Stimulation of Angiogenesis through Growth Factor Delivery from Synthetic Heparinised Hydrogels

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Declaration

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Abstract

Objectives: Vascular diseases are one of the leading causes of death. Due to minimal regenerative capability of the heart, alternative therapies have been sought after with engineered biomaterials being extensively investigated in this area. In this study, enzymatically degradable heparinised polyethylene glycol (PEG-Hep) hydrogels were synthesized and characterised for the binding and controlled release of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), as well as their bioactivity and angiogenic potential *in vivo*.

Methodology: VEGF and bFGF were combined into 4% (m/v) PEG-Hep hydrogels. The binding and release rates of VEGF and BFGF were analysed via an immunosorbent assay. Released growth factor bioactivity was measured using an XTT metabolic assay on human saphenous vein endothelial cells and human dermal fibroblasts. Neovascularisation was quantified in a subcutaneous rat angiogenesis model in which hydrogel growth factor combinations were implanted within porous polyurethane discs and analysed after a 4 week period. A novel hybrid hydrogel able to degrade proteolytically and hydrolytically was further developed, characterised and analysed as above.

Results: PEG-Hep hydrogels demonstrated substantial growth factor binding ability (500-600 ng) and allowed sustained release (10-20 ng/day) for up to 28 days. Bioactivity of the growth factors was retained throughout the release period. The degradation rate of the hydrogels could be controlled *in vivo* by varying the ratio of monomers capable of forming either hydrolytically or proteolytically degradable crosslinks. Qualitative and quantitative analysis demonstrated a pronounced and significant angiogenic response *in vivo ($p<0.05$).*

Conclusion: Heparinised PEG hydrogels show significant promise as controlled release vehicles for growth factors and warrant further examination in a myocardial infarction model.

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List of Abbreviations

ADSCs	Adipose Derived Stem Cells
Akt	Protein Kinase B
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
bFGF	Basic Fibroblast Growth Factor
BMSC	Bone Marrow Mesenchymal Stem Cells
BSA-PBS	Bovine Serum Albumin
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
EC	Endothelial Cells
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
FAK	Focal Adhesion Kinase
FGFR	Fibroblast Growth Factor Receptor
GAG	Glycosaminoglycans
HdFB	Human Dermal Fibroblast
Hep-Ac	Acrylated Heparin
HGF	Hepatocyte Growth Factor
HSVEC	Human Saphenous Vein Endothelial Cells
IGF-1	Insulin-Like Growth Factor 1
iso-PBS	Iso-Osmotic Phosphate Buffer Saline
MAPK	Mitogen-Activated Protein Kinase
MI	Myocardial Infarction
MMP	Matrix Metalloproteinases
MSC	Mesenchymal Stem Cells
PAA	Poly-Acrylic Acid
PDGF-B	Platelet-Derived Growth Factor-B
PEG	Polyethylene Glycol
PEG-AC	Polyethylene Glycol Acrylate
PEG-Hep	Heparinised Polyethylene Glycol

PEG-VS	Polyethylene Glycol Vinyl Sulfone
PI3K	Phosphoinositide 3-Kinase
PLGA	Poly-Lactide Poly-Glycolic Acid
PU	Polyurethane
PVA	Poly-Vinyl Alcohol
SDF-1	Stromal Derived Factor-1
SMC	Smooth Muscle Cells
TGF-B	Transforming Growth Factor Beta
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

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1. Introduction

Vascular disease is the leading global cause of death and has demonstrated an increasing trend in recent years within developing countries (Singelyn and Christman, 2010). The prevalence of vascular disease has been estimated at between 4.5% and 29% worldwide, and is highly associated with the male gender (Rao et al., 2007). The main cause of vascular disease is atherosclerosis, which is the thickening of the arterial wall as a result of invasion and accumulation of white blood cells, smooth muscle cells and fatty deposits, creating a fibro-fatty plaque within the blood vessels. Vessel blockages are commonly identified in the heart, lower limb and in the brain. The constant build-up of atherosclerotic plaque in vessels causes an imbalance in oxygen and nutrient supply due to prolonged constant reduction in blood flow resulting in ischemia and inflammation (Kurrelmeyer et al., 1998) (Miyahara et al, 2006).

