimal doses of IL-6 siRNA and eNOS pDNA through a collagen microsphere-in-hydrogel system will modulate the inflammatory response associated with the FBR and promote angiogenesis in a subcutaneous
In vivo assessment of dual gene delivery reservoir rat model. Furthermore, therapeutic nucleic acid delivery will result in changes in the ECM composition of the tissue, as measured by raman spectroscopy. The change in inflammation and angiogenesis in the tissue will lead to changes in the sGAG content and binding of sGAG to growth factors, specifically VEGF$_{165}$ and bFGF, two key angiogenic growth factors.

Therefore, the objectives that underpin this hypothesis are:

1. To optimise the dose of delivery of IL-6 siRNA through a collagen microsphere-in-hydrogel system to reduce the volume fraction of inflammatory cells following implantation in a subcutaneous model at seven days.
2. To optimise the dose of delivery of eNOS pDNA through a collagen microsphere-in-hydrogel system to increase the length and surface density of blood vessels following implantation in a subcutaneous model at 14 days.
3. To combine the optimal doses of IL-6 siRNA and eNOS pDNA in a collagen microsphere-in-hydrogel system and assess changes in volume fraction of inflammatory cells and surface and length density of blood vessels at 7 and 14 days in a subcutaneous rat model.
4. To examine changes in the ECM of the host tissue using Raman spectroscopy following treatment with IL-6 siRNA and eNOS pDNA loaded microsphere-in-hydrogel system, specifically in terms of the presence or absence of peaks associated with GAGs and proteoglycans (PGs)
5. Assess the changes in sGAG content, in terms of chondroitin sulphate (CS) and heparin sulphate (HS), and sGAG binding to VEGF$_{165}$ and bFGF as a result of treatment with IL-6 siRNA and eNOS pDNA microsphere-in-hydrogel system, and relate these changes with the volume fraction of inflammatory cells and length and surface density of blood vessels in the tissue.
4.2 Materials and methods

4.2.1 Extraction of collagen
Type 1 atellocollagen was isolated as previously described.[27] Briefly, bovine tendons were blended, washed in buffer (3.7mM Na$_2$HPO$_4$, 0.35Mm KH$_2$PO$_4$, 51Mm NaCl) and suspended in 0.5M acetic acid. The resulting solution was then pepsin treated (1 g pepsin: 100 g tendon) and filtered to remove insoluble collagen telopeptides. The soluble collagen was then purified by repeated salt precipitation (0.9M NaCl) and centrifugation, followed by dialysis against 0.01M acetic acid. (procedure described in detail in appendix A)

4.2.2 Fabrication of collagen microspheres
Hollow collagen microspheres were fabricated using the template method as previously described in the chapter 2.[28] Briefly, commercially available polystyrene beads (Gentaur, Chicago, Illinois) of defined size (1 μm), were sulfonated to impart a strong negative charge. Following sulfonation, beads were resuspended in 0.5M acetic acid. 5mg/ml of collagen solution was added to the beads at a weight ratio of 4:1 (beads: collagen). The resultant mixture was then stirred for four hours at room temperature. Crosslinking of the collagen coating was performed using pentaerythritol poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-PEG), with the active ester groups reacting with the free amino groups of collagen at a ratio of 1:4 (4S-PEG:free amino groups in the collagen). The mixture was agitated for two hours at room temperature. To produce a hollow sphere, the polystyrene core was dissolved by washing the coated beads with tetrahydrofuran (THF). The suspension of microspheres was diluted at a ratio of 1:1 with THF and agitated for one hour. The washing step was repeated twice to ensure complete removal of polystyrene. Hollow spheres were washed twice with ethanol and twice with water to ensure removal of any remaining THF. (see Appendix D)

4.2.3 Formation of polyplex
Polyplex were prepared by incubating the eNOS pDNA with a partially degraded polyamidoamine dendrimer (dPAMAM, Superfect™, Qiagen) (SF), as per the manufacturers
instructions. The weight ratio of the dPAMAM to peNOS used was 9:1, which was optimized in chapter 3.

4.2.4 Loading of microsphere reservoirs with polyplexes
Collagen hollow microspheres of 1 μm size were loaded with eNOS pDNA dPAMAM complexes, as previously described in chapter 2. Briefly, collagen microspheres were suspended in phosphate buffered saline (PBS). Complexes of peNOS and dPAMAM were added to the microspheres suspended in PBS and agitated on a mechanical shaker for four hours at room temperature. Microspheres were spun down and the supernatant removed.

4.2.5 Dual-release collagen scaffold fabrication
A dual release system was fabricated by combining hollow collagen microspheres with a collagen hydrogel. Microspheres loaded with peNOS polyplexes were mixed and re-suspended in a neutralized collagen/4S-PEG crosslinker solution containing IL-6 siRNA, prior to gelation.

4.2.6 In vivo implantation
All the animal procedures and treatments were approved by the ethics committee at the National University of Ireland, Galway. In addition, animal care and management followed the Standard Operating Procedures of the Animal Facility at the National Centre for Biomedical Engineering Science, National University of Ireland, Galway. Animals were allowed to acclimatize for at least seven days prior to any surgical procedures. In total 24 animals were used for the study, 12 for the dose study and 12 for the combinatorial study (n=6 for all groups).

Female Lewis rats were anaesthetized by isofluorane inhalation (5% induction reducing to 1-2% for maintenance during procedures). The dorsum of rats was shaved and swabbed with iodine to minimise the risk of bacterial contamination. Prior to gelation, the hydrogel solution was taken into a syringe and injected into the subcutaneous space. Four injections were performed per animal, with a minimal spacing of 1 cm between each.
4.2.7 In vivo IL-6 siRNA dose optimization study

Two doses of IL-6 siRNA was delivered from a collagen microsphere-in-hydrogel system. Either 1 or 5 μg siRNA was suspended within a neutralized collagen solution containing 4S-PEG as crosslinker. These doses were selected as these doses are in the range that have been reported in previous in vivo studies delivering interfering RNA.[29] A non-targeting control siRNA (NTC siRNA) was delivered via the same collagen system, as a control to assess the tissue response to an siRNA-loaded collagen delivery system. For consistency, unloaded collagen microspheres were suspended within the pre-gelling solution prior to it being taken into a syringe and injected subcutaneously through a 29-gauge needle. Samples were removed at seven days and processed for analysis (n=6 per group).

4.2.8 In vivo eNOS pDNA dose optimization study

Three doses of eNOS pDNA polyplexes were delivered from the collagen microsphere-in-hydrogel system. Either 5, 10 or 20 μg of eNOS pDNA complexed with dPAMAM was loaded within 1 μm collagen microspheres, and subsequently suspended within a neutralized collagen solution containing 4S-PEG as crosslinker. These doses were selected as these doses are in the range that have been reported in previous studies delivering pDNA via collagen scaffolds.[30] In addition, it was decided not to use a significantly higher dose given concerns about possible vector-mediated toxicity at high doses. The pre-gelling solution was taken into a syringe and injected subcutaneously through a 29-gauge needle. Samples were removed at 14 days and processed for analysis (n=6 per group).

4.2.9 Combination of IL-6 siRNA and eNOS pDNA optimal doses

The optimal doses (identified from the optimization studies) were combined and co-delivered using the collagen microsphere-in-hydrogel system. The optimal dose of IL-6 siRNA was loaded directly within the hydrogel phase of the system while the eNOS pDNA polyplexes were loaded within the collagen microsphere phase. These phases were mixed prior to gelation. The combined system was loaded into a syringe and injected.
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subcutaneously through a 29-gauge needle. Samples were retrieved at 7 and 14 days and processed for analysis (n=6 per group).

4.2.10 Tissue preservation and cryosectioning

Animals were sacrificed by CO₂ asphyxiation and the tissue immediately excised. Tissue was split and preserved for histological staining and immunostaining (10% buffered formalin for 24 hours), and protein and sGAG analysis (immediately frozen in -80°C).

For cryosections, tissue was cryoprotected by treatment with 30% sucrose for 24 hours, followed by embedding and freezing in optimal cutting temperature (OCT) medium. The tissue was then sectioned at 5 μm thickness. Sections were cut at four depths, a distance of 200 μm apart.

4.2.11 Histological staining

Standard hematoxylin and eosin (H&E) staining was performed on one section at each tissue depth. After staining, sections were dehydrated in increasing concentrations of ethanol, cleared in xylene and mounted using DPX mounting media. (Appendix EE)

4.2.12 Stereology

Three images per H&E stained section were taken at a magnification of 40X and used to quantify inflammation and angiogenesis stereologically using previous protocols.[31] (Appendix FF)

4.2.12.1 Inflammation

4.2.12.1.1 Volume fraction of inflammatory cells

Volume fraction (Vᵥ) of inflammatory cells was used to estimate the inflammatory response. A 192-point grid was overlaid on 40X images of H&E stained tissue sections. The number of macrophage and neutrophil nuclei intersecting points of the grid is counted (Pₚ), along with the total number of points on the tissue (Pₜ). The volume fraction of inflammatory cells (Vᵥ) is calculated using the formula below:
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\[ V_V = \frac{P_V}{P_T} \]

4.2.12.2 Angiogenesis

Angiogenesis was quantified using three different parameters: surface density \( (S_V) \), length density \( (L_V) \) and radial diffusion \( (R_{\text{DIFF}}) \).

4.22.121.1 Surface density \( (S_V) \)

Images were overlaid with a cycloid grid of radius 40 μm such that the grid was perpendicular to the main axis of the skin. The total number of intersections between blood vessels and the test line \( (I) \) was counted and the total length of test line \( (L_T) \) calculated. The following formula was used:

\[ S_V = \frac{2 \times I}{L_T} \]

4.22.121.2 Length density \( (L_V) \)

Images were rotated 90° and a cycloid grid of radius 40 μm applied. The total number of blood vessels and the test line \( (I) \) was counted and the total length of test line \( (L_T) \) calculated. The following formula was used:

\[ L_V = \frac{2 \times I}{(L_T \times T_s)} \]

Where \( T_s \) is the thickness of the section.

4.22.121.3 Radial diffusion \( (R_{\text{DIFF}}) \)

The distance between blood vessels, termed radial diffusion, was calculated using the following formula:

\[ R_{\text{DIFF}} = \frac{1}{\sqrt{\pi \times L_V}} \]

4.2.13 Immunofluorescent staining

Macrophages and blood vessels were identified by staining with anti-CD68 (Abcam, dilution 1:300) and anti-CD31 (Abcam, 1:30), respectively. Heat-mediated antigen retrieval was
performed in a pressure-cooker in the appropriate buffers as per the manufacturers instructions. A FITC-conjugated secondary antibody was used. The cell nuclei were stained with DAPI. (see Appendix GG)

4.2.14 RNA extraction from tissue

One mL of TRI Reagent® (Applera Ireland, Dublin, Ireland) was added to each tissue sample and incubated for five minutes at room temperature. The tissue was mechanically disrupted using a bead mill homogeniser (TissueLyserLT, Qiagen) for five minutes at least twice until the tissue was completely homogenised. Phase separation was performed by adding chloroform, and total RNA was purified using an RNeasy kit (Qiagen), according to the manufacturers instructions (Appendix Y).

4.2.15 Inflammatory and angiogenic RT-PCR arrays

Contaminant DNA was eliminated from RNA preparations using DNase I. The yield and quality of total RNA was determined according to the ratio of spectrophotometric absorbance values at wavelengths of 260 and 280 nm. cDNA synthesis was performed using DNase-treated RNA and random primers using an RT² First Strand Kit (Qiagen). The cDNA generated was used as a template for quantitative real-time PCR. A mastermix was prepared using a RT² SYBR™ Green Mastermix. This mixture was added to 96 wells in an RT² Profiler™ PCR Array, Rat Inflammatory cytokine and Rat Angiogenesis Array (PARB-0121Z; Qiagen). These PCR arrays contained RT² qPCR Primer Assays for a set of 84 cytokines related with inflammation and angiogenesis, respectively. The standard cycling conditions were as recommended by the PCR array supplier. Data were collected at the end of the annealing step. Fold changes in gene expression between the affected and control groups were calculated using the ΔΔCt method in the PCR array data analysis template. An examination of Ct value consistency for the housekeeping genes indicated that normalization was performed adequately. A similar evaluation of the built-in RNA controls verified an absence of genomic DNA contamination and inhibitors of either the reverse transcription or PCR (Appendix OO).
4.2.16 Protein extraction

Tissue samples were immediately frozen at -80 °C until needed. Samples were thawed on ice, chopped into small pieces and incubated in a lysis buffer containing a cocktail of protease inhibitors for five minutes. Samples were then mechanically disrupted in a bead mill homogeniser (TissueLyserLT, Qiagen, Hilden Germany) for five minutes at least three times until the tissue was completely homogenised and centrifuged at 15,000 g for 15 minutes. The protein fraction of the centrifuged sample was then extracted, aliquoted and stored at -80 °C until further use. (see Appendix LL)

4.2.17 Protein analysis

The protein content of the extracted samples was determined using the bicinchoninic acid (BCA) assay (Thermo Scientific. Appendix MM) and probed for 90 proteins simultaneously using a biotin label-based rat antibody array (RayBio®, Norcross, GA, USA) according to the manufacturer’s instructions. Briefly, samples were pooled according to the treatment group to a total of 1 mg/ml and dialysed overnight to remove remaining lysis buffer and the protein membranes were blocked using blocking buffer. Proteins were labelled with biotin using the amino groups present in proteins and incubated with the antibody labelled membranes overnight. Following this, HRP-conjugated streptavidin was reacted with the membranes and afterwards treated with the incubation buffer provided. Membranes were exposed using a Kodak™ Image Station 4000MM Pro (Kodak, Japan). Micrographs of the exposed membranes were digitally evaluated using ImageJ. The images obtained were imported into ImageJ and analysed using a protein array analyser plug-in by normalising to the given positive control signals on the membranes. Samples were analysed and normalised to the unloaded collagen system-treated controls at the appropriate timepoint (see Appendix NN).

4.2.18 Raman analysis of the host response to sphere-in-hydrogel implant

Tissue sections of thickness 30 μm were analysed via Raman spectroscopy. A custom-built Raman microspectroscopy system was used for all measurements as previously
All measurements were performed using a 60X water immersion objective (NA 1.2, Olympus) with the laser beam set at 85 mW. For the spectral detection, a spectrograph (Kaiser Optical Systems Inc., Ann Arbor, MI, USA) as well as a near-infrared-optimized and cooled CCD camera (Andor iDus, Belfast, Northern Ireland) were integrated at the output port of the system. Using an automated stage, 100 spectra were taken at 100 points throughout the tissue. An acquisition time of 100 seconds with 85 mW laser power were used for each spectrum. Raman spectra were acquired in the wavenumber range of 0-2000 cm$^{-1}$. Raman spectra were recorded using the Andor software package (Andor iDus). All measurements were performed from specimens on glass slides. The glass background signal was subtracted from the sample signal using the OPUS® software 4.2 (Bruker Optik GmbH, Ettlingen, Germany). Additionally, OPUS® software 4.2 was used to cut the Raman spectra into the spectral 400-1800 cm$^{-1}$ region and baseline correction performed. (see Appendix PP)

4.2.19 Principal component analysis (PCA)
PCA is a multivariate method utilized to analyze the variances in a spectral dataset. PCA is valuable to identify significant shifts in the spectra between sample groups. Prior to the PCA computation, all Raman spectra were imported to the UnscramblerX® 10.2 Software (CAMO, Oslo, Norway). PCA was performed on vector-normalized Raman spectra with a pre-defined list of Raman shift wavelengths primarily associated with GAGs, PGs and ECM components. Principal components (PCs) were calculated using the non-linear iterative partial squares (NIPALS) algorithm. As a result of the PCA, every spectrum is described by score values. Separation of score plots reveal differences between sample groups, while loadings indicate the region of the spectra where the primary differences occur.

4.2.20 sGAG extraction
Extraction of sGAG was performed as previously described.[26] Briefly, tissue samples were digested with proteinase K (200 μg/ml) and DNase (10 U per sample), followed by dilution to final volume of 2M NaCl and vigorously agitated for 10 minutes. Proteins were
precipitated and the supernatant was cleared by chloroform, followed by dialysis of the aqueous phase (Pierce, Slide-A-Lyzer Mini Dialysis Units 3,500 molecular weight cut-off) against the extraction buffer and then pure water. After freeze-drying, extracted sGAG were dissolved in water. (see Appendix HH)

4.2.21 sGAG quantification
Quantification of total sGAG was performed by means of a modified 1,9-dimethylmethylene blue (DMMB) assay, as previously described.[34] Briefly, 1 ml of the GAG-complexing DMMB solution was added to each sample, and samples were vigorously agitated. Then, samples were centrifuged (13,000g for 10 minutes) to sediment the solid GAG-DMMB complex, and supernatants were discarded. The GAG-DMMB pellet was then dissolved in 250 μl of the decomplexing solution by vigorous shaking, and absorbance of the resulting blue solution was measured at 656 nm. A standard curve, constructed with known amounts of standard CS ranging from 0 to 50 μg/ml was included in every assay.

To quantify relative amounts of HS and CS in the total extracted sGAG, samples were treated with chondroitinase A or nitrous acid, respectively, prior to the modified DMMB assay which was performed as above. Quality controls confirming total digestion of HS and CS by nitrous acid and chondroitinase, respectively, were included. (see Appendix II)

4.2.22 Heparin/sGAG binding competition assay towards growth factors
ELISA plates were coated overnight at 4°C with a 2 μg/ml BSA-heparin conjugate solution prepared, as described previously.[35] After washing the plate with 0.05% PBS-Tween 20, wells were saturated with 3% PBS-BSA for one hour at room temperature. A set amount of the growth factor (VEGF_{165} and bFGF) was added to the plate. Working growth factor concentrations were fixed at 30 ng/ml for VEGF_{165} and 6.25 ng/ml of bFGF, having been previously optimized. (see appendix JJ) These concentrations were used to determine the capacity of competing soluble extracted GAGs (0.001, 0.01, 0.1, 1, 10, 50 μg/ml in PBS) to inhibit binding of the growth factor to the immobilized heparin. For the test, growth factors and extracted GAGs were simultaneously added to the wells. After one hour of incubation at
room temperature, wells were washed, and the growth factor remaining bound to heparin was detected by incubation with the corresponding primary antibody followed by a peroxidase-labeled secondary antibody. Peroxidase activity measurement was performed with the 3,3',5,5'-tetramethylbenzidine substrate of the peroxidase activity detection kit, following the manufacturers indications. Heparin at a range of concentrations (0.01, 0.1, 1, 10, 100 and 1000 ng/ml) was used as a positive control of growth factor binding. (see Appendix KK)

4.2.23 Statistical Analysis
GraphPad Prism® (v.5 GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Analysis of variance (ANOVA) was used followed by Tukey’s post-hoc test to determine statistical significance between groups. All graphical data is presented as mean ± standard deviation of mean. p values of < 0.05 were considered statistically significant. Pearson’s test for correlation was performed with significance set at p < 0.05.