In cardiovascular cases, atherosclerosis, which limits blood supply to the heart, leads to discomfort and angina. Under critical conditions, atherosclerotic plaque develops a fibrous outer coating, which weakens and eventually ruptures, leading to blood clot formation and arterial rupture, completely diminishing blood supply to the heart and compromising myocardial cellular mechanisms. This results in myocardial infarction (MI), irreversible cell and tissue damage or death (Figure 1)(Sun and Jia, 2012). MI injuries lead to a substantial loss of cardiomyocytes, a cell type with minimal regenerative ability. Cardiomyocytes have been determined to have a renewal rate of approximately 1-2% per annum in the adult heart (Bergmann et al., 2009) (Senyo et al., 2013). Surviving cardiomyocytes therefore need to work harder to maintain the sufficient cardiac output in the heart (Perricone and Vander Heide, 2014). Such injuries in the heart cause strain and severe cases become pathological (Kurrelmeyer et al., 1998). This may lead to complications in myocardial perfusion, systolic function and severe haemodynamic deterioration (Orlic et al., 2001).

Several therapies have been used to re-infuse blocked blood vessels prior to MI injury. These therapies include administration of pharmacological adjuvants such as antiplatelet therapeutics that help maintain vessel patency, among others (Gerczuk and Kloner, 2012). These modern therapies, although useful in some patient cases, have proven to be inadequate in long-term treatment of MI damage.

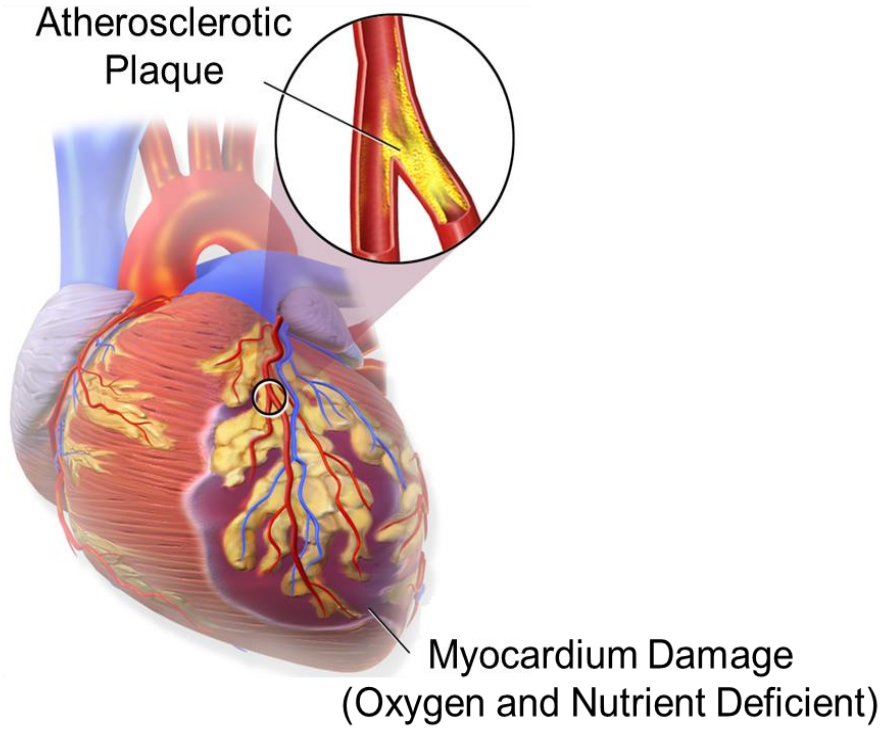

Figure 1: Schematic Diagram of Myocardial Infarction Progression

Development of MI occurrence via atherosclerotic build-up in the lining of the coronary artery, followed by arterial damage and shortage of oxygen and nutrients. Modified from (Kurrelmeyer et al., 1998).

The preferred treatment for patients who have experienced a myocardial infarction is coronary angioplasty or thrombolytic stenting (Nallamothu and Bates, 2003). This therapy, although reliable, is only useful if a patient is able to have the procedure performed within approximately 6 hours after MI occurrence (Epstein, 2007). This is a narrow time frame for doctors to use angioplasty and coronary stenting to open blocked arteries of heart MI patients. If the patient is unable to get treatment within this timeframe, the damage is extensive and such procedures will not be helpful. The majority of patients in developing countries are unable to get treatment within such limited time due to a lack of resources and therefore require alternative therapies (Gibbons et al., 1993).

The pathological damage and increased stress that occurs after MI often progresses to heart failure, a severe stage which reduces the quality of a patient's life (Falk et al.,

1995). Over the past two decades, treatment of chronic heart failure has made considerable progress. Pharmacological therapies such as beta-receptor blockers, angiotensin-converting enzyme inhibitors, aldosterone antagonists, and resynchronization therapy are aimed at reducing progression to heart failure (Neubauer, 2007). However, even with the best of modern therapy, stopping the progression of heart failure has not been successful, with an annual mortality rate of 10% (Dayer and Cowie, 2004, Abraham et al., 2002). The only proven cure for heart failure is heart transplantation, but this presents a problem as the opportunities for patients to receive a donor heart are very low, and transplant patients require frequent monitoring, with dependence on immunosuppressant drugs for the rest of their lives (Armitage et al., 1990). This suggests that present therapies are inadequate, and there is a need for alternative therapies with regenerative ability.

2. Therapeutic Strategies for Vascular Diseases

Three significant alternative therapies, stem cell delivery, growth factor therapy and cardiac hydrogel injection that are being explored in the context of MI are briefly delineated below. Then approaches towards optimising a naturally occurring process for overcoming ischemia, namely angiogenesis, are discussed with a particular emphasis on growth factor delivery in conjunction with hydrogel injection.

2.1. Stem Cell Therapies

The delivery of stem cells through intra-myocardial injection is the subject of intensive investigation. Major research focus has been on the adult stem cell populations such as mesenchymal stem cells (MSCs), (ADSCs), and bone marrow derived stem cells (BMSCs) with many laboratory based studies and clinical trials performed over the last 15 years (Karantalis and Hare, 2015). The pluripotent embryonic and induced pluripotent stem cells though also explored, are impeded in their translation as an injectable source due to safety concerns such as potential for teratoma and tumour formation though they have proven ability to differentiate towards cardiomyocytes and other desirable cell types (Mauritz et al., 2011). The use of the adult stem cell populations was originally proposed based on their possible potential to generate new cardiomyocytes in the damaged heart (Orlic et al., 2001) but later research showed

that direct regeneration of cardiac tissue through stem cell differentiation most probably does not occur (Murry et al., 2004).

It is now understood that the limited therapeutic effect observed most probably results from paracrine mechanisms mediated by factors released from stem cells that play essential roles in repairing ischemic tissue and promoting angiogenesis (Gnecchi et al., 2008). This has most convincingly been demonstrated by the use of conditioned media from cultured adult stem cells (Uemura et al., 2006) (Kwak and Mach, 2005). The release of biologically active substances in a temporal and spatial manner from these cells is probably enhanced by environmental stimuli in the infarct such as ischemia, again informed by studies on conditioned media where exposure to hypoxia greatly stimulated the therapeutic effect (He et al., 2015). The cytokines and factors released influence the microenvironment by acting on a range of different cardiac cell types including cardiomyocytes and endothelial cells leading to tissue protection, repair, and regeneration (Maltais et al., 2010) (Kamihata et al., 2001). The factors may also exert autocrine actions modulating the biology of stem cells, allowing for self-renewal and proliferation (Takahashi et al., 2006). Stem cells are able to produce a wide range of cardio protective and/or angiogenic factors such as vascular endothelial factor (VEGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), insulin-like growth factor 1 (IGF-1), epidermal growth factor, keratinocyte growth factor, angiopoietin-1 (Ang-1), stromal derived factor-1 (SDF-1), macrophage inflammatory protein-1 alpha and erythropoietin (Kinnaird et al., 2004). However, a major limitation experienced in stem cell therapy is the poor performance of injected stem cells due to their low cell engraftment and survival (Sheng et al., 2012) and this is believed to be at least part of the reason for the disappointing results thus far from clinical trials (Gyöngyösi et al., 2015) (Fisher et al., 2015). Here hydrogels are being extensively explored for their ability to entrap cells at the point of cardiac delivery (Davies et al., 2016).