4.3 Results and discussion

4.2.1 IL-6 siRNA dose optimization: inflammation
IL-6 siRNA was incorporated within the collagen sphere-in-hydrogel system to modulate the inflammatory response that occurs following implantation of a foreign material within the body. The inflammatory response is a key factor that determines the success or failure of a biomaterial implant.[36] Excessive inflammation results in the rejection of a biomaterial, which may then be degraded or encapsulated within a fibrotic capsule, essentially allowing the body to exclude the implant. This severely hampers the function of the implant. Thus, a strategy to reduce the local
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Figure 4-1: Schematic illustrating the assembly and application of the collagen sphere-in-hydrogel system in the subcutaneous space, and the workflow of the study. From dose optimisation (1) to combining the optimal dose (2) and analysis of the effect of treatments on the composition of the ECM (3). (V_V : volume fraction of inflammatory cells, L_V : length density of blood vessels)
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Inflammatory response to biomaterial implants will be useful. IL-6 is a pro-inflammatory cytokine produced by fibroblasts as well as inflammatory cells such as macrophages.[37,38] It is involved in the progression of inflammation and the foreign body reaction to implanted biomaterials.[7] Thus, delivery of IL-6 targeting siRNA is proposed as an approach to reduce the inflammatory response. Samples were implanted subcutaneously and removed after seven days. Samples were fixed and prepared for histological staining. The seven day timepoint was chosen as an early timepoint appropriate to assess the inflammatory response following implantation of a foreign material. Two doses of IL-6 siRNA were examined (1 and 5 μg), along with a non-targeting control siRNA (NTC siRNA). Delivery of NTC siRNA is used as a baseline to assess the tissue response to siRNA delivered via a collagen delivery system. In each case, siRNA was delivered via a crosslinked collagen sphere-in-hydrogel system, and thus the tissue response can be attributed to both the implanted collagen and the delivered siRNA. Thus, the delivery of NTC siRNA via the collagen sphere-in-hydrogel system can be considered as a baseline tissue response to the delivery of the siRNA-loaded sphere-in-hydrogel system, and thus any improvement in tissue response is related to the incorporation of the IL-6 targeting siRNA.

Stereological examination of H&E stained tissue sections revealed that incorporation of IL-6 siRNA within the collagen delivery system resulted in a significant reduction in the volume fraction of inflammatory cells. Treatment with 1 and 5 μg IL-6 siRNA resulted in a reduction of 52% and 42%, respectively, compared with the NTC siRNA. The volume fraction of inflammatory cells following the delivery of NTC siRNA is considered as the ‘standard inflammatory response’ to an RNA-loaded collagen delivery system. Thus, the reduction observed in volume fraction of infiltrating inflammatory cells, primarily neutrophils and macrophages, can be attributed to the delivery of IL-6 targeting siRNA. Importantly, in this optimization study the delivery of an IL-6 targeting siRNA did not significantly affect angiogenesis in the tissue. It was observed that the surface density of
Figure 4-2: Optimization of IL-6 siRNA dose. (A) No effect was observed on length density of blood vessels between groups. (B) Volume fraction of inflammatory cells was reduced following treatment with scaffold and IL-6 siRNA at doses of 1 and 5 μg compared with the scaffold loaded with non-targeting control (NTC) siRNA. Representative H&E stained sections of (C) NTC siRNA, (D) 1 μg IL-6 siRNA and (E) 5 μg IL-6 siRNA treated-groups, respectively. Data represents mean ± SD (n=6). Analysis performed using one-way ANOVA, p<0.05. * denotes statistical significance versus scaffold & NTC siRNA group. Scale bar indicates 25 μm.
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blood vessels was unchanged in both the 1 or 5 μg IL-6 siRNA doses (6.8 and 8.5 mm$^{-1}$, respectively) compared with the collagen system containing NTC siRNA (7.3 mm$^{-1}$). While IL-6 is primarily associated with inflammation, it is also linked with angiogenic processes both in disease and development.[39,40] However, in this study it was observed that delivery of an IL-6 targeting siRNA does not significantly impact the surface density of blood vessels at seven days following delivery from a collagen system, when compared to delivery of a NTC siRNA. Considering that the goal is to combine delivery of IL-6 siRNA and eNOS pDNA, an angiogenic factor, it is critical that IL-6 siRNA does not negatively impact angiogenesis.

4.2.2 eNOS pDNA dose optimization: angiogenesis

One of the primary causes of the failure of implanted biomaterials is a lack of vascularization of the implant. Delivery of angiogenic signals such as growth factors is one avenue that has been explored. However, issues such as the short in vivo half-life of growth factors have reduced the effectiveness of this approach.[41] Gene therapy offers an opportunity to bypass this issue by providing a continuous supply of pro-angiogenic signals. Delivery of the eNOS gene via biomaterial systems has previously been shown to increase vascularization in a diabetic wound model.[42,43] The 14 day timepoint was chosen as an appropriate time to observe blood vessel formation for a couple of reasons. The first of which is the release profile of the eNOS polyplexes from the microsphere phase of the sphere-in-hydrogel system, which is more delayed compared with the release from the hydrogel phase. In addition, following tissue injury (implantation) there will be a tissue response, usually characterized by a short-term increase in blood vessel formation. In order to differentiate between blood vessels formed due to the foreign body response following the implantation procedure and those due to the delivery of the eNOS gene itself, 14 days was chosen as an appropriate timepoint. Previous efforts aimed at the delivery of the eNOS gene from biomaterial systems have utilized various vectors, including lipoplexes and viral vectors.[42,43] However, in this project, a dPAMAM transfection reagent was used as a
vector. In all cases, the efficiency of delivery and transfection is dependent on the vector used, and thus dose optimization is required. The use of three doses of eNOS pDNA, all complexed at the same weight-to-weight ratio with dPAMAM, has been explored: a low dose (5 μg), a medium dose (10 μg) and a high dose (20 μg).

After sacrifice, tissue explants were removed at 14 days, fixed, sectioned and stained with H&E. Following stereological examination it was found that delivery of the lowest dose of eNOS pDNA, 5 μg, resulted in a statistically significant increase in the surface density of blood vessels compared with a collagen sphere-in-hydrogel system without loaded eNOS pDNA. An almost doubling of the surface density of blood vessels was observed (8.6 mm⁻¹ compared with 4.7 mm⁻¹). The medium dose, 10 μg, showed a further increase in the length density of blood vessels with an almost three-fold increase observed over that of the control (12.9 mm⁻¹). The 10 μg eNOS pDNA dose was significantly higher than that of both the unloaded collagen sphere-in-hydrogel system, but also the system loaded with 5 μg eNOS pDNA. It was expected that the highest dose of 20 μg of eNOS pDNA would further increase the length density of blood vessels. However, there was no change observed in the length density between the 10 μg and 20 μg treated groups (12.9 mm⁻¹ and 13.2 mm⁻¹, respectively), suggesting a peak in the dose response. The 10 μg eNOS pDNA group had the highest length density of blood vessels, but also had the lowest volume fraction of inflammatory cells. This is not surprising considering that NO has anti-inflammatory properties.[44] Examining all three eNOS pDNA treated groups, only the 10 μg eNOS pDNA treated group had a statistically significant reduction in the volume fraction of inflammatory cells compared with the scaffold alone control (43% reduction). This indicates that with all three doses of eNOS pDNA the level of NO produced was not pro-inflammatory such that it could activate the pro-inflammatory NF-κB pathway.[44] The NF-κB pathway reacts to cellular stress and induces the transcription of numerous pro-inflammatory genes including TNF-α, IL-1β and IL-6.[45] Considering the increase observed in length density of blood vessels and the reduction in volume fraction of
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Figure 4-3: Optimization of eNOS pDNA dose. (A) Length density of blood vessels was increased following treatment with eNOS pDNA at doses of 5 μg, 10 μg and 20 μg compared with the control scaffold alone. (B) No difference was observed in the volume fraction of inflammatory cells between groups; although amongst the eNOS pDNA treated groups, a trend towards an increase in volume fraction in the 20 μg eNOS pDNA treated group was observed. Representative H&E stained sections of (C) scaffold alone, (D) scaffold & 5 μg eNOS pDNA, (E) scaffold & 10 μg eNOS pDNA and (F) scaffold & 20 μg eNOS pDNA, respectively. Data represents mean ± SD (n=6). Analysis performed using one-way ANOVA, p<0.05. *denotes statistical significance versus scaffold alone group. # denotes statistical significance versus 5 μg eNOS pDNA treated group. Scale bar indicates 25 μm.
inflammatory cells, the 10 μg eNOS pDNA treated group has been identified as the optimal dosing regimen of eNOS pDNA from the collagen sphere-in-hydrogel system.

4.2.3 Co-delivery of IL-6 siRNA and eNOS pDNA

4.2.12.1 Inflammation

Following on from the selection of the optimal doses of anti-inflammatory IL-6 siRNA (1 μg) and the pro-angiogenic eNOS pDNA (10 μg) to modify the host response to the implanted collagen sphere-in-hydrogel system, these two optimal doses were combined, co-delivered and the tissue response, in terms of inflammatory response and angiogenesis, examined at both 7 and 14 days. Inflammation was examined stereologically in terms of volume fraction of inflammatory cells, with immunostaining performed to confirm the relative presence of macrophages (CD68-positive cells).

Stereological examination of serial H&E stained sections was performed to assess differences in inflammatory and angiogenic parameters. It was observed that at the seven day timepoint combined delivery of IL-6 siRNA and eNOS pDNA resulted in a volume fraction of inflammatory cells on a similar level to that of the IL-6 siRNA delivery alone. That is, a reduction of 46% and 34% in volume fraction for delivery of IL-6 siRNA alone or IL-6 siRNA and eNOS pDNA, compared with the collagen system alone.

At 14 days, a similar pattern emerged to that which was exhibited at seven days. That is, that the addition of IL-6 siRNA to the collagen delivery system significantly reduced the volume fraction of inflammatory cells. Compared with the collagen delivery system alone there was a reduction in the volume fraction of inflammatory cells of 65% with the addition of IL-6 siRNA and 69% with delivery of IL-6 siRNA eNOS pDNA (0.041). Delivery of eNOS pDNA alone via the collagen delivery system resulted in a reduction in volume fraction of 40% compared with the unloaded collagen delivery system. Interestingly however, this was not to the same level as when IL-6 siRNA was delivered, and there was a significant difference between the group receiving eNOS pDNA alone and the two that received IL-6
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**Figure 4-4**: Effect of dual delivery on inflammation. A reduced volume fraction of inflammatory cells was observed in groups treated with IL-6 siRNA, with or without eNOS pDNA at both 7 and 14 days. Representative images reveal a reduction in the presence of CD68+ cells in IL-6 siRNA treated groups, alone or in combination with eNOS pDNA. Data represents mean ± SD (n=6). Analysis performed using one-way ANOVA, p<0.05. *denotes statistical significance versus scaffold alone group at same timepoint. # denotes statistical significance versus eNOS pDNA treated group at 14 days. Scale bar indicates 100 μm.
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siRNA, either without or with eNOS pDNA. Thus, it is clear that at 14 days the addition of IL-6 siRNA, with or without eNOS pDNA, significantly reduces the volume fraction of inflammatory cells when compared with either the collagen delivery system itself or the collagen delivery system combined with eNOS pDNA.

In addition to comparing between groups at each timepoint, it is also interesting to observe differences within groups between timepoints. That is, to observe the change in volume fraction over time to assess the progression of the inflammatory response. In that respect, it can be seen that the inflammatory response increases over time in the control group, with an increase of 65% observed from 7 to 14 days. In comparison with this, the volume fraction of inflammatory cells in the IL-6 siRNA alone treated group remains relatively stable over time (0.044 and 0.047 at 7 and 14 days) the eNOS pDNA treated group at 7 and 14 days. In the group receiving dual delivery of IL-6 siRNA and eNOS pDNA there is also a small change observed between 7 and 14 days, with a reduction of 24% in volume fraction of inflammatory cells seen over time. It can be seen that in the control group, the inflammatory response is progressing and the volume fraction of inflammatory cells increases by 64% from 7 to 14 days. However, in the IL-6 siRNA treated groups, there is no change observed in volume fraction of inflammatory cells between 7 and 14 days. In the eNOS pDNA treated groups no significant difference is observed in volume fraction of inflammatory cells between 7 and 14 days.

To confirm differences in the relative presence of inflammatory cells between the various treatment groups, immunofluorescent staining was performed. Macrophages, a key cell in the inflammatory process, were stained with a CD68 antibody. Representative images are shown in figure 4(B). It can be seen that there are less CD68-positive cells in the IL-6 siRNA treated group and the IL-6 siRNA and eNOS pDNA dual-treated group compared with the control group. In addition, there is a reduction in CD68-positive cells in the eNOS pDNA treated group, but this is less than the groups in which IL-6 siRNA is delivered. This is observed at both the 7 and 14 day timepoints.
4.2.12.2 Angiogenesis

The effect of co-delivery of IL-6 siRNA and eNOS pDNA on angiogenesis was also examined stereologically on H&E stained sections, in this case in terms of surface density, length density and radial diffusion. In addition, endothelial cells (CD31-positive cells) were stained as confirmation of the presence of blood vessels.

At seven days there was a statistically significant increase in the length density of blood vessels in the eNOS pDNA treated group and the IL-6 siRNA and eNOS pDNA dual-treated group when compared with the control group, with an almost two-fold increase observed (2617 and 3018 mm$^{-2}$, respectively, compared with 1681 mm$^{-2}$). There was no significant difference between the eNOS pDNA treated group and the IL-6 siRNA and eNOS pDNA dual-treated groups. No significant difference was observed between the IL-6 siRNA treated group and the control group (1237 mm$^{-2}$). Further data on the perfusion of the tissue was acquired by measuring the radial diffusion distance, revealing the average distance between adjacent blood vessels. It was observed that eNOS pDNA treated groups, with or without IL-6 siRNA, had a significantly reduced radial diffusion distance (21% and 25% reduction, respectively) compared with the control. The radial diffusion distance in the IL-6 siRNA treated group was not significantly different to the control.

A similar effect was observed at 14 days to those at 7 days. Once again, the length density of blood vessels was greatest in the eNOS pDNA treated groups, both without and with the addition of IL-6 siRNA (2568 mm$^{-2}$ and 2910 mm$^{-2}$). The length density of both of these groups were significantly greater than that of the control, with an almost three-fold increase observed (939 mm$^{-2}$). The IL-6 siRNA treated group was not statistically different to the control (1244 mm$^{-2}$). Radial diffusion distance was also statistically different between eNOS treated groups (42% and 47% reduction without and with IL-6 siRNA, respectively) compared with the control, while the IL-6 siRNA treated group was not significantly different.
Figure 4-5: Effect of dual delivery on angiogenesis. An increase in surface and length density of blood vessels was observed following treatment with eNOS pDNA, alone or in combination with IL-6 siRNA. Conversely, a reduction was observed in the radial diffusion distance following eNOS pDNA delivery, either alone or in combination with IL-6 siRNA. Representative images reveal an increase in CD31+ cells following eNOS pDNA delivery, with or without co-delivery of IL-6 siRNA. Data represents mean ± SD (n=6). Analysis performed using one-way ANOVA, p<0.05. *denotes statistical significance versus scaffold alone group at same timepoint. # denotes statistical significance between groups. Scale bar indicates 100 μm.
It was important to observe the changes in individual groups between 7 and 14 days, to assess changes in angiogenesis over time as a result of each treatment. In the control group a reduction of 44% is observed in length density between 7 and 14 days. This is attributed to an initial increase in blood vessels due to the foreign body response. However, over time many of these immature vessels will regress, as seen with the reduction in length density at 14 days. In contrast, the eNOS pDNA treated groups had a higher surface density at day seven, but this remained constant at 14 days, with no changes observed.

To confirm the presence of blood vessels, immunofluorescent staining was performed for CD31, an endothelial cell marker. Representative images are shown in figure 5 (D). It can be seen that there is a greater presence of CD31-positive endothelial cells in both eNOS pDNA treated groups compared with either the control or the IL-6 siRNA treated groups. This effect is present at both the 7 and 14 days.

4.2.4 Analysis of tissue expression of inflammatory and angiogenic factors following treatment with sphere-in-hydrogel system

A protein blot array was used to analyze the relative expression patterns of 90 proteins simultaneously. This rat antibody protein array provided a means by which to extensively analyse the relative protein expression of a range of key inflammatory mediators and angiogenic factors. Pooled samples from each group were analyzed to determine the global effect of the various treatment groups. The data is compared with the control at each timepoint, and thus the mediators that indicate differential expression relative to the control are shown. The data is presented in diagrammatic form in figure 6. For the purpose of clarity, the inflammatory and angiogenic factors have been grouped.

The primary goal of incorporation of IL-6 siRNA into the collagen sphere-in-hydrogel system is to reduce the expression of IL-6 and modify the inflammatory response. Figure 6 shows that the delivery of IL-6 siRNA resulted in a reduction in IL-6 protein expression at seven days. In addition, the protein expression of a range of other inflammatory mediators are also reduced. These include two primary mediators of inflammation, IL-1α and TNF-α,
as well as cytokine-induced neutrophil chemoattractant 2 and 3 (CINC-2/3), macrophage derived chemokine (MDC), macrophage inflammatory protein-2 (MIP-2), interferon-inducible protein-10 (IP-10) and granulocyte-macrophage colony-stimulating factor (GMCSF). Given that many of these factors are involved in the attraction and trafficking of inflammatory cells towards the implantation site, their reduction is in line with the reduction in volume fraction of inflammatory cells seen from stereological evaluation. In addition to a reduction in inflammatory cytokines, a reduction was also observed in the protein expression of bFGF, FGF-binding protein (FGF-bp), VEGF and VEGF-C, which are all angiogenic factors. These findings relate with the observation that there is a slight reduction in the surface and length density of blood vessels at seven days following treatment with IL-6 siRNA. Although there was no significant difference in these parameters when they were measured, the reduction in these angiogenic factors implies an alteration of the angiogenic process following IL-6 siRNA incorporation into the collagen sphere-in-hydrogel system.

Addition of eNOS pDNA to the collagen sphere-in-hydrogel system resulted in an increase in the surface and length density of blood vessels relative to the unloaded, control sphere-in-hydrogel system. This is reflected in the increased protein expression of a number of angiogenic factors, amongst them bFGF, FGF-bp, CXCR4, PDGF-AA, VEGF and Neuropillin-2 (NRP-2). CXCR4 is the receptor for stromal-derived factor-1 (SDF-1), a key pathway in neo-angiogenesis, involved in the recruitment of endothelial precursors.[46,47] NRP-2 is a receptor for VEGF, and has been shown to interact with VEGF receptor-2 (VEGFR-2) and -3 (VEGFR-3). Conversely, an increase was observed in the protein expression of three inflammatory cytokines, CINC-2/3 and MIP-2. This increase in CINC-2/3 and MIP-2 shows the link between angiogenesis and inflammation, implying that increased angiogenesis will also result in an increase in inflammatory factors. This, along with the reduction in angiogenic factors following IL-6 siRNA treatment, again implies the need for a combined therapy that modulates both inflammation and angiogenesis.
The combination of IL-6 siRNA delivery and eNOS pDNA from the collagen sphere-in-hydrogel system resulted in a modulation of the protein expression of both inflammatory and angiogenic factors at seven days. As shown in figure 6, there is both a reduction in IL-6, TNF-α and GMCSF, as would be expected from the reduced volume fraction of inflammatory cells. In addition, there is an increased protein expression of angiogenic factors, similar to that of the eNOS pDNA treated group. The combined effect of IL-6 siRNA and eNOS pDNA is clearly visible in terms of protein expression, with the reduction in inflammatory factors and increase in angiogenic factors similar to that of the individual treatments alone. The combinatorial effect of dual delivery of IL-6 siRNA and eNOS pDNA through the collagen sphere-in-hydrogel system is seen, as compared with the same system delivering either factor separately.

A similar observation was made at 14 days. Delivery of IL-6 siRNA via the collagen sphere-in-hydrogel system reduced IL-6 protein expression, along with the protein expression of IP-10 and resistin-like molecule-beta (RELM-β). An increase in interleukin-4 (IL-4) and -13 (IL-13) was also observed. Increased IL-4 and IL-13 expression are indicative of an alternative macrophage activation state. Alternatively activated macrophages are more regulatory in nature compared with classically activated macrophages, and are indicative of an anti-inflammatory and wound healing state in the tissue.[48] This is further emphasized by an increased expression of angiogenic factors (VEGF and VEGF-C) and receptors (NRP-2), characteristic of alternative macrophage activation. However, in contrast with the groups that have received eNOS pDNA treatment, the absence of an increase in PDGF-AA expression implies that any blood vessels formed are likely to be immature and regress over time.

At 14 days, delivery of eNOS pDNA resulted in an increase in a range of angiogenic factors similar to that observed at seven days, with the exception of FGF and NRP-2, which are unchanged compared with the control. There was also an increase in osteopontin (OPN) and matrix metalloproteinase-8 (MMP-8) protein expression. Both OPN[49,50] and MMP-8 [51]
Figure 4-6: Change in protein expression. Alterations in protein expression detected via protein blot array between the treatment groups and the control at 7 and 14 days. The proteins have been grouped in terms of proteins with functions primarily associated with inflammation or angiogenesis.
have been proven to play important roles in angiogenesis both in vitro and in vivo. Increased FADD expression is also observed, along with a reduction in IL-10 expression. The reduction in IL-10 expression, coupled with no change in IL-4 and IL-13 expression, indicates that macrophages may still be in a more pro-inflammatory phenotype compared with the control. This is despite the reduction observed in the volume fraction of inflammatory cells seen from stereological evaluation, and poses an interesting dilemma as to whether it is better to have less infiltration of inflammatory cells, or to change their activation state to that of a more regulatory phenotype. Studies have shown that changes in the activation state of macrophages can dramatically alter the tissue response and therapeutic benefit of biomaterials therapies in the ischemic heart[52] and injured peripheral nerve.[53] Altered macrophage phenotype has also been shown to play a role in the vascularization of tissue engineered scaffolds. [54]

The combined delivery of IL-6 siRNA and eNOS pDNA through the collagen sphere-in-hydrogel system resulted in a number of substantial changes in protein expression. As with delivery of IL-6 siRNA alone, there was a reduction in IL-6 expression. An increased expression of IL-4, IL-10 and IL-13 was also observed. Increased protein expression of IL-10, along with that of IL-4 and IL-13, suggests an alternative macrophage activation, in which the macrophages are more regulatory rather than pro-inflammatory in nature. As with the eNOS pDNA group, there was also an increase in FADD, an anti-inflammatory mediator. In terms of angiogenic factors, FGF-bp, CXCR4, PDGF-AA, VEGF, VEGF-C, OPN, NRP-2 and MMP-8 are significantly upregulated, as would be expected with the increase observed in surface and length density of blood vessels.

Taken together, this change in protein expression in the IL-6 siRNA and eNOS pDNA combined group versus the control implies reduction in inflammatory cell infiltration at 7 days (reduced IL-6, TNF-α and GMCSF) along with a change in the macrophage activation state at 14 days (increased IL-4, IL-10 and IL-13). The protein expression of angiogenic factors is quite consistent throughout the two timepoints, with factors such as VEGF and
PDGF-AA prominent. This consistency is matched in terms of surface and length density of blood vessels, which remains relatively stable between the two timepoints.

4.2.5 **Inflammatory and angiogenic gene expression in subcutaneous tissue following treatment with sphere-in-hydrogel system**

In order to assess changes related to inflammatory and angiogenic gene expression at 14 days, inflammatory and angiogenic PCR arrays were performed. These arrays were performed with RNA extracted from tissue treated with the sphere-in-hydrogel system loaded with IL-6 siRNA and eNOS pDNA. RNA was extracted from the tissue by the Trizol method and pooled. DNase treatment and the PCR reaction was performed to form cDNA (as described by the manufacturers). Evaluation of the built-in RNA controls confirmed the absence of genomic DNA as well as inhibitors of the reverse transcription and PCR reactions. The housekeeping genes ACTB, B2M, HPRT1, LDHA and RPLP1 were chosen as internal controls to normalize the gene expression in each group. The data is expressed in figure 7, in terms of (A) inflammatory and (B) angiogenic factors. A five-fold change in gene expression was chosen as a cut-off. Factors enclosed by a blue circle are upregulated and factors enclosed by a red circle are downregulated by treatment with the IL-6 siRNA and eNOS pDNA loaded sphere-in-hydrogel system compared with the unloaded sphere-in-hydrogel system.

In total, 12 inflammatory factors were observed to be increased. This includes factors and receptors such as CCR2, CCR8, CCR10, CXCL2 and IL-17. Many of these factors are associated with angiogenesis as well as inflammation, exemplifying the fact that modulating inflammation and angiogenesis is a balancing act. A plethora of pro-inflammatory factors are downregulated, including IL-6, IFN-β, IFN-γ, TNF-α and IL-1β. In addition, a reduction in the expression of a number of receptors is also observed, such as CCR3, CXCR2, CXCR5 IL-1R1, IL-6R. In total, 22 factors show a reduction in gene expression following treatment with the combined IL-6 siRNA and eNOS pDNA loaded collagen sphere-in-hydrogel system.
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In terms of effect on angiogenic factors, overall an increase in angiogenic gene expression is observed, with a total of 16 factors upregulated and 4 downregulated. Upregulated factors include connective tissue growth factor (CTGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), VEGF and PDGF-A and –B, all pro-angiogenic factors. In addition, an increase in eNOS mRNA is observed, as expected with delivery of eNOS pDNA. The downregulation of a number of factors was also observed, including ephrin A1, endoglin, JAG-1 and thrombospondin-1. The reduction of ephrin A1, endoglin and JAG-1 gene expression implies a change in the angiogenic process by dual delivery of IL-6 siRNA and eNOS pDNA. Meanwhile, the reduction of thrombospondin-1 as it is controlled by eNOS expression.[55] The increases observed in expression of integrins are explained by the fact that angiogenesis is regulated by integrins.[56] In particular, the αV subunit have been studied in the context of cancer-related angiogenesis.[57,58] The β3 subunit has also been heavily implicated in angiogenesis, but its expression was observed to be unchanged in this study. Overall, the increases observed in VEGF and PDGF-A and –B mRNA expression in particular imply an increase in angiogenesis and the formation of a mature vasculature.

Comparing the gene and protein expression data, the overall trend of a reduction in inflammatory mediators and an increase in angiogenic factors is consistent. However, differences tend to emerge with regards to the exact molecules that are up- and downregulated. Despite this, consistency between gene and protein analysis is seen with regards to a reduction in IL-6 expression, and also an increase in VEGF and PDGF expression.

A note of caution must be attached to the interpretation of both the protein and gene analysis. That is, that the samples analysed were pooled samples (n=6), and that the possibility exists that individual samples (so-called outliers) may mask effects of the treatment. In addition, cut-offs have been used to analyse the data, which can lead to the possibility of subtle increases or decreases being missed in the data. However in this case,
Figure 4-7: Altered gene expression. PCR arrays revealed changes in the gene expression of (A) inflammatory and (B) angiogenic factors in tissue treated with the collagen sphere-in-hydrogel system loaded with IL-6 siRNA and eNOS pDNA at 14 days. Gene expression is compared with the expression in tissue treated with the unloaded, control sphere-in-hydrogel system.
although a difference was observed between the individual factors up- and downregulated, a reduction in the expression of pro-inflammatory factors and an increase in the expression of pro-angiogenic factors is observed.

4.2.6 *Raman spectroscopic analysis of host response*

Raman spectroscopy is a vibrational technique that generates a molecular fingerprint of the tissue based on the detection of wavelength shifts caused by vibrations of chemical bonds. Raman spectroscopy is a label-free technique requiring minimal sample preparation prior to analysis. In this study, 30 μm tissue sections were analysed to detect changes in the tissue, primarily in terms of changes in the peaks associated with GAGs and PGs present in the ECM. Samples were analysed from all groups at both the 7 and 14 day timepoints. PCA was performed to assess changes in ECM content as a result of treatment with the collagen sphere-in-hydrogel system. Specific bands were chosen (see table 4-1) that relate to changes in the GAG and PG content of the tissue. In addition, bands that have previously been related to inflammatory signals in tissue were included in the analysis (see table 4-2). From this analysis, score plots revealed a separation of the different groups compared with the control group at each timepoint. In addition, loadings revealed in which bands these differences occurred. By plotting the intensity of the bands from each group, differences between groups can be identified. This analysis revealed a number of changes that were related in particular to the GAG content and also the sulfation of GAGs. This revealed increased chondroitin-4-sulfate (C4S) in the IL-6 siRNA treated-group, while chondroitin-6-sulfate was increased in all three treatment groups (IL-6 siRNA, eNOS pDNA and the combination of both) compared with the control. There was also an increase in chondroitin sulfate proteoglycans (CSPGs) in the IL-6 siRNA and eNOS pDNA combined group compared with the control (sphere-in-hydrogel system alone). The effect of ECM changes, particularly that of GAGs present in the ECM, on inflammatory and angiogenic processes is well established. The inverse is also true, that inflammation and angiogenesis can effect
Table 4-1: **Raman peaks.** Peaks that were assessed in Raman spectral analysis, and the ECM components with which they are associated.

<table>
<thead>
<tr>
<th>Raman shift (cm⁻¹)</th>
<th>Component</th>
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<tbody>
<tr>
<td>1340</td>
<td>GAGs</td>
</tr>
<tr>
<td>1065</td>
<td>CS &amp; PGs</td>
</tr>
<tr>
<td>1003</td>
<td>Total Protein</td>
</tr>
<tr>
<td>1001</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>939</td>
<td>C4S</td>
</tr>
<tr>
<td>921</td>
<td>Proline (2)</td>
</tr>
<tr>
<td>882</td>
<td>C6S</td>
</tr>
<tr>
<td>853</td>
<td>C4S</td>
</tr>
<tr>
<td>817</td>
<td>Proline (1)</td>
</tr>
<tr>
<td>640</td>
<td>GAGs</td>
</tr>
<tr>
<td>617</td>
<td>GAGs</td>
</tr>
<tr>
<td>510</td>
<td>CSPG</td>
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<tr>
<td>490</td>
<td>CSPG</td>
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<table>
<thead>
<tr>
<th>Raman shift (cm⁻¹)</th>
<th>Component</th>
</tr>
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<tbody>
<tr>
<td>1542</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>1270</td>
<td>Inflammation</td>
</tr>
<tr>
<td>1217</td>
<td>Inflammation</td>
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</tbody>
</table>

**Table 4-2: Raman peaks.** Peaks assessed in Raman spectral analysis associated with inflammation.
Figure 4-8: **Seven day analysis of Raman spectra.** Analysis of the difference between scaffold alone and scaffold & IL-6 siRNA & eNOS pDNA spectra at seven days. The score plot reveals differences between the groups, while the loadings show where in the spectrum these differences primarily occur.
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**Figure 4-9: Fourteen day analysis of Raman spectra.** Analysis of the difference between the scaffold alone and scaffold & IL-6 siRNA & eNOS pDNA treated groups at 14 days. The score plot reveals differences between the two groups while the loadings show primarily in which peaks these differences occur.
Figure 4-10: **Raman intensity analysis of selected ECM components at seven days.** Analysis of the difference in intensity in specific peaks between groups. The intensity of the scaffold alone group is set at 1, and all other groups shown relative to this. Data represents mean ± SD (n=6). Analysis performed using one-way ANOVA, p<0.05. *denotes statistical significance versus scaffold alone group.
Figure 4-11: **Raman intensity analysis of inflammation at seven days.** Analysis of the difference in intensity in specific peaks between groups. The intensity of the scaffold alone group is set at 1, and all other groups shown relative to this. Data represents mean ± SD (n=6). Analysis performed using one-way ANOVA, p<0.05. * denotes statistical significance versus scaffold alone group.
Figure 4-12: Raman intensity analysis of selected ECM components at 14 days. Analysis of the difference in intensity in specific peaks between groups. The intensity of the scaffold alone group is set at 1, and all other groups shown relative to this. Data represents mean ± SD (n=6). Analysis performed using one-way ANOVA, p<0.05. *denotes statistical significance versus scaffold alone group.
Figure 4-13: **Raman intensity analysis of inflammation at 14 days.** Analysis of the difference in intensity in specific peaks between groups. The intensity of the scaffold alone group is set at 1, and all other groups shown relative to this. Data represents mean ± SD (n=6). Analysis performed using one-way ANOVA, p<0.05. *denotes statistical significance versus scaffold alone group.
components of the ECM. Thus, changes that occur in the ECM following biomaterial-mediated therapeutic delivery is not unexpected. In this case, changes particularly with regard to GAG sulfation and also GAG and PG content were observed.

4.2.7 Changes in content and activity of sGAG present in ECM

The ECM is a dynamic niche with many components including proteins, GAGs and growth factors. This niche directs cell behavior by altering ECM content and activity. GAGs are an important part of the ECM and have long been linked with changes in cell behavior, specifically related with changes in inflammation and angiogenesis. Thus, changes in inflammation and angiogenesis can result in differential sGAG content of the ECM. This may manifest itself as either a difference in the total sGAG content of the ECM or the relative composition of the sGAG in the ECM. Raman spectroscopy revealed changes in particular with both sulfation and content of GAGs and PGs. Using the DMMB assay, the total sGAG content can be measured, and using a subtraction method, the relative amount of CS and HS can be determined. With regards to the total sGAG content extracted from tissue, no difference was observed between the treatment groups at either the 7 or 14 day timepoints. However, this must be taken with the caveat that the DMMB assay measures only sGAG, and one GAG which is closely related with changes in inflammation is hyaluronic acid (HA), a GAG which is not sulfated. Low molecular weight HA or HA fragments can act as damage-associated molecular patterns (DAMPs) and trigger an immune response, in this case related to the foreign body response. However, any changes in HA, either in terms of content or length, cannot be detected by this method.

The relative quantities of CS and HS were also measured, using a subtractive method. This involves degradation of CS using chondroitinase, allowing HS to be measured using DMMB, and degradation of HS by nitrous acid, allowing for quantification of CS by DMMB. It was found that there was no difference in the ratio of CS to HS between groups at either timepoint, (Figure 13 (B)). That is, the composition of the sGAG in the ECM was unchanged following treatment with IL-6 siRNA, eNOS pDNA or the combination of both,
Figure 4-14: No difference in sGAG profile of tissue. No changes were seen between groups either in the total sGAG content of the tissue or the ratio of chondroitin sulfate (CS) to heparin sulfate (HS) in the tissue, at either 7 or 14 days.
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at 7 and 14 days. However, this does not rule out changes in the function and activity of the sGAG in the ECM of the treated tissue. ELISA competition assays were used as a means to assess the relative binding capacities of extracted sGAGs towards two angiogenic factors, VEGF$^{165}$ and bFGF. This assay is based on the ability of the extracted sGAG to inhibit the binding of a growth factor to immobilized heparin. It was found that there were changes in the growth factor binding of the extracted sGAG between groups. At seven days, there was a significant increase in the binding of sGAG with both growth factors tested in eNOS pDNA treated groups. For VEGF$^{165}$ binding, it is noticeable that there is a significant increase in sGAG binding at seven days in the eNOS pDNA treated samples versus the control or IL-6 siRNA treated samples (45.6% and 50.1% for eNOS pDNA groups without and with IL-6 siRNA, respectively, compared with 31.5% and 22.2% for the control and IL-6 siRNA treated groups, respectively). Binding to bFGF shows a similar effect, with an increase observed in the eNOS pDNA treated groups (42.6% and 51.5% without and with IL-6 siRNA, respectively) compared with the control (29.5%) and the IL-6 siRNA alone treated groups (23.6%). Previous studies have shown that the binding of growth factors by sGAGs and sGAG-mimetics potentiates and increases the activity of growth factors.[26,59] Thus, the binding studies indicate a potential ‘priming’ of the sGAG content of the ECM toward a more angiogenic capacity.