As the potentially therapeutically beneficial effects appear to be derived from cell secreted bioactive components such as growth factors, this has given further impetus towards optimising delivery of the growth factors themselves as this might allow for a more controllable and pharmaceutical approach towards protecting the heart post-MI.

2.2. Growth Factor Therapies

Direct delivery of proteins has generated much attention as a potentially simpler and more defined route for introducing a range of growth factors provided by the paracrine effect to the damaged cardiac tissue. The delivery of growth factors via gene therapy has also been extensively explored but is beyond the scope of this thesis. In particular, growth factors have been investigated due to their potential for stimulating a variety of protective mechanisms such as therapeutic angiogenesis (see below), cytoprotection of surviving cardiomyocytes and attraction, differentiation and proliferation of endogenous stem cells (Urbich et al., 2005) (Gnecchi et al., 2008). The use of VEGF, bFGF, HGF and erythropoietin for treatment of MI has progressed to clinical trials, but experimental designs have varied considerably over the years, showing significant variations in terms of delivery route and dosage of the specific growth factor being administered. This variability has certainly contributed to contradictory findings thus far (Pascual-Gil et al., 2015). Undoubtedly, although the use of proteins has been set back by aspects such as short half-lives and rapid clearance from the site of delivery through blood flow over a period of minutes, in a similar manner to that observed for stem cells (Davies et al., 2008). Once again hydrogels are being actively explored as a means of overcoming the above issues.

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Although cell and protein based therapies are powerful strategies to therapeutically improve ischemic damage, more research is required to advance what has been achieved and combat the current disadvantages. It is necessary to improve the efficacy of these novel strategies to reach their full potential, and specialised delivery mechanisms such as biomaterials have been studied towards achieving these goals.

3. Hydrogel Injection

Hydrogels have a potential direct application for MI therapy apart from their utility as delivery vehicles for stem cells and growth factors. Initial research was directed towards the potential ability of injectable hydrogels to mechanically support the damaged heart and thus limit increased stress in the wall (Dai et al., 2005) (Singelyn et al., 2009). Upon infarction, there is a dilation of the ventricle due to ineffective pumping of the blood resulting in an increased volume of blood retained in the heart.

Though compensatory in the short term due to enhanced cardiac output as a result of the Frank-Starling mechanism, this dilation and the concomitant wall thinning in the infarct region increases wall stress. This increased wall stress can be seen to result in part from the Law of Laplace ($Stress = Pressure\ x\ {radius}/{2\ x\ Wall\ Thickness}$) and is believed to contribute strongly to the relentless progression towards heart failure after MI. Injectable hydrogels can reduce wall thinning and thus stress, contributing to increased function and preservation of cardiac geometry (Wall et al., 2006) (Kadner et al., 2012) (Dobner et al., 2009). Research into the potential of hydrogels to serve as mechanical supports is on-going but as observed for so many alternative therapies for MI, translation to the clinic is challenging. The reasons for poor translation are undoubtedly complex and beyond the scope of this text (Bayés-Genís et al., 2016). In addition to hydrogel's ability to reinforce cardiac tissue as mentioned above, they have a range of other capabilities such as the potential to mimic the extracellular matrix (ECM) and create a desirable homing environment (De Mel et al., 2008). Over the last decade, the number of studies using biomaterials in combination with cell and protein therapies has increased exponentially. This is because biomaterial-based delivery mechanisms are essential in enhancing outcomes of stem cell and protein therapy in vascular diseases (Lam and Wu, 2012). Biomaterials are desirable for their biocompatible and biodegradable qualities, with the ability to promote cell migration by reproducing aspects of a natural tissue environment, improving cell behaviour by producing a 3D environment, increasing viable cell retention to facilitate the paracrine effect and enabling prolonged delivery of growth factors by controlling their release and protecting them from degradation (Stabenfeldt et al., 2010) (Kim et al., 2008) (Cheng and García, 2013).