However, the increased growth factor binding only appears to be a temporal change, as by 14 days the eNOS pDNA treated samples returned to baseline for bFGF binding (24.9% without and 22.5% IL-6 siRNA). Interestingly, for VEGF$^{165}$, there was a greater reduction in binding with the eNOS pDNA treated samples. That is, VEGF$^{165}$ binding by sGAG was reduced to levels below that of the control (18% and 6% for eNOS pDNA and IL-6 siRNA and eNOS pDNA dual treatment respectively, compared with 24% for the control). Similarly, sGAG binding of bFGF was unchanged between samples at 14 days. This is due to the temporal nature of the angiogenic process. The early stages of angiogenesis, such as pericyte detachment, increased vascular permeability and endothelial cell proliferation, are
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Figure 4-15: Growth factor binding of sGAGs extracted from tissue samples changes with eNOS pDNA treatment. Treatment with eNOS pDNA increased the ability of sGAG extracted from the tissue to bind VEGF_{165} and bFGF at 7 days, either alone or in combination with IL-6 siRNA treatment. Data represents mean ± SD (n=3, 2 pooled samples). Analysis performed using one-way ANOVA, p<0.05. *denotes statistical significance versus scaffold alone group at 7 days. ** denotes statistical significance compared with scaffold & IL-6 siRNA & eNOS pDNA group at 14 days. # denotes statistical significance compared with scaffold & eNOS pDNA and scaffold & IL-6 siRNA & eNOS pDNA groups at 7 days.
In vivo assessment of dual gene delivery reservoir

primarily governed by VEGF and bFGF activity.\textsuperscript{[60]} However, in later stages, other factors such as platelet-derived growth factor (PDGF) and transforming growth factor-\(\beta\) (TGF-\(\beta\)) are more dominant in processes such as pericycle attachment and maturation and basement membrane deposition. Thus it appears that the sGAG content of the ECM, changes its affinity towards growth factors over time and potentiates angiogenic activity. In addition to the factors tested, it is likely that the sGAG affinity towards multiple factors changes over time. With changes observed in angiogenesis and inflammation in the tissue over time, this may include a range of other growth factors as well as cytokines that can influence the progression of both angiogenesis and the inflammatory response. The importance of changes in ECM compositions as well as activity are known in tumour progression, where dynamic changes in the ECM influences inflammation and angiogenesis in the tumour microenvironment, promoting tumour growth.\textsuperscript{[19]} Similar effects have been observed with the ECM during cardiovascular disease, where the balance between inflammation and angiogenesis has shifted.\textsuperscript{[18]}

4.4 Conclusions

A 4S-PEG crosslinked collagen sphere-in-hydrogel system has been optimized for the delivery of IL-6 siRNA (from the hydrogel phase) to modulate inflammation and eNOS pDNA (from the microsphere phase) to increase angiogenesis in an \textit{in vivo} subcutaneous model. The doses of IL-6 siRNA and eNOS pDNA to be delivered through the collagen sphere-in-hydrogel system were optimized individually to reduce the volume fraction of inflammatory cells and increase the surface density of blood vessels. Dual delivery of the optimal IL-6 siRNA and eNOS pDNA doses through the collagen sphere-in-hydrogel system resulted in a reduction in the infiltration of inflammatory cells and an increase in blood vessels at 7 and 14 days. Protein and gene expression analysis revealed an overall reduction in the expression of inflammatory cytokines and an increase in the expression of angiogenic factors. Raman spectroscopy revealed a change in the ECM of the treated tissue, particularly with regard to bands associated with GAG content and sulfation. Analysis of the total sGAG
content and ratio of CS:HS following treatment revealed no changes between groups at both 7 and 14 days. However, further investigation revealed a change in the binding capacity of tissue-extracted sGAG to growth factors VEGF165 and bFGF, which was in line with the increase in blood vessels observed following treatment with eNOS pDNA, whether with or without co-delivery of IL-6 siRNA.
Figure 4-16: **Mechanism of inflammation and angiogenesis changes.** Changes in inflammation and angiogenesis were observed to be controlled at the levels, as depicted in this figure. Gene and protein analysis revealed changes in inflammatory and angiogenic factor expression. In addition, changes in the sGAG extracted from the subcutaneous tissue manifested itself as a change in sGAG affinity towards growth factors.
4.5 References


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Summary and Future Directions

Chapter Five

Summary and Future Directions

A portion of this chapter has been previously published in:

5.1 Introduction

The use of biomaterials in recent times have progressed from inert implants to delivery systems for a wide range of therapeutics. Biomaterial scaffolds have been embraced as reservoirs for a range of biological therapeutics.[1–3] These include protein and nucleic acid therapeutics as well as differentiated and progenitor cell types. However, most pathological states are complex and require a multi-factorial, temporal approach to ensure a satisfactory outcome.[4–7] With that in mind, a collagen-based delivery system was developed with the ability to delivery two separate nucleic acids in a temporal manner, first an anti-inflammatory siRNA targeting interleukin-6 (IL-6) followed by a pro-angiogenic pDNA encoding endothelial nitric oxide synthase (eNOS). The study involved a number of phases. In the first phase, a reservoir system composed of collagen was fabricated using a template method. This reservoir was characterised for use as a gene delivery depot. In the second phase a dual delivery system was assembled following the selection of an optimal sized microsphere reservoir. In combination with a collagen hydrogel, a multi-modal delivery system was characterised for its ability to deliver IL-6 siRNA and eNOS pDNA in vitro. In the final phase of the project, the assembled collagen system was assessed in vivo for its ability to modulate inflammation and angiogenesis in a subcutaneous rat model. The effect of changes in inflammation and angiogenesis on the local ECM was also explored.

5.2 Summary

5.2.1 Phase I (Chapter Two)

The objective of phase I was to develop an extracellular matrix (ECM)-based reservoir for the delivery of nucleic acids. This was achieved by using the template method which has been used previously to fabricate chitosan and elastin hollow spheres.[8–10] A collagen coating was formed around the polystyrene bead which acted as a template. This collagen coating was crosslinked followed by dissolution of the sacrificial polystyrene core using tetrahydrofuran (THF). Hollow spheres were loaded with pDNA polyplexes and the release profile assessed. In addition, cell viability and transfection was also assessed.
Hollow sphere formation was confirmed via TEM, while the removal of polystyrene was observed from FTIR spectra. Loading and release of partially degraded polyamidoamine (dPAMAM, Superfect®, Qiagen) complexed pDNA was assessed using the commercially available Cy3® dye. Loading efficiency was greater than 80% and the complexes showed a delayed released profile from the collagen microspheres over seven days in vitro. Transfection was observed using the luciferase assay. Transfection from polyplex loaded spheres was on a level similar to that of non-loaded polyplexes. In addition, polyplexes released over five days were shown to be still bioactive and capable of transfecting 3T3 fibroblasts in vitro. Importantly, it was shown that loading of polyplexes within hollow collagen microspheres ameliorated the toxic effects of polyplexes at high doses. This was shown with polyplexes formed using both dPAMAM and poly (ethylenimine) (PEI) (branched, MW=25,000).

5.2.2 Phase II (Chapter Three)

The objective of phase II of this project was to fabricate a collagen-based reservoir from which a multi-modal release of nucleic acids can be achieved. This initially involved the selection of the optimal size of collagen microsphere for use as a reservoir system. It was important to select an appropriate size of microsphere to avoid uptake by activated macrophages.[11,12] Uptake studies identified 1 μm collagen spheres as the optimal size. These microspheres were embedded within a crosslinked collagen type I hydrogel to generate a multi-modal release system. This multi-component system (spheres and hydrogel) was assessed for its effect on cellular metabolic activity and proliferation with HUVEC and primary fibroblasts. Degradation studies in the presence of collagenase showed no negative impact on stability of the crosslinked collagen hydrogel following the incorporation of 1 μm collagen spheres.

Prior to functionalisation of the system with nucleic acids, the specificity of the IL-6 siRNA was confirmed with primary fibroblasts by RT-PCR. In addition, eNOS pDNA was optimised for delivery using the dPAMAM transfecting agent via RT-PCR and the Griess
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assay. Nucleic acid release studies using labelled siRNA and pDNA showed a staggered release profile, with release from the hydrogel phase occurring quicker than that from the microsphere phase. In order to discount the possibility that the release profile was different due to differences between siRNA and pDNA, release studies were performed with two doses of pDNA, embedded within either the hydrogel or microsphere phase of the assembled system. This confirmed that the difference in release profile was due to the phase of loading in the system (in the microspheres or the hydrogel), rather than the difference between siRNA and pDNA. The activity of nucleic acids released from the collagen system was confirmed by PCR, with gradual knockdown of IL-6 mRNA observed along with a prolonged increase in the expression of eNOS mRNA.

5.2.3  Phase III (Chapter Four)

The final phase of the project was to test the collagen system in an in vivo model. The objective was to modulate the inflammatory response and to enhance angiogenesis following implantation in a subcutaneous rat implantation model. Prior to combining IL-6 siRNA and eNOS pDNA delivery in vivo, the dose of each nucleic acid was optimised in vivo. Two doses of IL-6 siRNA were examined (1 and 5 μg) , along with a non-targeting control siRNA (NTC). The effect of delivery on the volume fraction of inflammatory cells was examined at seven days. Three doses of eNOS pDNA were delivered (5, 10 and 20 μg), and the effect on surface density, length density and radial diffusion distance of blood vessels examined at 14 days. Doses of 1 μg of IL-6 siRNA and 10 μg of eNOS pDNA were determined to be optimal from this preliminary in vivo dose study. These doses were then delivered in a combinatorial manner using the assembled microsphere-in-hydrogel collagen system and tissue explanted at 7 and 14 days. The four groups examined were:

1. Collagen hydrogel & microspheres
2. Collagen hydrogel & microspheres with 1 μg IL-6 siRNA (in hydrogel)
3. Collagen hydrogel & microsphere with 10 μg eNOS pDNA (in microspheres)
4. Collagen hydrogel & microspheres with 1 μg IL-6 siRNA (in hydrogel) & 10 μg eNOS pDNA (in microspheres)

Animals were sacrificed and the tissue response to each group was assessed in terms of stereological quantification of inflammation and angiogenesis. The volume fraction of inflammatory cells as well as surface, length density and radial diffusion of blood vessels were quantified on histological sections, with immunostaining performed for confirmation. Protein blot arrays and gene arrays revealed changes in both the proteomic and gene expression profiles following treatment. Delivery of IL-6 siRNA reduced inflammatory protein expression, delivery of eNOS pDNA increased pro-angiogenic protein expression, while co-delivery of IL-6 siRNA and eNOS pDNA had a combinatorial effect, with a reduction in inflammatory cytokine expression and an increase in pro-angiogenic protein expression observed. A similar effect was seen by gene arrays, with dual delivery of IL-6 siRNA and eNOS pDNA reducing the expression of pro-inflammatory factors and increasing the expression of pro-angiogenic factors. Raman spectroscopic analysis of the tissue sections showed differences between the groups, particularly with regard to GAG content and sulfation at seven days. Changes in the sulfated glycosaminoglycan (sGAG) content of the ECM were then analysed by extracting the sGAG and running a modified dimethyl methylene blue (DMMB) assay. In terms of sGAG content, no differences were observed with regards to either total sGAG content or the ratio of heparin sulfate to chondroitin sulfate between samples. However, differences were observed in the growth factor binding capacity of sGAG extracted from the eNOS pDNA treated tissue. Increased binding to VEGF_{165} and bFGF was observed, but only at the seven day timepoint. This implies a temporal change of the sGAG in the tissue, although the possibility that the sGAG changes its capacity to bind different factors as time progresses was not tested and cannot be discounted.
5.3 Limitations

5.3.1 Phase I (Chapter Two)

Choice of transfecting reagent is a vital consideration when developing a gene-based therapy. However, no clear consensus exists with regards to what is the most suitable vector system for nucleic acid delivery, with differences in efficacy dependent on cell types and tissues being transfected. In this project a commercially available dPAMAM reagent was used. dPAMAM is a partially degraded polyamidoamine dendrimer that can complex and deliver pDNA to a number of cell lines in vitro. Despite its widespread use in vitro, dPAMAM has a number of drawbacks including cytotoxicity.[13] As with all non-viral transfection reagents, transfection levels are lower compared with viral vectors. Non-viral delivery systems are slowly bridging this gap, but this is an iterative process involving small and gradual improvements in transfection reagents rather than massive improvements.[14] In this context, since this project has begun, a number of newer non-viral transfection reagents have become commercially available that have proved themselves to be superior to dPAMAM in terms of transfection efficiency and toxicity. These include the commercially available Xfect® and Turbofect®, which have formulations optimised for both in vitro and in vivo applications. In addition, exosomes have emerged as a new class of vector with potentially greater transfection capabilities. Exosomes are used in cell-cell communication, and are therefore ideal as cell-derived carriers for the delivery of nucleic acids.[15–17] These newer delivery vectors are likely to improve the transfection from the hollow collagen microspheres. Since dPAMAM was also used in the later stages of the project (chapters 3 and 4), this limitation is also applicable to phase II and III.

The template method was used to form collagen hollow spheres. A coating of collagen is formed around a polystyrene bead, which is then removed by washing with a solvent, THF. FTIR measurements showed that the characteristic peaks of polystyrene, present prior to a following coating with collagen, were removed by THF treatment. However, FTIR is a surface measurement, with a depth typically on the order of about 1 μm. Thus FTIR is
incapable of determining if there is polystyrene below this depth remaining within the microsphere. Thus, if the polystyrene bead was partially dissolved by THF treatment, it may not show up on the FTIR analysis. In order to minimise the possibility of this occurring, multiple THF washes were performed. In addition, analysis revealed a change in the zeta potential of the microspheres following THF treatment, consistent with the removal of polystyrene. Despite this, the possibility that small amount of polystyrene were not removed cannot be discounted.

The microsphere-based gene reservoir system was assessed for its ability to deliver bioactive polyplexes in 2D culture. However, this set-up is not representative of the \textit{in vivo} situation. Thus, delivery of polyplex-loaded microspheres in a 3D environment is more representative of \textit{in vivo} applications, allowing one to study both the release kinetics and the cell-microsphere interactions in a 3D system. Despite this, 3D culture systems do not offer a perfect environment to assess transfection, as they cannot recapitulate fully the ECM microenvironment \textit{in vivo}.

5.3.2 \textit{Phase II (Chapter Three)}

Batch-to-batch variability and differences between commercially available collagen solutions complicates matters with regards to collagen hydrogel formation. Therefore, it is necessary to standardise methods for collagen hydrogel fabrication, such as has been done for fibrin glues using a double-barrelled syringe approach. This would also allow for the mixing of therapeutics or crosslinkers into either phase prior to gelation. This has been proposed as a part of future projects emanating from this work (Project I – Section 5.4.1).

5.3.3 \textit{Phase III (Chapter Four)}

Phase III involved the \textit{in vivo} testing of the system developed in phases I and II. In phase II, the system was functionalised with an anti-inflammatory siRNA targeting IL-6 and a pro-angiogenic pDNA encoding eNOS, and in phase III the system was assessed in an \textit{in vivo} model. The \textit{in vivo} model involved subcutaneous delivery of the system via injection, and
assessment of the tissue response at 7 and 14 days. Doses of IL-6 siRNA and eNOS pDNA were optimised seperately prior to them being combined. While there was a level of inflammation present in the tissue microenvironment, this was due to the foreign body response to the implanted biomaterial, and is not a model of inflammation. Similarly, assessment of any angiogenic therapy is most appropriate and applicable in an ischemic tissue environment, which was not the case. Ideally, the system needs to be assessed in a model in which inflammation and angiogenesis are compromised. As it was, the study gave an overview of the overall tissue response to the biomaterial, and a proof-of-concept assessment of the functionalisation, confirming the capability of the system to modulate inflammation and promote angiogenesis. Future studies will focus on the analysis of the effectiveness of the system in a number of disease models, as will be outlined in section 5.4.1. These models include critical limb ischemia (CLI), myocardial infarction (MI) and diabetic wound healing.

In phase II, the *in vitro* release profile of the microsphere/hydrogel system was characterised. However, this release profile is likely to be different *in vivo*, with the presence of enzymes and proteases capable of degrading the implanted collagen system that accelerate release. A definitive link between the *in vitro* and *in vivo* release profile will aid in future optimisation of the system. An *in vivo* release study using labelled siRNA and pDNA and *in vivo* imaging techniques will help to ascertain a correlation between *in vitro* and *in vivo* release kinetics, and enable further development and optimisation of the system *in vitro* via crosslinking and responsive linker systems.

### 5.4 Future Directions

Based on the results a number of projects are proposed that are based on further modification or application of the collagen hydrogel/microsphere system that has been developed. The first project involves the application of the collagen hydrogel/sphere system in a number of pre-clinical models in which inflammation and angiogenesis are compromised. The second project describes the development of more reactive and progressive delivery systems that can react to the stimuli around the pathological states.
The third project involves the use of the collagen hydrogel/sphere system in combination with progenitor cells, to study both the interaction between released factors and also the effect of these combined factors on progenitor cell fate and function. The final project describes the identification of therapeutic targets specific to the biomaterial itself and its release profile via protein and RNA arrays. These projects are outlined below.

5.4.1 Project I – Pre-clinical assessment

Overall, this project has focused on the development and establishment of a collagen system with the ability to deliver two separate doses of nucleic acids in a temporal manner. This has been achieved and verified in vitro using labelled siRNA and pDNA and in vivo in a subcutaneous rat model. However, the delivered siRNA and pDNA was simply to modulate the tissue response to the biomaterial itself, in this case a collagen hydrogel with embedded collagen spheres. This is vital for proof of concept and verification of the potential of the system. Thus, the system has been established and its ability to deliver nucleic acids confirmed. The next step for this system is its application and the assessment of its efficacy in a wide number of disease states including CLI, MI and diabetic wound healing, as can be seen in figure 5-1. Each of these pathologies involve an excessive inflammatory response and reduced perfusion, thus a strategy to deliver anti-inflammatory and pro-angiogenic nucleic acids in a temporal manner will prove efficacious. In this project, the hydrogel/microsphere system has been used to deliver IL-6 siRNA and eNOS pDNA.
Figure 5-1: Clinical targets for composite hydrogel/sphere system. Conditions such as (A) diabetic wound healing, (B) myocardial infarction (MI) and (C) critical limb ischemia (CLI) all involve dysregulation in inflammation and angiogenesis, and thus will benefit from the temporal delivery of anti-inflammatory and pro-angiogenic therapeutics.
However, the nucleic acids to be delivered may be altered to suit specific pathologies. In addition, therapeutic biomolecules such as proteins may also be delivered in place of nucleic acids if preferred. Hollow collagen microspheres have been shown to be capable of loading and delivering bioactive growth factors,[18] while collagen hydrogels have previously been used to deliver a wide range of biological therapeutics, notably miRNA[19] and stem cells.[20,21]

A key factor in the translation of this system is the ease of use and injectability of the system, which has been verified in vivo in a subcutaneous model in this project. However, a separate hydrogel was assembled at the time of implantation for each animal, requiring a certain level of expertise and experience. This requirement introduces the possibility for human error, while the possibility of gelation occurring prior to injection, especially given the elevated temperatures in an operating theatre cannot be discounted. In an effort to overcome these issues, optimisation and standardisation of the assembled collagen system is necessary. This will involve a double syringe set-up, akin to that used to form fibrin hydrogels. Using such a set-up will eliminate the possibility of gelation occurring prematurely, as the collagen solution will not be neutralised or the crosslinking solution added until directly prior to injection and implantation. A possible set-up of this double syringe system is shown in figure 5-2.

5.4.2 Project II – Responsive delivery systems

Multi-modal scaffolds have mostly relied on methods such as differential physical entrapment and degradation to modulate the temporal release of therapeutics, as well as on interactions that delay the release of one factor over another. In this project, collagen microspheres were embedded within a collagen hydrogel to create a multi-modal release profile. This system is quite crude, and does not fully appreciate the dynamic nature of biological processes of pathology and disease in which the end of one phase often triggers the next. In the case of an ECM-based system, degradation occurs naturally dependent on
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Figure 5-2: Hydrogel delivery. A potential double-barreled syringe set-up to standardise collagen hydrogel and microsphere system formulation and delivery for future in vivo applications.
Figure 5-3: More control over therapeutic release. Systems that react to specific stimuli such as radical oxygen species (ROS) and matrix metalloproteinases (MMPs) can trigger the release of therapeutics and respond dynamically to changes in pathology. In addition, the release profile will depend on the magnitude of the pathological state, thus helping to overcome issues related to therapeutic dose.
matrix metalloproteinase (MMP) activity, and this directly affects release kinetics. However, systems that are more reactive and responsive in nature can prove quite useful in this respect. Biomaterial scaffolds have previously incorporated MMP-cleavable linkers[22–25] to modulate biodegradability. By utilizing this technology, intrinsic properties associated with specific phases of pathology can be used as triggers that release different factors which will allow the biomaterial to dynamically progress with the pathology. For example, MMP, radical oxygen species (ROS) or pH-sensitive linkers can be used to release anti-inflammatory molecules, while low oxygen tension can be used to trigger the release of angiogenic factors in ischemic conditions. Figure 5-3 depicts systems capable of on-demand release of multiple factors, in this case reacting to ROS and MMP activity. Linking systems can be used to bind therapeutics to scaffolds with linker systems sensitive to stimuli such as ROS and MMPs, resulting in the release of therapeutics. Mimicking, or even hijacking, natural processes to engineer responsiveness into biomaterial systems can produce sensitive, flexible and truly reactive biomaterial delivery systems. This helps to overcome issues with regard to optimizing the release of the appropriate dose, as the release rate relates to the intensity of the response, whether it be inflammatory, ischemic or another. Zisch et al have shown that ‘cell-demanded’ VEGF (MMP-dependent) results in the formation of a more regular vasculature compared with passive VEGF release via diffusion.[26] This emphasises the need to involve the host system in the release of any loaded therapeutic. In this way, these systems can be considered both multi-modal as well as responsive in nature, and may react to match the progression of specific disease states.

5.4.3 Project III – Biomaterial-based target identification

Biomaterial scaffolds are often used in combination with therapeutics or ‘targets’ which have been previously identified or validated in basic biological studies of disease. However, these studies do not take into account or consider the consequences of a biomaterial present in the microenvironment, both in terms of positive and negative impact. For example, a biomaterial implanted in the myocardium following MI may strengthen the myocardial wall
resulting in reduced remodelling, but may also induce an increased inflammatory response depending on the material itself and its degradation products. Therefore, it will prove useful to identify therapeutic targets using a biomaterial-specific approach. That is, to perform protein and RNA arrays on diseased models both in the presence of and without a biomaterial. With the addition of a positive control sample which does fully regenerate (e.g. foetal healing), the positive and negative effects of biomaterial implantation may be elucidated.

Another step in the direction of developing biomaterial-centric therapeutics is to choose factors of the regenerative process which match the timing of release from biomaterial delivery systems. Following characterisation of the *in vivo* release profile of therapeutics from the biomaterial, a study to identify regenerative factors which will compliment the temporal nature of the release of therapeutics from biomaterial can be performed. Similar to the study previously proposed, but importantly with timepoints which correspond with the release profile of therapeutics from the biomaterial delivery system. This approach may seem overly-focused on the biomaterial rather than the biologic, but in order to fully exploit biomaterials as therapies it is necessary to design the therapies and select appropriate biologics which complement the biomaterial.

*Project IV – Determine temporal interaction between factors in multi-modal system*

The addition of progenitor cells to the collagen system is an avenue which may prove useful in the context of directing cell fate and functionality. For example, for bone tissue engineering, cells may be directed to an osteoblast phenotype through the addition of plasmids encoding for combinations of VEGF, BMP, TGF-β etc., or using microRNAs. The combination of plasmids or microRNAs may be altered, and the system may act essentially as a screen to assess the differentiation of incorporated progenitor cells and also the interaction between and the suitability of the incorporated signals. The timing of presentation of these factors may be altered using modified crosslinking, and as such the
Figure 5-4: Biomaterial-specific target identification. RNA and protein arrays can be used to identify specific complimentary therapeutic targets that will aid in key regenerative processes when combined with biomaterials systems for the treatment of diabetic wounds, critical limb ischemia (CLI) and myocardial infarction (MI).
Figure 5-5: Interplay between factors and progenitor cells. The collagen hydrogel/sphere system may be used to study the temporal interplay between different factors, and their effect on progenitor cells, in terms of differentiation and function.
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osteogenic factors (BMP-2), depending on the tissue requirements and its pathological state. In this way, the system can be temporal interaction of factors, a key but often overlooked facet of biological systems, may be studied.

Priming of cells to produce paracrine factors, acting as so called ‘factories’ may also be directed using the collagen system developed in this project. By altering relative doses of the incorporated factors, and by modifying the timing of release of each, cells can be first primed to release angiogenic factors (VEGF, FGF, PDGF), and may then be switched to release anti-inflammatory (IL-10) or anti-apoptotic factors (heat shock protein 27), or pro-used to assess the interplay between various molecules as a function of time, and how these factors effect cellular behaviour and differentiation.

5.4 Conclusions

In conclusion, a composite collagen microsphere-in-hydrogel system has been developed as a reservoir of nucleic acids. This system is capable of delivering nucleic acids in a delayed manner, with the timing of release dependent on where the nucleic acid is loaded in the system i.e. in the microsphere phase or in the hydrogel phase. The system was shown to release bioactive nucleic acids over 14 days in vitro, knocking down IL-6 mRNA expression and increasing eNOS mRNA expression. In a subcutaneous in vivo model, the ability of the system to modulate inflammation and promote angiogenesis was observed, following optimisation of the respective doses of IL-6 siRNA and eNOS pDNA. A resultant change in the gene and protein expression of various inflammatory and angiogenic factors was observed, with a shift towards a reduction in inflammatory factors and an increase in angiogenic factors observed following dual-delivery of IL-6 siRNA and eNOS pDNA compared with the control. Raman spectroscopy revealed a change in the composition of the ECM of the tissue following a reduction in inflammation and increase in angiogenesis, specifically related to GAG content and sulfation. Assessment of the total sGAG content of the tissue and the relative content of HS:CS revealed no changes between treatments. However, growth factor binding studies revealed an alteration in the binding of the extracted...
sGAG to growth factors. Specifically, it was found that sGAG extracted from tissue treated with eNOS pDNA had an increased affinity for the growth factors which were tested, VEGF\textsubscript{165} and bFGF.

Thus it can be seen that the delivery of IL-6 siRNA and eNOS pDNA through the collagen sphere-in-hydrogel system modifies inflammation and angiogenesis \textit{in vivo}. This is seen through a reduction in volume fraction of inflammatory cells and an increase in length density of blood vessels. However, we also see how these changes are effected at three distinct levels of molecular control. That is, at the level of gene expression, protein expression and at the changes in the binding of growth factors to sGAG present in ECM.
5.5 References


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Appendices
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A Collagen extraction

1. Frozen tendons are cut into small pieces using a blade. The resulting small pieces are put through the blender with ice.

2. The blended tendon is washed three times in the cold room, gently stirring using the following buffer solution for two hours, at a ratio of 1 g tendon: 50 ml buffer:
   - 3.7 mM Na$_2$HPO$_4$
   - 0.35 mM KH$_2$PO$_4$
   - 51 mM NaCl

3. Suspend the washed tendon in 0.5 M acetic acid for a week in the cold room (1 g tendon : 50 ml acetic acid).

4. Add pepsin at a ratio of 1 g Pepsin: 100 g tendon (wet weight) at room temperature for one hour, then leave for two days in the cold room.

5. Filter through a sieve to remove all the big pieces of tendon that did not come into solution.

6. Add 0.9 M NaCl to the filtered solution, stirring manually every two hours (if possible) for two days. Any mechanical stirring used should be as light as possible to ensure that the precipitated collagen does not break up into pieces which are too small to collect.

7. The precipitated collagen should collect at the top of the solution. Collect as much as possible of the collagen and as little as possible of the liquid. Centrifuge at 11000 rpm for 20 minutes to remove as much liquid as possible. Weigh the collagen.

8. Re-suspend in 1 M acetic acid (1 g collagen: 50 ml acetic acid).

9. Add 0.9 M to the re-suspended collagen solution, stirring manually every two hours (if possible).
10. The precipitated collagen should collect at the top of the solution. Collect collagen (by centrifugation or filter using the sieve) and as little liquid as possible. Centrifuge at 11000 rpm for 20 minutes to remove as much liquid as possible. Weigh collagen.

11. Re-suspend in the minimum volume of 1 M acetic acid (1 g collagen : 50 ml acetic acid). [May take a few days]

12. Centrifuge at maximum speed for 30 minutes to remove any insoluble collagen/dirt/sediment, which will pellet out.

13. Add 0.9 M NaCl, stirring overnight.

14. Centrifuge at 11000 rpm for 20 minutes, collect collagen, re-suspend in minimum volume 0.5 M acetic acid.

15. When collagen comes fully in solution, dialyse in 1 mM acetic acid (10 L), stirring and changing the solution every 1.5-2hrs. Split the collagen and use a number of dialysis membranes, as a precaution incase of spillage or contamination.

16. Purity and concentration of collagen may be determined using the Sircol™ assay.

B Sircol assay

1. Adjust concentration of isolated collagen to 1 mg/ml using 5 mM hydrochloric acid. Rationale for this is that the collagen standard supplied with the Sircol™ kit is also 1 mg/ml and will thus ensure better relativity. Note: The isolated sample does not contain 1 mg/ml of pure collagen as there are trace amounts of fat tissue and non-digested proteins. The Sircol™ assay will determine the actual collagen content within this isolate.

2. Label 1.5 ml eppendorfs appropriately for 0, 10, 20, 30, 40 and 50 μg of standard and test samples. Both standards and test samples should be prepared in triplicate.

3. Add 0, 10, 20, 30, 40 and 50 μl of collagen standard into appropriate tubes.

4. Add 0, 10, 20, 30, 40 and 50 μl of isolated collagen into appropriate tubes.
5. There should now be a total of 36 tubes, half containing collagen standards and the other half containing samples of isolated collagen.

6. Adjust the volume of all tubes to 100 μl with distilled water.

7. To each tube, add 1 ml of Sircol™ dye reagent (Sirius Red in picric acid).

8. Place tubes in an eppendorf rack and place rack on mechanical shaker for 30 minutes. The dye should bind to the collagen and precipitate out of the solution.

9. Transfer the tubes to a micro-centrifuge and centrifuge at 10,000 g for 10 minutes.

10. The un-bound dye can be removed by gently tipping the contents gently onto absorbent paper.

11. The remaining un-bound dye can be removed with a cotton bud. Be careful not to touch the pellet during this procedure.

12. Add 1 ml of alkali reagent (0.5 M sodium hydroxide) to each tube.

13. Re-cap the tubes and centrifuge the contents until the precipitate has become dissolved (may take up to 15 minutes).

14. Remove 200 μl from each tube and place in a separate well of a clear 96 well plate.

15. Place the plate in a plate reader and measure absorbance at 550 nm for 0.1 seconds per well.

16. Remove the average blank reading from all readings.

17. By plotting collagen reference samples against isolated collagen samples the collagen content can be determined.

C Sulfonation of polystyrene beads

1. Dry polystyrene beads in vaccum oven at 40°C. Note: Do not freeze-dry polystyrene particles.

2. In a round bottom flask, add sulfuric acid to dried polystyrene beads in a ratio of 1 g polystyrene beads: 35 ml sulfuric acid. Sonicate to ensure homogenous distribution.

   Note: Care should be taken with concentrated sulfuric acid.
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3. Heat at 40°C under constant stirring for 18 hours.
4. Centrifuge at 7000g to retrieve sulfonated polystyrene particles.
5. Wash with ethanol (x4) and water (x4) to remove any residual sulfuric acid.
6. Remove and dry a small aliquot (20-50 μl) to determine the concentration of polystyrene particles in mg/ml.
7. Store until required for collagen hollow sphere preparation.

D Fabrication of collagen microspheres

1. Centrifuge polystyrene beads and discard water. Re-suspend in 0.5 M acetic acid and sonicate to ensure homogenous distribution and no aggregates.
2. Add collagen solution (5 mg/ml) in a weight ratio of 4:1 (polystyrene beads: collagen). Adjust final concentration of collagen to 1 mg/ml. Leave under constant stirring for four hours at room temperature.
3. Centrifuge and discard supernatant. Re-suspend in 1X PBS.
4. Add pentaerythritol poly(ethylene glycol) ether tetrascuccinimidyl glutarate (4S-PEG) in 1X PBS to crosslink the collagen coating. Add 4S-PEG such that the active ester groups in the 4S-PEG react with the amino groups of the collagen at a ratio of 1:4 (4S-PEG:Free amino groups in the collagen). For 5 mg of collagen, this equates to 8.5 mg of 4S-PEG. Leave under agitation for two hours at room temperature.
5. Centrifuge and wash with water. Re-suspend in water.
6. Add tetrahydrofuran (THF) at a ratio of 1:1 (water: THF).
7. Centrifuge and repeat to ensure complete removal of polystyrene.
8. Wash with ethanol (x2) and water (x2) to ensure complete removal of any remaining THF.
9. Remove and dry a small aliquot (20-50 μl) to determine the concentration of collagen hollow spheres.
10. Store at room temperature in ethanol until required.
Appendices

E Characterisation of collagen microspheres

E.1 Materials and methods

E.1.1 Analysis of surface coating
The process of coating polystyrene (PS) beads with type I collagen and removing the core by washing with tetrahydrofuran (THF) was characterized by SEM imaging. Images were taken of PS beads prior to coating, following coating and following removal of the PS core.

E.1.2 Zeta potential analysis of surface coating during fabrication process
To characterize the surface coating of microspheres, zeta potential was analysed using a zeta sizer (Malvern, Nano-ZS90). Microspheres at various stages of fabrication were analysed to determine the charge and to confirm coating. The four stages at which microspheres were assessed for charge analysis were: PS beads pre-coating, PS beads following collagen coating, PS beads collagen coated and crosslinked, and finally hollow crosslinked collagen microspheres (THF treated to remove PS). Zeta analysis was performed in acetic acid, in which collagen is positively charged.

E.1.3 Size analysis
Size of samples was determined by using ImageJ and measuring the diameter of 60 spheres from SEM images. In order to obtain high accuracy, the analysis was repeated for three different batches of prepared spheres. The average size of spheres obtained using the 500nm template was 440nm, and showed only a small variance. The reduction in size can be explained by the fact that the analysis was performed on dried microspheres and images taken in the vacuum environment of the SEM, which would contribute to shrinkage.

E.1.4 Sample preparation for scanning electron microscopy (SEM) and transmission electron microscopy (TEM)
Preparation of samples for SEM analysis consisted of fixation with 3% glutaraldehyde in freshly prepared 0.2 M sodium cacodylate buffer for one hour. After washing the samples in
sodium cacodylate buffer, the samples were resuspended in osmium tetraoxide for two hours in the fumehood. After a series of washings in the buffer, samples were dehydrated by immersion in a graduated series of ethanol in H₂O and hexamethyldisilazane (HDMS) in ethanol solutions of 30%, 50%, 80%, 95% and 33%, 50%, 66%, 100%, respectively. The samples were let dry on the sample holder. TEM samples were embedded in an epoxy-based resin (Agar Low Viscosity Resin kit). The embedding process consisted of incubations of samples in a mixture of Resin/Propylene Oxide 50:50 for four hours and then replaced with a mixture 75:25 overnight and lastly in pure resin for six hours. The final step consisted of thermo-crosslinking at 65°C for 48 hours which allows the resins to acquire the hardness needed to cut cross-sections of the embedded samples.

E.2 Results and discussion

E.2.1 Analysis of surface coating

The analysis of the coating process was performed using both SEM and zeta potential. As can be seen from SEM images (Fig. E-1), the spheres maintain their shape and size during the coating process. The spherical structure is maintained throughout the process. Following removal of the PS core, the hollow nature of the spheres becomes apparent under the vacuum of the SEM chamber (Fig. E-1 (C)).

Charge analysis of the coating process was carried out to observe changes in charge during the various stages of sphere fabrication. The analysis was performed in acetic acid in which collagen has a positive charge. As expected, there was a transition from a negatively charged particle to a positively charged particle when the polystyrene template was coated with collagen (Fig. E-2). There was a reduction in the positive charge when the coating was crosslinked, owing to the reduction in free amines due to the crosslinking mechanism. Following removal of the negatively charged polystyrene core there was an increase in the positive charge observed. It must be stressed that all measurements were performed in acetic acid, as the pH can effect the charge.


Appendices

E.2.2 Size analysis

Microsphere size analysis was performed to assess the consistency of the template method to produce spheres of defined size. SEM images were analysed using ImageJ. The average size of microspheres fabricated using the 500 nm polystyrene template was 440 nm, as illustrated in Fig. E-4. The reduction in size relative to the size of template used can be explained by the fact that the particles are dried prior to imaging and also that the images are taken in the vacuum conditions of the SEM chamber. As mentioned, analysis was performed using 60 spheres from three separate preparations of spheres, thus confirming the reproducibility and consistency of the method.