This review focuses on recent developments in the use of hydrogel biomaterials for fuelling therapeutic angiogenesis, with the assistance of angiogenic growth factors. In order to achieve optimal results, growth factors may be locally delivered and controllably released at the site of injury through altering and reconstructing the scaffold chemical composition.

4. Therapeutic Angiogenesis

4.1. Angiogenesis

Angiogenesis is the development of new blood vessels from existing vasculature. It is a normal and vital process required for growth, development, and wound healing (Rak and Weitz, 2003). The growth of new blood vessels by sprouting and extension from pre-existing vasculature is referred to as sprouting angiogenesis (Hellström et al., 2007) (Risau, 1997). The mechanism of physiological angiogenesis involves a highly organized sequence of cellular events, which include vascular initiation, formation, maturation and remodelling, controlled and modulated to meet the tissue requirements (Staton et al., 2009). Capillary endothelial cells (EC) are an essential component in these 4 sequential steps.

The activation of EC for vascular initiation is acquired through the blockage of a cell stabilizing signal that occurs through the Angiopoietin-1 (Ang-1) and Tie receptor tyrosine kinases. Angiopoietin-2 (Ang-2) plays a critical role in destabilizing vessels by promoting pericyte and smooth muscle cells (SMC) detachment (Yancopoulos et al., 2000). Ang-1 acts as an agonist for Tie-2 properties. The Ang-1 and Tie-2 pathway is required for maturation of blood vasculature and EC formation (Ekland et al., 2013). This is followed by EC sprouting and migration controlled by VEGF and Notch signalling pathways, which dissemble cell adhesion proteins such as vascular endothelial cadherin, claudins and occludin with the consequent loosening of inter-endothelial cell contacts (Vestweber, 2000, Carmeliet, 2000). During migration, ECs break down the ECM, proliferate and reorganize into a new vascular sprout driven by tip cells down a VEGF gradient. Activation of Notch receptor in the stalk cells (cells behind the tip) reduces the ability of these cells to respond to VEGF, but allowing controlled proliferation for the extension of capillaries behind the invading tip (Prestwich et al., 2006) (Rossant and Howard, 2002). During vessel sprouting, ECM is remodelled via degradation mediated by proteinases such as matrix metalloproteinases (MMPs) and plasminogen activators with additional control by inhibitors of matrix metalloproteinases and plasminogen activator inhibitor thus promoting vessel maturation (Chun et al., 2004, GE, 2005).

After new vessel formation, mural cells help stabilize the immature vasculature. The recruitment of pericytes expressing platelet-derived growth factor receptor (PDGFR-β) by EC secreting platelet-derived growth factor-B (PDGF-B) (Jain, 2003) (Van Hinsbergh et al., 2006). Ang-1, secreted by perivascular cells also plays a prominent

role in vessel maturation by assisting interaction of ECs with both mural cells and the ECM (Uemura et al., 2002). These mural cells then allow for stable vessel formation (Davies et al., 2008).

4.2. Relevance of Therapeutic Angiogenesis

Therapeutic angiogenesis is appealing as a therapy because it aims to augment the development of new blood vessels from pre-existing vessels by exogenous administration of appropriate growth factors in order to supply blood flow to affected ischemic tissue (Mitsos et al., 2012). In the damaged heart it is hypothesised that increased angiogenesis will support poorly perfused areas of the surviving myocardium and thus limit infarct expansion, attenuating the progression towards heart failure (Hao et al., 2007).