E.2.3 Imaging

SEM imaging confirmed the formation of a mono-dispersed non-aggregated population of spheres. This was performed following fixation with glutaraldehyde and drying through an ethanol gradient containing HDMS, and the difference is apparent between these samples (Fig. E-3) and those that were simply air-dried prior to imaging in the vacuum chamber of the SEM (Fig. E-1 (C)). The formation of a non-aggregated population is important for the application of the spheres as a drug delivery system. Aggregated spheres have lower loading capacity due to a reduced surface area, while large aggregates affect the injectability through small-bore needles.

TEM imaging gave an indication of the interaction between microspheres and 3T3 fibroblasts in vitro. Sections revealed that a small number of microspheres were internalized into cells, but that a large number of the cells were not internalized and remained external to the cells, as can be seen in Fig E-5. This was observed with 1 μm microspheres only, and it is likely that there will be differences between different sizes of spheres, while differences may also exist between different cell types.
E.3 Conclusions

Hollow collagen microspheres can be fabricated in a reproducible, consistent and non-aggregated form using the template method.
Figure E-1: Steps of hollow microsphere fabrication. SEM images of the coating process showing (A) polystyrene beads, (B) collagen coated polystyrene beads and (C) hollow collagen microspheres following treatment with tetrahydrofuran (THF) to dissolve the polystyrene template.
Figure E.2: Charge analysis of surface coating in acetic acid. Zeta potential of microspheres throughout the fabrication process with (A) 500 nm and (B) 5 μm microspheres. It was observed that the negatively charged microspheres gained a positive charge when coated with collagen. This positive charge was reduced by crosslinking of the amines, but then further increased by dissolution of the negatively charged polystyrene template by tetrahydrofuran (THF) treatment.
Figure E-3: Isolated collagen hollow microspheres. SEM image showing a non-aggregated population of uniformly sized collagen hollow microspheres.
Figure E-4: Size analysis of collagen hollow microspheres formed with a 500 nm polystyrene template. The difference in size between the template and the hollow microspheres may be accounted for by the fact that the analysis is done on SEM images, in which the hollow microspheres are subject to a vacuum.
Figure E-5: TEM images revealing the interaction between collagen hollow spheres and 3T3 fibroblasts. Imaged (B) shows some microspheres being internalized by the cells, while others are not.
F Preparation of collagen type I hydrogel crosslinked with 4S-succinimyld glutarate terminated poly (ethylene glycol)

1. Prepare type I collagen solution, 10X PBS, 1X PBS, 2 M NaOH and leave on ice.
2. Prepare solution of 4S-PEG in 1X PBS. 4S-PEG is stored at -20°C under argon and in the dark.
3. Add 100 µl 10X PBS to 900 µl collagen solution.
4. Neutralise solution by adding 2 M NaOH dropwise until pH reaches about 7.2. Too far above or below this will stop gel formation. Monitor pH carefully using pH strips with a narrow range (6-8).
5. Add 4S-PEG crosslinking solution (typically 50-100 µl) such that the free amines in the collagen react with the 4S-PEG in an optimal 4:1 ratio and mix without forming bubbles.
6. Keep on ice until ready for use. Gelation typically occurs within eight minutes.

G Preparation of composite collagen hydrogel/microsphere scaffold

1. Fabricate collagen microspheres as described in appendix D.
2. Neutralize collagen solution, add crosslinker and keep on ice as described in appendix F.
3. Centrifuge collagen microspheres and form a pellet at the bottom of an eppendorf tube. Remove supernatant in which collagen microspheres were suspended and add neutralised collagen solution containing crosslinker to the eppendorf tube.
4. Re-suspend the collagen microspheres within the collagen gelling solution to form a homogenous dispersion of microspheres within the gelling solution. Keep on ice to prevent premature gelation.
5. Take the microspheres/gelling solution into a pipette or syringe and proceed with experiment.
Appendices

H Thawing liquid nitrogen frozen cells

1. Find location of cells on inventory prior to opening liquid nitrogen container.
2. Wearing protective gloves and face shield, remove box that contains cells and locate and remove vial containing your cells. Remove cells from inventory.
3. Warm tube in palm of hands or in water-bath. Make sure lid of vial does not go below level of water.
4. When cells have thawed add to a 15 ml falcon tube. Gently add 10 ml of pre-warmed media.
5. Centrifuge at 1500 rpm for 5 minutes. Remove and discard media.
6. Re-suspend cells in 10 ml of pre-warmed media. Transfer the contents to a cell culture flask.
7. Place tissue culture flask in incubator at 37°C and 5% CO₂. Change media every two to three days (depending on cell type).

I Changing media

1. Remove media from flask. Note: when pipetting, do not let liquid get up to the cotton at the top of the pipette as this will break the pipette boy.
2. Place waste media in waste container.
3. Pipette in new pre-warmed media (T25: 3-5 mL media, T75: 8-10 mL media, T175: 25-30 mL media).
4. Return flask to incubator.

J Splitting cells

1. When cells are 80% confluent they need to be split.
2. Remove waste media and place in waste container.
3. Add 10 ml of pre-warmed Hanks balanced salt solution (HBSS). Gently agitate the flask.
Appendices

4. Remove the HBSS and discard into waste container.

5. Add Trypsin-EDTA such that the surface of the flask is completely covered (typically 3-5 ml).

6. Place in incubatory. After 5 minutes check under light microscope if cells have detached. If not, gently tap flask to detach remaining cells.

7. Add pre-warmed media to bring total volume up to 10 ml. Transfer to 15 ml falcon tube and centrifuge at 1500 rpm for 5 minutes.

8. Remove supernatant, being careful not to disturb the cell pellet.

9. Re-suspend cell pellet in 5-10 ml media (depending on desired concentration).

10. Remove 50 µl from cell suspension and add 50 µl trypan blue.

11. Use haemocytometer to count live cells (exclusion of blue) and dead cells (blue) on grid.

12. Average cell number on either side of haemocytometer, and multiply by two (to account for dilution by trypan blue) and 10,000 to calculate cell density per ml.

13. Cells may be further diluted to be seeded at required density.

**K Freezing cells**

1. Repeat steps 1-8, appendix J.

2. Re-suspend cells in freezing media and count as previous (steps 10-12, appendix I).
   
   Freezing media constitutes 45 ml FBS, 5 ml DMSO. Typically 500,000 to 1,000,000 cells are frozen down per vial. This is dependent on the cell type.

3. Place vials in Mr. Frosty™ freezing container and place in -80°C freezer. After 24 hours, remove cells from Mr. Frosty™ freezing container and place in liquid nitrogen storage. Add to cell inventory.
L  Culture and differentiation of THP-1 monocytes

1. Seed cells at 300,000 per ml. Use culture flasks for non-adherent cell lines (green caps). The media is composed of RPMI with 10% FBS, 1% L-glutamine and 1% P/S.

2. Every two days fresh media must be added to maintain cell density at 300,000-500,000 cells per ml.

3. Spin down and replace with fresh media every seven days.

M  Differentiation of THP-1 monocytes

1. Culture THP-1 monocytes as has been described in appendix L.

2. Monitor the number of cells by counting regularly. Use for differentiation when the density is at 800,000-1,000,000 cells per ml.

3. Add PMA to the medium for a final concentration of 100 ng/ml.

4. Spin down the cells and re-suspend in differentiation medium at a density of 300,000 cells per ml. Incubate cells in incubator at 37°C and 5% CO₂.

5. After 24 hours, observe under the microscope that the cells have differentiated and attached to the plate.

N  Activation of differentiated THP-1 monocytes

1. Culture and differentiate THP-1 monocytes as has been described in appendices K and L, respectively.

2. Replace differentiation media with media containing 100 ng/ml lipopolysaccharide (LPS).

3. One hour after addition of LPS proceed with experiment.

O  FITC-labelling of collagen microspheres

1. Make a stock solution of 2 mg/ml FITC in DMSO. Note: Protect FITC from light.
2. Centrifuge spheres at 12,000 rpm in micro-centrifuge, remove supernatant and re-suspend in PBS.

3. Add equal volume of FITC stock solution such that final concentration of FITC is 1 mg/ml. Note: This should be enough to label a large quantity of spheres as the FITC is always in excess.

4. Leave in the dark at room temperature for 4 hours.

5. Centrifuge spheres at 12,000 rpm in micro-centrifuge, remove supernatant and re-suspend in PBS.

6. Repeat step 5 until the supernatant is clear. Typically at least five washing steps are required.

7. Store in PBS in the dark until required.

P Isolation of cardiac fibroblasts from neonatal rat pups

1. Pre-warm all media, including HEPES buffer and PBS, to 37 °C. Also prepare an ice-cold aliquot of PBS.

2. Sacrifice two to three day old rats and excise hearts from all pups. Store the excised hearts in calcium and magnesium free PBS on ice.

3. Squeeze hearts gently with forceps to expel the blood from the lumen. Transfer hearts into fresh ice-cold PBS. Move ventricles to a dry six-cm Petri dish and mince tissue as small as possible with a scalpel blade.

4. Transfer minced neonatal heart tissue into 20 ml of warm digestion buffer (HEPES with 65 U collagenase/ml) in a falcon tube and incubate for 5 minutes in a 37 °C water bath. Mix either with a micro stirrer or by gentle shaking.

5. Let the cells settle for five minutes, remove supernatant, add new pre-warmed digestion buffer and repeat enzyme treatment six to seven times.

6. Let cells settle down and wash once with pre-warmed HEPES buffer containing 5 % horse serum. Eventually, spin cells down for one minute at 340 g.
7. Resuspend cells in adhesion medium (20 ml per 10 hearts) and plate suspension on uncoated 10 cm dishes. Incubate for 1 - 1.5 hours in incubator at 37 °C/5 % CO₂. Repeat this step. During this time, fibroblasts will stick and spread on the plate whereas cardiomyocytes remain suspended.

8. Collect and count the cells to determine cell density. Expect a yield of 0.8 - 1 x 10⁶ cardiomyocytes per heart. Culture cells as normal, changing media every two to three days.

Q Agarose gel electrophoresis of pDNA complexes

1. Add 0.7 g agarose in 100 mL TAE buffer (for a large gel) or 0.35 agarose in 50 mL TAE buffer (for a small gel).

2. Add about 2 mL extra liquid to account for boiling and mix in an Erlenmeyer flask.

3. Place in microwave and heat until boiling.

4. Remove from microwave. Be careful as flask will be very hot.

5. Allow to cool until flask can be held comfortably in hand.

6. Add 10 μL SYBR®Safe dye (Do not add until the solution is sufficiently cool).

7. Pour immediately into plate, add comb, and remove any bubbles with a pipette tip. Leave to cool and set.

8. Prepare complexes at various polymer-to-nucleic acid weight-to-weight ratios. Two controls which should always be present are uncomplexed pDNA and pDNA complexed with a known commercially available transfecting agent. Polyethylenimine (PEI) is a good positive control in this case.

9. Mix the blue loading dye with the sample prior to loading into each well, taking note of which sample is in each well.

10. Run gels at 80-100V and leave the current on ‘Auto’. Check that bubbles are forming along the wires at the bottom.
11. Check after 15 minutes – dye should have moved slightly out of the wells. Check that the direction is right and that all seems to be in order. If you leave it much after this, it is too late to rectify it.

12. Check every half hour/hour. Depending on the gel, dye, etc. it can take 3-4 hours to run. Make sure to stop it by the time that the dye reaches about ¾ of the way along the gel.

13. Put under the imager and observe under UV light – save images.


R Transfection protocol

1. Twenty-four hours prior to transfection, seed cells well plate. The cell number and size of well-plate will be determined by the cell type and analysis to be performed.

2. For a typical transfection experiment, use 1 µg of pDNA per well. Make up complexes in sterile epindorf tube.

3. Adjust volume to 50 µl with serum free media.

4. Gently vortex sample for five seconds and let stand at room temperature for a further ten minutes.

5. Remove media from cells and add the complexed DNA.

6. Adjust volume to standard culture volume with standard cell media and place in cell culture media.

7. At selected time periods (usually every two days), remove media from the cells and store at -20°C for analysis. If PCR analysis is to be performed, add Trizol reagent and freeze at -80°C.

S Luciferase assay

1. Prepare luciferase dye (1X) from stock solution supplied in kit (100X). Use the supplied buffer (1X) for diluting. Note: This dye is sensitive to light so protect with tin foil.
2. Prepare a fresh white or black plate (be consistent) 96 well plate.

3. Place 100 µl of PBS into a single well.

4. Add 10 µl of cell culture media which is removed from transfected cells.

5. Prepare plate reader for sample loading (ideally a plate reader with injection needles should be used but if not available use a multi channel pipette and test one column per test run). Plate reader settings should be set to luminescence (1.0 seconds).

6. Add luciferase dye, being careful not to make any bubbles.

7. Close plate reader lid and read sample.

**Alamarblue® metabolic activity assay**

1. Remove media from cells in culture.

2. Add HBSS into the required number of wells in a sterile 24/96 well plate.

3. Prepare alamarBlue® working solution in Hanks’ balanced salt solution (ratio 1:9 respectively). Add into well plate. Ensure to have control wells without cells containing (a.) alamarBlue® working solution and (b.) HBSS alone.

4. Incubate for two hours at 37 °C.

5. After incubating for two hours, transfer 200 µl of the dye into a clear 96 well, flat bottomed plate.

6. Measure the absorbance at 550 nm and 595 nm (0.5 seconds per well).

7. Subtract the absorbance values of HBSS only from the absorbance values of the alamarBlue® in media. This gives the absorbance of alamarBlue® in media only. Denote these values $A_{OLW}$; absorbance of oxidized form at lower wavelength, and $A_{OHW}$; absorbance of oxidized form at higher wavelength.

Calculate correlation factor:

$$R_0 = \frac{A_{OLW}}{A_{OHW}}$$
Appendices

8. To calculate the percent of reduced alamarBlue®

\[ \text{% reduction} = \left( \frac{A_{LW}}{A_{HW} - R_0} \right) \times 100 \]

9. To calculate the relative % difference in metabolic activity:

\[ \text{Metabolic activity relative to control} = \left( \frac{A_{LW} - (A_{HW} \times R_0)}{A_{LW} - (A_{HW} \times R_0)_{control,passive}} \right) \times 100 \]

**PicoGreen® proliferaton assay**

1. Remove the media and gently rinse the cells with HBSS. Add 250μl of DNase free water. Freeze-thaw cells three times (-80°C for 15 minutes minimum and thaw at room temperature until it is completely defrosted). If a collagen scaffold/hydrogel has been used, transfer the construct to an eppendorf tube and digest it by 1mL of proteinase K at a concentration of 1mg/mL at 56°C overnight. Use the digestion product directly or freeze it at -20°C.

2. Prepare 1X TE buffer (initial solution at 20X), 2μg/mL DNA stock solution (100μg/mL DNA standard) and 50ng/mL DNA stock solution (2μg/mL DNA stock solution). Note: Prepare the standard curve with DNase free water.

3. Prepare a standard curve using the volumes in table U-1 below.

4. Transfer 100μl of each sample in the 96 well plate.

5. Make up the diluted PicoGreen® solution: 5.376mL 1X TE + 27μL concentrated PicoGreen® solution.

6. Add 100μl diluted PicoGreen® solution to each sample in the 96 well plate.

7. Incubate at room temperature in the dark (cover with foil) for two to five minutes.

8. Read the plate for fluorescence (excitation: ~480nm; emission: ~520nm).

9. Plot the standard curve and determine the concentration of DNA as a function of the standard curve.
Appendices

Table U-1: Volumes for DNA standard curve.

<table>
<thead>
<tr>
<th>Final DNA concentration (ng/ml)</th>
<th>Volume of DNAse free water</th>
<th>Volume of 2 μg/ml DNA stock solution (μl)</th>
<th>Volume of 50 ng/ml DNA stock solution (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>500</td>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>90</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>95</td>
<td>5</td>
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<td>0</td>
<td>20</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

V  Griess assay

1. Re-suspend powdered Griess reagent in bottle by adding 250 ml distilled water. Reagent should be stable for use for three months following re-suspension.

2. Prepare a 100 mM solution of sodium nitrate (NaNO₂) with distilled water. Dilute 1:1000 with media (of cell type being tested) to prepare a 100 μM solution and use to prepare a standard curve according to table V-1 below.

3. Add an equal volume of re-suspended Griess reagent to sample/standard curve (50-100 μl). Be careful not to form bubbles when adding Griess reagent.

4. Allow colour to develop for 5-10 minutes in the dark (cover with tinfoil).

5. Measure the absorbance in the plate reader at 540 nm.

6. Determine experimental values from standard curve.
Table V-1: Dilutions for preparation of Griess assay standard curve.

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Volume of 100 μM NaNO2 solution (μl)</th>
<th>Volume of media (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>25</td>
<td>250</td>
<td>750</td>
</tr>
<tr>
<td>12.5</td>
<td>125</td>
<td>875</td>
</tr>
<tr>
<td>6.25</td>
<td>62.5</td>
<td>937.5</td>
</tr>
<tr>
<td>3.125</td>
<td>31.25</td>
<td>968.75</td>
</tr>
<tr>
<td>1.56</td>
<td>15.6</td>
<td>984.4</td>
</tr>
<tr>
<td>0.78</td>
<td>7.8</td>
<td>992.2</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>1000</td>
</tr>
</tbody>
</table>

**Rhodamine phalloidin staining**

1. Remove media from cells and gently wash with HBSS.
2. Add the appropriate volume of 4% paraformaldehyde (PFA) in PBS.
3. Incubate at room temperature for 15 minutes.
4. Remove 4% PFA (dispose of in an appropriate manner) and wash three times with PBS.
5. Add 0.1% Triton-X and leave at room temperature for five minutes.
6. Remove 0.1% Triton-X and wash with PBS three times.
7. Add rhodamine phalloidin solution (diluted 1:100 in PBS) and leave at room temperature for 30 minutes. Note: protect from light.
8. Remove rhodamine phalloidin solution and wash three times with PBS.
9. Add DAPI solution (diluted 1:1000 in PBS) for 30 minutes at room temperature.
10. Remove DAPI solution and wash three times with PBS.
11. Add Vectashield® mounting media and place coverslip over slide. Seal slide with nail polish and proceed to image.

X Labelling of pDNA

1. Resuspend pDNA in Nuclease-free water at 1 mg/ml.

2. Add 100 μl of Reconstitution Solution to the dry Cy™3 labeling reagent. Perform all manipulations in the dark as the Cy™3 dye is light sensitive.

3. In a sterile, nuclease-free tube, assemble the reagents in the order shown in Table X-1, making sure to add the labelling reagent last. Mix well by vortexing. Note: reaction volumes may be scaled up.

Table X-1: Volumes required for labelling of pDNA.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free Water</td>
<td>35</td>
</tr>
<tr>
<td>10 X Labelling Buffer</td>
<td>5</td>
</tr>
<tr>
<td>pDNA solution (1 mg/ml)</td>
<td>5</td>
</tr>
<tr>
<td>Cy™3 Labelling Reagent</td>
<td>5</td>
</tr>
<tr>
<td>Total Volume</td>
<td>50</td>
</tr>
</tbody>
</table>

4. Incubate the reaction mix at a constant temperature of 37 °C for one hour in the dark.

5. Prepare columns for purification. Briefly vortex each column to re-suspend the resin.

6. Loosen column one fourth and remove the stopper from bottom. Place column in 1.5 ml epindorf tube and centrifuge at 735 g for one minute. Discard buffer collected in tube.

7. Place the column in a new 1.5 ml eppendorf tube. Slowly apply 50 μl of sample to the centre of each column, being careful not to disturb the resin bed.

8. Centrifuge column at 735 g for two minutes. The purified sample is collected in the epindorf tube. Store at -20°C in the dark until required.
Appendices

Y RNA isolation

1. Add 1 ml of TRI Reagent® to wells containing scaffolds/cells.
2. Homogenize samples containing scaffold using a tissue rupture, being careful not to contaminate any adjacent wells. Make sure scaffolds have been completely homogenized. Using a 1 ml pipette tip, aspirate the solution.
3. Store homogenate for five minutes at room temperature to dissociate nucleoprotein complexes. Remove the TRI Reagent® solution to a sterile 1.5 ml eppendorf.
4. Add 200 μl of chloroform per 1 ml of TRI Reagent®. Shake vigorously for 15 seconds by inversion. Incubate for 15 minutes at room temperature.
5. Centrifuge at 12,000 g for 15 minutes at 4 °C. Following the centrifugation, three phases will appear; a lower red phenol-chloroform phase, an interphase, and an aqueous phase (translucent). The mRNA is located within the aqueous phase.
6. Remove the clear upper aqueous phase (~ 650 μl) to a sterile eppendorf. Be careful not to touch the interface. Leave a little of the upper phase to avoid contact with the interface.
7. Slowly add an equal volume of 70% ethanol and mix by inversion.
8. Add 700 μl sample from previous step to RNeasy™ column.
9. Centrifuge at 8,000 g for 15 seconds and discard the collected solution.
10. Repeat step 10 and 11 for remaining sample.
11. Add 350 μl of RW1 buffer to centre of column, centrifuge at 8,000 g for 15 seconds. Discard the collected solution.
12. Transfer column to new 1.5 ml eppendorf. Add 500 μl RPE to centre of column, centrifuge at 8,000 g for 15 seconds. Discard the collected solution.
13. Add 500 μl of RPE buffer to centre of column, centrifuge at 8,000 g for 15 seconds. Discard the collected solution and centrifuge for a further two minutes at 8,000 g.
14. Transfer column to a new 1.5 ml eppendorf. Add 30 μl RNase-free water onto the column, incubate at room temperature for one minute and centrifuge for one minute at 8,000 g.

15. Add a further 30 μl RNase-free water onto the column, incubate at room temperature for one minute and centrifuge at 8,000 g for one minute. Split the collected sample into three for storage purposes.

16. Determine the concentration using the NanoDrop™ and freeze at -80 °C.

17. Dilute RNA 1:50 or 1:100 in water RNase Free. Measure the absorbance at 260 nm. (Calibrate the spectrometer with water). The purity is determined from the ratio between A260 and A280. The ratio A260/A280 should be above 1.8 to indicate pure RNA.

Z Synthesis of cDNA

1. Add up to 1 μg of RNA and 0.5 μg random primers in a pre-chilled tube and bring volume up to 5 μl with nuclease-free water.

2. Denature the target RNA and primers by incubation at 70 °C for 5 minutes. Chill on ice for 5 minutes.

3. Prepare reverse transcription mix as per table Z-1 below. Begin by adding the biggest volume. Add the reverse transcriptase last.

Table Z-1: Recipe for reverse transcriptase mix. Volumes are shown for one sample and must be scaled up depending on number of samples.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Reaction (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease free H₂O</td>
<td>5.6</td>
</tr>
<tr>
<td>ImProm-II™ 5 X Reaction Buffer</td>
<td>4</td>
</tr>
<tr>
<td>MgCl₂, 25 mM</td>
<td>2.4</td>
</tr>
<tr>
<td>dNTP mix (10mM each dNTP)</td>
<td>1</td>
</tr>
<tr>
<td>Recombinant Rnasin ribonuclease inhibitor</td>
<td>1</td>
</tr>
<tr>
<td>ImProm-II ™ reverse transcriptase</td>
<td>1</td>
</tr>
<tr>
<td>Final volume reaction mix</td>
<td>15</td>
</tr>
</tbody>
</table>
4. Gently vortex to mix.

5. Add 15 μl to each tube containing RNA and random primers. Gently vortex to mix.

6. Run samples on program outlined in table Z-2 below.

Table Z-2: PCR machine program for reverse transcription of RNA to cDNA.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annealing</td>
<td>25 °C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Extension</td>
<td>42 °C</td>
<td>60 minutes</td>
</tr>
<tr>
<td>Heat inactivation of reverse transcriptase</td>
<td>70 °C</td>
<td>15 minutes</td>
</tr>
<tr>
<td>End</td>
<td>4 °C</td>
<td>Indefinitely</td>
</tr>
</tbody>
</table>

7. When program has finished proceed with PCR or store cDNA at -20°C.

**AA RT-PCR**

1. Dilute cDNA template so as to obtain a final concentration of 20ng per well.

2. Make sure that the cDNA concentration does not exceed 100ng/reaction and 10% of the final volume.

3. Add the components listed in Table AA-1 below to prepare master mix.

Table AA-1: Recipe for one RT-PCR reaction. Must be scaled up for multiple samples.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per Reaction (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 X Quantifast SYBR™ Green PCR Master mix</td>
<td>12.5</td>
</tr>
<tr>
<td>Forward primer, pmol</td>
<td>0.25</td>
</tr>
<tr>
<td>Reverse primer, pmol</td>
<td>0.25</td>
</tr>
<tr>
<td>Template cDNA</td>
<td>1.49</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>10.51</td>
</tr>
<tr>
<td>Final volume</td>
<td>25</td>
</tr>
</tbody>
</table>

4. For a triplicate reaction, add cDNA template in triplicate and also add a negative control by replacing cDNA with nuclease free water.
5. Mix the master mix by pipetting and add to each well to obtain final volume of 25 μl.

6. Add the plastic cover provided by supplier on the PCR plate.

7. Centrifuge 1 minute at 1400 rpm. Place the plate in the machine.

8. Open the step one software and enter the details to map the plate on the software.

9. Choose Sybr™ Green filter for each well.

10. Choose the endogenous control from the plate and enter in the software.

11. Set up the steps in accordance to the gene and melting temperature ($T_m$) of the primers. A general program is detailed in Table AA-2 below.

Table AA-2: A typical program for RT-PCR.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Ramp Rate</th>
<th>No. Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial step</td>
<td>50 °C</td>
<td>2 minutes</td>
<td>Maximal/fast</td>
<td>1</td>
</tr>
<tr>
<td>PCR initial activation step</td>
<td>95 °C</td>
<td>5 minutes</td>
<td>mode</td>
<td>1</td>
</tr>
<tr>
<td><strong>Two step cycling</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>10 seconds</td>
<td>Maximal/fast</td>
<td>40</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60 °C</td>
<td>30 seconds</td>
<td>mode</td>
<td></td>
</tr>
<tr>
<td><strong>Final Step</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>15 seconds</td>
<td>Maximal/fast</td>
<td>1</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60 °C</td>
<td>20 seconds</td>
<td>mode</td>
<td></td>
</tr>
<tr>
<td>Final denaturation</td>
<td>95 °C</td>
<td>15 seconds</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**BB Subcutaneous implantation in a Lewis rat model**

1. At time 0, weigh rats and conduct health check.

2. Anaesthetize using isofluorane inhalation (5% induction reducing to 1-2% for maintenance).

3. Maintain the animals under anaesthetic with a maintenance dose of 1-2% isofluorane and room air inhaled through a mask.

4. Shave the dorsum of the rats and swab the area of injection with 4% chlorohexidine or 10% iodine to control bacterial contamination and minimize any potential risk of infection.
Appendices

5. Four injections of the in situ gelling system may be made per back. Use a 22 gauge needle to inject a sample volume of 50ul, and mark the injection site to make it easier to monitor and retrieve samples post sacrifice. Make two injections either side of the midline, with a spacing of at least 1cm between injections. Randomise samples within and between animals to account for variability between animals. Be sure to note the location of each sample within an animal.

6. Post-operatively, place animals on a heating pad or in an incubator until able to regulate their own temperature and fully recovered from anaesthesia.

7. If any signs of infection are observed in an animal, administer antibiotic therapy (Baytril - enrofloxacin) daily for three to four days. Monitor the infection and ensure that it clears up.

8. Mark each individual animal with an individual tail mark. House animals in two’s or three’s.

9. Monitor the animals on a daily basis and fill out animal distress scoring chart (appendix CC). Any animal reaching a score of 12 or more is to be humanely sacrificed to minimize suffering.
Appendices

CC  Animal distress scoring chart

Table CC-1: Animal distress scoring sheet used to analyse animal behavior and well-being post biomaterial-implantation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symptom</th>
<th>Score</th>
<th>Date/Time</th>
<th>Date/Time</th>
<th>Date/Time</th>
<th>Date/Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>Normal</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lack of grooming</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Piloerection, ocular and nasal discharge</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Piloerection, hunched up</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Above and eyes half closed</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural behaviour</td>
<td>Normal</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Minor changes</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Less mobile, but alert</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Restless or still, not alert</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydration</td>
<td>Normal</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abnormal pinch test</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory signs</td>
<td>Normal respiratory rate and pattern</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased respiratory rate</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased respiratory rate and abdominal breathing</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decreased respiratory rate and abdominal breathing</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Marked abdominal breathing and cyanosis</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Provoked behaviour</td>
<td>Normal</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Minor depression or exaggerated response</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moderate change in expected behaviour</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Very weak and pre-comatose</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>0-19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DD  Animal sacrifice and tissue processing

1. Prepare aliquots of 10% buffered formalin.
2. Identify animals by markings on tail.
3. Sacrifice animals by CO₂ asphyxiation.
4. Shave dorsum of rat.
5. Prepare labeled tubes with formalin for each sample. In addition, prepare labelled empty tubes and place on ice.
6. Make an incision into the skin and proceed to cut around the four injection sites (if they can be identified).
7. Remove patch of skin and identify the four implants.
8. Cut out the implants and split each sample in two. Place one sample in formalin and place the other sample on ice. Note: Be sure to have tubes label clearly and accurately.
9. Store tissue in formalin at 4°C for 24 hours. Place tissue which had been on ice in freezer at -80°C.

**EE**  **Histological staining**

**EE.1** *Hematoxylin & eosin*

1. Stain in hematoxylin for eight minutes.
2. Wash in running tap water for eight minutes.
3. Differentiate in 1% acid alcohol for 30 seconds.
4. Note: prepare the 1% acid alcohol fresh and be strict with the 30 seconds time).
5. Wash slides in running H₂O for five minutes.
6. Blue in 0.2 % ammonia H₂O or saturated lithium carbonate for 30 seconds to one minute.
7. Wash in running tap water for five minutes.
8. Rinse in 95% EtOH – dip ten times.
9. Counterstain in eosin solution for 30 seconds – 1 minute.
10. Dehydrate:

   i. 95% ethanol for 5 minutes
   ii. 100% ethanol for 5 minutes twice
Appendices

iii. Xylene for 5 minutes twice.


**FF  Stereology**

**FF.1  Taking images**

1. Place stained slide under bright field light at 1.25X and identify the tissue.
2. Switch to the 10X magnification and identify the implant site as best you can (it may be partially or fully degraded).
3. Switch to 40X magnification and take three pictures at random in the vicinity of the implant.

**FF.2  Surface density**

1. Choose a cycloid grid in the command box.
2. Calibrate all images for this measurement. A microscope slide with markings of known dimensions has been captured in ImagePro® previously, with images at every objective and saved on the computer.
3. Open the calibration folder. Select the appropriate objective lens folder, and open an image.
4. Scroll down ‘measurements’ toolbar, and select ‘calibration wizard’.
5. In the pop-up menu, select ‘set calibration’. Choose the objective lens, and measure the size of the circle on the open image.
6. In the next command, enter the actual measurement of the circle (given in the name of the image). Save this calibration, in your working folder. This file must be re-opened every time ImagePro® is re-opened.
7. Open the calibration file. Go to ‘measurements’ and ‘set system’.
8. Apply a cycloid grid of radius 20 μm, spacing 40 μm and set margins to 20 μm.
9. Overlay the grid.
Appendices

11. As the length of the cycloid grid is twice its height, each arc is 40 μm. Therefore, multiply the number of arcs by 40 to calculate the length of test line.

12. Count the number of intersections between blood vessels and the cycloid grid.

13. Use the following formula to calculate surface density:

\[ S_V = 2 \times \frac{I}{L_T} \]

\( S_V \) = surface density

\( I \) = number of intersections

\( L_T \) = total length of test line

**FF.3 Length density**

1. Rotate each image by 90°.

2. Apply the same grid and settings as for surface density.

3. Count the number of intersections between blood vessels and the cycloid grid.

4. Use the following formula to calculate length density:

\[ L_V = 2 \times \frac{I}{(L_T \times T_S)} \]

\( L_V \) = length density

\( I \) = number of intersections

\( L_T \) = total length of test line

\( T_S \) = section thickness
Appendices

Figure FF-1: Haemotoxylin and Eosin (H&E) stained subcutaneous rat tissue with overlayed cycloid grid to quantify angiogenesis.

**FF.4 Radial diffusion**

1. Radial diffusion is calculated following calculation of the length density using the following formula:
   \[
   R_{\text{DIFF}} = \frac{1}{\sqrt{\pi \times L_V}}
   \]
   
   \(R_{\text{DIFF}} = \text{radial diffusion}\)
   
   \(L_V = \text{length density}\)

**FF.5 Volume fraction**

1. Open image and select ‘grid mask’ command.
2. Choose a line grid with 40 x 40 pixel spacing and margins of 20 pixels. Click ‘apply’.
3. Make sure cells/objects of interest are not bigger than the area enclosed by four grid points.
4. Record both the number of inflammatory cells that hit grid points and also the number of grid points that hit the tissue of interest.
5. Calculate the volume fraction of inflammatory cells using the following formula:

\[ V_V = \frac{P_I}{P_T} \]

\( V_V \) = volume fraction of inflammatory cells

\( P_I \) = number of grid points which hit inflammatory cells

\( P_T \) = number of grid points which hit the tissue of interest

Figure FF-2: Haemotoxylin and Eosin (H&E) stained subcutaneous rat tissue with overlayed point grid to quantify inflammatory volume fraction.

GG Immunostaining

1. Prepare the following solutions:

**Antigen retrieval buffers**

1. **Tris-EDTA buffer** (10 mM Tris-Base, 1 mM EDTA, 0.05% Tween20, pH 9.0)

   - Tris Base 1.21 g
   - EDTA 0.37 g
   - ddH₂O 1000 ml
Appendices

- Tween- 20 0.5 ml

II. Citrate buffer (10mM Citric acid, .05% Tween-20, pH 6.0)

- Citric acid (anhydrous) 1.92 g
- ddH₂O 1000 ml
- Tween-20 0.5 ml

**Blocking buffers**

I. Goat block

- 1% BSA (stabilizer) 0.5 g or 1.67 ml
- 0.1% Triton x100 50 µl
- 0.1% cold-water fish skin gelatin 50 µl
- 0.05% Tween-20 25 µl
- Add PBS up to 50 ml

2. Immerse the slides in a staining dish and cover with the appropriate buffer (if both buffers are to be used, use the tris-EDTA buffer first, allow to cool down, wash once with PBS and then repeat with the citrate buffer).

3. Half fill the pressure cooker with water and place the staining dish in the pressure cooker.

4. Cook in the microwave for approximately eight minutes.

5. Allow the staining dish to stand at room temperature for approximately 15 minutes.

   Following this place on ice for 15 minutes or until cool.

6. Was three times with PBS.

7. Incubate for 30 minutes in 1% TritonX-100 in PBS (skip this step if the antigen is on the membrane).

8. Wash three times with PBS.

9. Use PapPen™ to draw circles around tissue sections on each slide.
Appendices

10. Block the tissue for 30 minutes at room temperature. Note: Use serum from the species in which the secondary antibody is raised i.e if secondary antibody is raised in goat, use goat serum in blocking buffer.

11. Wash three times with PBS.

12. Wash three times with 0.05% PBS-Tween.

13. Incubate overnight at 4°C with primary antibody at appropriate dilution. Ensure that the slide will not dry out by using a humidity chamber.

14. Wash three times with 0.05% PBS-Tween.

15. Incubate secondary antibody at appropriate dilution for 30 minutes at room temperature in the dark.

16. Wash three times with PBS.

17. Incubate in DAPI solution at room temperature for 10-15 minutes in the dark.

18. Wash three times with PBS.

19. Mount the slides with Prolong Gold Anti-fade solution. Put the slides at 4°C overnight in the dark and seal the edges of the glass coverslips the next day.

**HH Extraction of sulfated glycosaminoglycans (sGAGs)**

1. Weigh out tissue samples of about 25 mg. Note and record the exact weight of each sample.

2. Prepare the following buffer:
   - Tris-HCL 50 mM
   - NaCl 10 mM
   - MgCl₂ 3 mM
   - 1 % Triton X-100
   - Adjust the pH to 7.9
3. Add proteinase K at a concentration of 200 μg/ml in previously prepared buffer. Ensure the tissue sample is completely covered by the solution. Incubate at 56°C for 24 hours.

4. Incubate at 90°C for 30 minutes (to de-activate proteinase K).

5. Centrifuge at 12,000 rpm for 15 minutes. Collect supernatant.

6. Add 10 U of DNase per sample to the collected supernatant and incubate at 37°C overnight.

7. Add same volume of 4 M NaCl (to make a 2 M NaCl solution) and quickly vortex.

8. Add trichloroacetic acid (TCA) such that there is a final concentration of 10%.

9. Vortex and centrifuge at 12,000 rpm for 10 minutes.

10. Collect the supernatant and add the same volume of chloroform.

11. Vortex quickly and collect the aqueous phase.

12. Dialyse sample in the following buffer:
   - Tris-HCl 50 mM
   - CH₃COOH Na 50 Mm
   - CaCl₂ 2 mM
   - Adjust pH to 7

13. Change the buffer three times during dialysis (minimum four hours).

14. Dialyse samples in water with three changes (minimum four hours).

15. Freeze-dry the samples.

16. Re-suspend in water (volume of about 200 μl, depending on amount of sGAG). Proceed to sGAG quantification.

II Quantification of sulfated GAGs

II.1 Quantification of total sulfated GAGs

1. Prepare the following solutions:

   DMMB dye solution
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- 16 mg DMMB
- 25 ml ethanol
- 200 ml 1 M guanidine hydrochloride
- 2 g sodium formate
- 2 ml formic acid (98%)
- 750 ml water

**Decomplexation solution**

- 3.4 g sodium acetate tri-hydrate
- 191.06 g guanidine hydrochloride
- 50 ml propan-1-ol
- 500 ml water

2. Prepare a standard curve with chondroitin A as per table below:

   **Table II-1: Standard curve for sGAG quantification by DMMB.**

<table>
<thead>
<tr>
<th>Total sGAG amount (μg)</th>
<th>Volume of 0.1 mg/ml chondroitin A solution (μl)</th>
<th>H₂O (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.25</td>
<td>2.5</td>
<td>97.5</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>70</td>
</tr>
</tbody>
</table>

3. Place 20 μl of extracted sGAG solution into a 1.5 ml epindorf tube.
4. Add 1 ml of DMMB dye solution to each tube (samples and standards).
5. Vortex for 30 minutes.
6. Centrifuge samples at 12,000 rpm and 8°C for 10 minutes.
Appendices

7. Carefully remove all of the supernatant from the tubes and discard. Be careful not to touch the walls of the tubes.

8. Add 300 μl of the decomplexation solution and vortex for 30 minutes.

9. Remove 200 μl and read absorbance at 650 nm in plate reader.

II.2 Quantification of heparin sulfate

1. Prepare DMBB dye solution and decomplexation solution as described in appendix FF.1.

2. Prepare a standard curve of heparin sulfate. Use 0.1 mg/ml stock solution and make up standard curve as defined in table FF-1.

3. Prepare a 3 mg sample chondroitin sulfate as a control to ensure total degradation by chondroitinase ABC.

4. Place 40 μl of extracted sGAG solution into a 1.5 ml eppendorf tube.

5. Add 30,000 U of chondroitinase ABC (3 μl from 10,000 U/ml stock).

6. Incubate at 37°C for 90 minutes.

7. Add 1 ml of DMBB dye solution to each tube (samples and standards).

8. Vortex for 30 minutes.

9. Centrifuge samples at 12,000 rpm and 8°C for 10 minutes.

10. Carefully remove all of the supernatant from the tubes and discard. Be careful not to touch the walls of the tubes.

11. Add 300 μl of the decomplexation solution and vortex for 30 minutes.

12. Remove 200 μl and read absorbance at 650 nm in plate reader.

II.3 Quantification of chondroitin sulfate

1. Prepare DMBB dye solution and decomplexation solution as described in appendix II.1.

2. Prepare a standard curve of chondroitin sulfate. Use 0.1 mg/ml stock solution and make up standard curve as defined in table II-1.
3. Prepare a 3 mg sample of heparin sulfate as a control to ensure total degradation of HS by method described below.

4. Place 40 μl of extracted sGAG solution into a 1.5 ml epindorf tube.

5. Add 100 μl of 33% acetic acid and 100 μl of 0.25 M NaNO₂.

6. Incubate at room temperature for one hour. Observe the formation of bubbles in the tube.

7. Add 1 ml of DMMB dye solution to each tube (samples and standards).

8. Vortex for 30 minutes.

9. Centrifuge samples at 12,000 rpm and 8°C for 10 minutes.

10. Carefully remove all of the supernatant from the tubes and discard. Be careful not to touch the walls of the tubes.

11. Add 300 μl of decomplexation solution and vortex for 30 minutes.

12. Remove 200 μl and read absorbance at 650 nm in plate reader.

**Optimisation of growth factor/primary antibody concentration for GAG/Heparin competition assay**

1. Coat an ELISA plate with 100 μl per well of heparin-protein complex in 50 mM Tris-HCL, 12.7 Mm EDTA (pH = 7.4). Incubate overnight at 4°C.

2. Wash three times with 0.05% PBS-Tween 20.

3. Block the plate with 3% PBS-BSA (300 μl per well). Incubate for one hour at room temperature.

4. Wash three times with 0.05% PBS-Tween 20.

5. Add 50 μl per well of growth factor solution in 1% PBS-BSA containing a number of concentrations (e.g. 0, 0.125, 0.25, 0.5, 1, 2). Add 50 μl per well of PBS.

6. Incubate plates for one hour at room temperature.

7. Wash three times with 0.05% PBS-Tween 20.
Appendices

8. Add 100 μl per well of primary anti-growth factor antibody (at two concentrations that are to assessed) diluted in 1% PBS-BSA to each well. Incubate the samples for one hour at room temperature.

9. Wash three times with 0.05% PBS-Tween 20.

10. Add 100 μl per well of secondary antibody diluted in 1% PBS-BSA. Incubate for one hour at room temperature.

11. Wash three times with 0.05% PBS-Tween 20.

12. Detect the peroxidase activity by adding 100 μl per well of the detection mixture (1:1 mixture of the two components of the TMB substrate kit).

13. Incubate the plate for a maximum of 15 minutes at room temperature.

14. Stop the reaction by the addition of 100 μl per well of 2M sulfuric acid.

15. Read the plate at 450 nm.

16. Determine the optimal amount of growth factor and antibody concentration by selecting values that give an absorbance value of around 1.

Figure JJ-1: Optimisation curves for concentration of vascular endothelial growth factor (VEGF165) protein and antibody. In this case a VEGF amount of 1.5 ng per well was selected, and a primary antibody concentration of 1:500. (n=3)
Appendices

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Figure JJ-2: Optimisation curves for concentration of fibroblast growth factor-2 (FGF-2) protein and antibody. In this case an FGF amount of 0.3125 ng per well was selected, and a primary antibody concentration of 1:2000. (n=3)

KK  GAG/Heparin competition assay towards growth factor

1. Coat an ELISA plate with 100 μl per well of heparin-protein complex in 50 mM Tris-HCl, 12.7 Mm EDTA (pH = 7.4). Incubate overnight at 4°C.

2. Wash three times with 0.05% PBS-Tween 20.

3. Block the plate with 3% PBS-BSA (300 μl per well). Incubate for one hour at room temperature.

4. Wash three times with 0.05% PBS-Tween 20.

5. Add 50 μl of GAG sample per well, at the dilutions shown in table KK-1. Heparin is used as a positive control, at the dilutions shown in table KK-2 below. In addition a positive and negative control of binding are necessary (50 μl of PBS in each set of wells).

6. Add 50 μl of the growth factor (VEGF₁₆₅ or bFGF, in 1% PBS-BSA) in the predetermined amount to each well, except the negative control well. To this well, simply add 1% PBS-BSA with no growth factor.

7. Incubate the samples for one hour at room temperature.

8. Wash three times with 0.05% PBS-Tween 20.

9. Add 100 μl per well of primary anti-growth factor antibody (at the pre-determined concentration) diluted in 1% PBS-BSA to each well. Incubate the samples for one hour at room temperature.
Appendices

Table KK-1: Dilutions of sGAG required for GAG/Heparin growth factor binding competition assay.

<table>
<thead>
<tr>
<th>sGAG concentration (μg/ml)</th>
<th>Volume to be added (μl)</th>
<th>Total amount of sGAG added (ng/well)</th>
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Table KK-2: Heparin dilutions required for GAG/Heparin growth factor binding competition assay.

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<th>Heparin concentration (ng/ml)</th>
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<tr>
<td>0.01</td>
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10. Wash three times with 0.05% PBS-Tween 20.

11. Add 100 μl per well of secondary antibody diluted in 1% PBS-BSA. Incubate for one hour at room temperature.

12. Wash three times with 0.05% PBS-Tween 20.

13. Detect the peroxidase activity by adding 100 μl per well of the detection mixture (1:1 mixture of the two components of the TMB substrate kit).

14. Incubate the plate for a maximum of 15 minutes at room temperature.
Appendices

15. Stop the reaction by the addition of 100 μl per well of 2M sulfuric acid.

16. Read the plate at 450 nm.

LI. Protein extraction from tissue

1. Retrieve protein samples from -80°C storage and keep on icebox.

2. Place approximately 1 gram of tissue into eppendorf suitable for TissueLyserLT bead mill (Qiagen) with 1 ml lysis buffer (RIPA buffer) and one stainless steel bead. The TissueLyser LT functions by vertically oscillating the rupture chamber at a high speed to move the stainless steel bead up and down rapidly within the eppendorf to rupture and homogenise the associated tissue.

3. Place eppendorfs into the pre-cooled bead mill chamber and begin rupture for one minute. Check samples to see if complete homogenisation has taken place. Otherwise repeat rupture until complete homogenisation occurs, taking care to keep the eppendorfs cool.

4. Spin samples in a centrifuge at 10,000 rpm for 5 minutes at 4°C. Remove supernatant and quantify total protein content using BCA protein assay.

MM BCA assay for protein quantification

1. Prepare BSA standards according to manufacturers instructions.

2. Prepare the working reagent by mixing 50 parts BCA reagent A with 1 part BCA reagent B. Note: 200 μl is required per sample and standard.

3. Pipette 25 μl of each sample or standard into wells of a 96 well-plate. Note: Dilution of samples may be necessary depending on concentration.

4. Add 200 μl of working reagent to each well and shake on agitator for 20 seconds to ensure complete mixing. Cover plate and incubate at 37°C for 30 minutes.

5. Allow plate to cool to room temperature, then proceed to read absorbance at 562 nm on plate reader.
Appendices

6. Compare values with standard curve to determine concentration, ensuring to take any dilutions into account.

**NN Protein blot array**

**NN.1 Method**

1. The extracted protein must be dialyzed with a Dialysis tube (Item A) prior to the biotin labeling procedure. Recommended loading is 2.5~3.0 ml of extracted protein into a dialyzer and dialyzing with at least 500 ml 1X PBS buffer (pH = 8) at 4 °C.

2. After three hours, change the 1 x PBS buffer and dialyze again. Allow at least three hours for each dialysis step, stirring gently. (Dialysis may be left overnight)

3. Immediately before use, briefly spin down the Labeling Reagent tube (Item B). Add 100 μl 1X PBS into the tube, pipette up and down or vortex to dissolve the powder to prepare 1X Labeling Reagent solution. Add an appropriate amount of prepared Labeling Reagent (7.2 μl of 1X Labeling Reagent is required for labeling 1 mg protein) into tube with sample in step 2, mix well immediately.

4. Incubate the reaction solution at room temperature for 30 min with gentle shaking. Gently tap the tube to mix the reaction solution every 5 min.

5. Add 5 μl Stop Solution into the above reaction solution and then use the spin column to remove free biotin. Twist off the spin column’s bottom closure and loosen the cap. Place the column into a 50 ml collection tube. Centrifuge column at 1,000 g for three minutes to remove storage solution.

6. Add 5 ml 1X PBS into column, centrifuge at 1,000 g for three minutes to 1X PBS. Repeat twice to wash the column.

7. Place the column in a new collection tube and slowly load the sample to the center of the compact resin bed. Centrifuge the column at 1,000 g for three minutes to collect sample. Discard column following use.
Appendices

8. Place each membrane into the provided tray ("-" mark is on the antibody printed side).
   Note: The printed side should be facing upward. Add 2.5 ml Blocking Buffer and
   incubate at room temperature for one hour to block membranes.

9. After one hour, decant Blocking Buffer from each container. Add 2.5 ml of sample
   into each array membrane, and cover with the lid. Incubate at room temperature with
   gentle shaking for 2 hours. Dilute sample using Blocking Buffer.

10. Decant the samples from each container, and wash three times with 3 ml of 1X Wash
    Buffer I at room temperature with shaking and five minutes per wash. Dilute 20X
    Wash Buffer I with deionized or distilled water.

11. After each wash, decant the 1X Wash Buffer I from each container. Wash three times
    with 3 ml of 1X Wash Buffer II at room temperature with gentle shaking, as with 1X
    Wash Buffer I.

12. Add 2.5 ml of 500 fold diluted HRP-conjugated streptavidin (e.g. add 10 ml of HRP-
    conjugated streptavidin to 5 ml of Blocking Buffer) to each membrane.
    Note: Mix tube containing 500X HRP-Conjugated Streptavidin well before use since
    precipitation may form during storage.

13. Incubate at room temperature with gentle shaking for two hours.

14. Add 2.5 ml of Detection Buffer C and 2.5 ml of Detection Buffer D into a tube (for
    detecting 2 membranes). Mix both solutions and drain off excess wash buffer. Place
    membrane protein side up ("-" mark is on the protein side top left corner) on a clean
    plastic plate or its cover (provided in the kit).

15. Pipette 2.2 ml of the mixed Detection Buffer on to each membrane and incubate at
    room temperature with shaking for 2 minutes. Ensure that the detection mixture is
    evenly covering the membrane without any air bubbles. Gently place the membrane
    with forceps, protein side up, on a piece of plastic sheet ("-" mark is on the protein
    side top left corner).
16. Cover the array with another piece of plastic sheet. Gently smooth out any air bubbles. Avoid putting any pressure on the membrane and work as quickly as possible.

17. The signal can be detected directly from the membrane using a chemiluminescence imaging system or by exposing the array to x-ray film with subsequent development. Expose the membranes for 40 Seconds. Then re-expose the film according to the intensity of signals. If the signals are too strong (background too high), reduce exposure time (eg, 5–30 seconds). If the signals are too weak, increase exposure time (eg, 5–20 min or overnight). Or re-incubate membranes overnight with 1X HRP-conjugated streptavidin, and repeat detection on the second day. Save membranes at -20 °C to -80 °C for future reference.
### NN.2 Results at seven days

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<tr>
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<th>eNOS pDNA Treatment</th>
<th>IL-6 siRNA and eNOS pDNA Treatment</th>
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**NN.3 Results at 14 days**

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**OO RT² profiler PCR array**

**OO.1 Method**

1. Homogenize samples in Trizol and proceed to RNA extraction (described in Appendix Y).
2. Perform DNA digestion step and PCR reaction as described by manufacturers.
3. Briefly centrifuge the RT² SYBR Green Mastermix to bring the contents to the bottom of the tube.
4. Prepare the PCR components mix in a 5 ml tube or a loading reservoir depending on the RT² Profiler PCR Array format.
Appendices

5. Dispense the PCR components mix into the RT²Profiler PCR Array depending on the RT²Profiler format.
6. Carefully remove the RT²Profiler PCR Array from its sealed bag.
7. Add PCR components mix to each well of using an 8-channel multi-pipette.
8. Carefully seal the RT²Profiler PCR Array with the optical adhesive film.
9. Centrifuge for one minute at 1000 g at room temperature to remove any bubbles. Visually inspect the plate from underneath to ensure no bubbles are present in the wells.
10. Place the RT²Profiler PCR Array on ice while setting up the PCR cycling program.
11. Proceed in real-time cycler as normal.

OO.2 Inflammatory gene expression after IL-6 siRNA and eNOS pDNA dual treatment at 14 days

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### Appendices

**OO.3 Angiogenic gene expression after IL-6 siRNA and eNOS pDNA dual treatment at 14 days**

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PP  Raman spectral analysis of tissue

1. Cut sections of 30 μm thickness for Raman analysis.
2. Prior to analysis, wash sample three times to remove residual OCT on the slide.
3. Hydrate sample with PBS, place coverslip over sample and seal slide with nail polish to prevent sample drying out.
4. Open ‘Andor’ program to control camera for spectrum.
5. Cool camera to -80°C.
6. Start calibration using 60X air lens and silicon waifer.
7. Open hyper terminal to control laser.
8. Open ‘Cell beam’ program for live camera.
9. Enter the following commands in hyper terminal:
   
   **LA ON**
   
   **EN 2**
   
   **SET POWER 85**
   
10. Ensure both laser shutter and detection shutters are open. Note: Ensure laser light is off when looking into microscope.
11. Focus microscope on silicone waifer.
12. In ‘Andor’ program, setup acquisition, readout mode, multi-track. Load the calibration file (Kallibreun), and set exposure time to one second.
13. Take a spectrum of silicone waifer.
15. Change units to Raman shift (783.55).
16. The silicone peak should now be at about 522, instead of 240.
17. Focus sample so intensity reaches about 10,000.
Appendices
18.

Change sample and lens (60X water). Select area to scan using ‘Switchboard’
program, and set pause to 100 seconds. Set exposure time to ten seconds and number
of accumulations to ten.

19.

Start ‘Andor’ and ‘Switchboard’ programs at same time. Note: Spectra must be taken
in the dark.

20.

Run should take about three hours (100 spectra, 100 seconds each). After it has
completed, save file name (sample numbering, including timepoint) and date.

QQ

QQ.1

pDNA and siRNA sequences

pCMV-GLuc

GACGGATCGGGAGATCTCCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCTGATGCCGCATAGTT
AAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGCAAAATTTAAGCTACA
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Sequence 3: UCGGAGCCUAAAUACAUA
Sequence 4: CAUAUCAGUUUGGGGCAU

QQ.3 ON-TARGETplus SMARTpool siRNA, IL-6

RR Journal publications and conference proceedings

RR.1 Journal Publications

Appendices


RR.2 Conference presentations


Appendices


Appendices


RR.3 Grants awarded

1. EMBO travel grant, European Molecular Biology Organisation. (With Prof. Dr. Katja Schenke-Layland, IGB Fraunhofer). Total: €8,083.00.

2. DAAD travel grant, Deutscher Akademischer Austauschdienst. (With Prof. Dr. Katja Schenke-Layland, IGB, Fraunhofer). Total: €3,200.00.
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4. SFI/EI Technology Innovation Development Award (TIDA), Science Foundation Ireland/Enterprise Ireland. (With Prof. Abhay Pandit and Dr. Honorata Kraskiewicz). Total: €94,023.00.