5. Angiogenic Growth Factors

As detailed above, a wide range of growth factors are involved in regulating vessel growth. Apart from those described above, others that have been seen to be pro-angiogenic include acidic fibroblast growth factor and bFGF, IGF-1, angiogenin, transforming growth factor beta (TGF-β), tumour necrosis factor alpha, HGF, neuregulin, granulocyte-colony stimulating factor, and SDF-1 (Awada et al., 2014) (Tang et al., 2009, Dunn et al., 2000) (Jay and Lee, 2013, Kucia et al., 2004). In past experiments, although factors such as TNF-α, PDGF-BB and EGF directly stimulated the formation of new vessels by promoting the sprouting of endothelial cells and their growth, these proteins mainly triggered the release of VEGF or the upregulation of VEGFR-2 gene (Giraudo et al., 1998, Hoeben et al., 2004). Though VEGF is clearly a critical and dominant component of the angiogenic pathway, bFGF has also been identified as a potent angiogenic stimulant because it is activated by multiple ligand-receptor specificities, and triggers a network of signal pathways that promote endothelial cell growth, migration, and survival from pre-existing vasculature (Presta et al., 2005) (Pike et al., 2006) (Tabibiazar and Rockson, 2001).

5.1. VEGF

VEGF is a member of a family of 6 structurally related proteins; VEGF-A to VEGF-E and placental growth factor (PIGF) (Mitsos et al., 2012). It exists as a glycosylated disulphide-linked homodimer (Figure 2A) and there are 4 major isoforms of VEGF-A that range from 121 to 206 peptide residues in humans. These isoforms are $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, where $VEGF_{165}$ is the dominant isoform (Muller et al., 1997). Although these isoforms behave identically in solution, they differ in their ability to bind to different glycosaminoglycan molecules in the ECM. $VEGF_{165}$ and $VEGF_{189}$ bind the heparin molecule with high affinity, and are sequestered to the cell surface or within the ECM bound to proteoglycans, while $VEGF_{121}$ is freely diffusible and does not bind heparin (Shibuya, 2001).

VEGF is produced by a variety of cell types, including keratinocytes, macrophages, mast cells, and smooth muscle cells (Nör et al., 1999). It mediates angiogenic effects by binding to specific VEGF receptors on the surface of endothelial cells (Muller et al., 1997). VEGF binds to 3 primary receptors; VEGFR-1, VEGFR-2 and VEGFR-3, and various co-receptors. Of the primary receptors, VEGFR-1 and VEGFR-2 are mainly associated with angiogenesis (Yancopoulos et al., 2000). VEGFRs also interact with bFGF receptors and with distinct co-receptors such as neuropilins and heparin glycosaminoglycans (GAG) important for enzyme, GF, and ECM protein interaction for the modulation of biological processes *in vitro* and *in vivo* {Ornitz, 2000} (Hicklin and Ellis, 2005). VEGFR are classified as type 5 of receptor tyrosine kinases whose extracellular domains consist of seven immunoglobulin-like (Ig-like) domains (Stuttfeld and Ballmer‐Hofer, 2009). VEGF receptors are activated upon ligand-mediated dimerization, which is stabilized by the Ig-like domains, leading to downstream signal transduction (Figure 2B). In VEGFR downstream pathway, several signaling proteins such as Akt (protein kinase B), focal adhesion kinase (FAK), p38 mitogen-activated protein kinase (MAPK), endothelial nitric oxide synthase (eNOS) and phosphoinositide 3-kinase (PI3K) are activated directly or indirectly via receptor binding (Jiang et al., 2000) (Eliceiri et al., 1998). These in turn stimulate angiogenesis by inducing vascular permeability and vasodilation, EC proliferation, migration, and upregulation of the expression of MMP, which initiate the dissolution of the extracellular matrix (Ferrara, 2002). VEGF was shown to stimulate endothelial production of nitric oxide (NO) and prostacyclin (PGI2), two vasodilatory agents that induce permeability of vessels, promoting the deposition of proteins in the interstitium, thus stimulating downstream

pathways and facilitating angiogenesis. These agents are also known to increase cell-to-cell interactions by opening junctions between adjacent EC and promote vascular smooth muscle cell proliferation necessary for vessel maturation (Hicklin and Ellis, 2005, Lamalice et al., 2007).

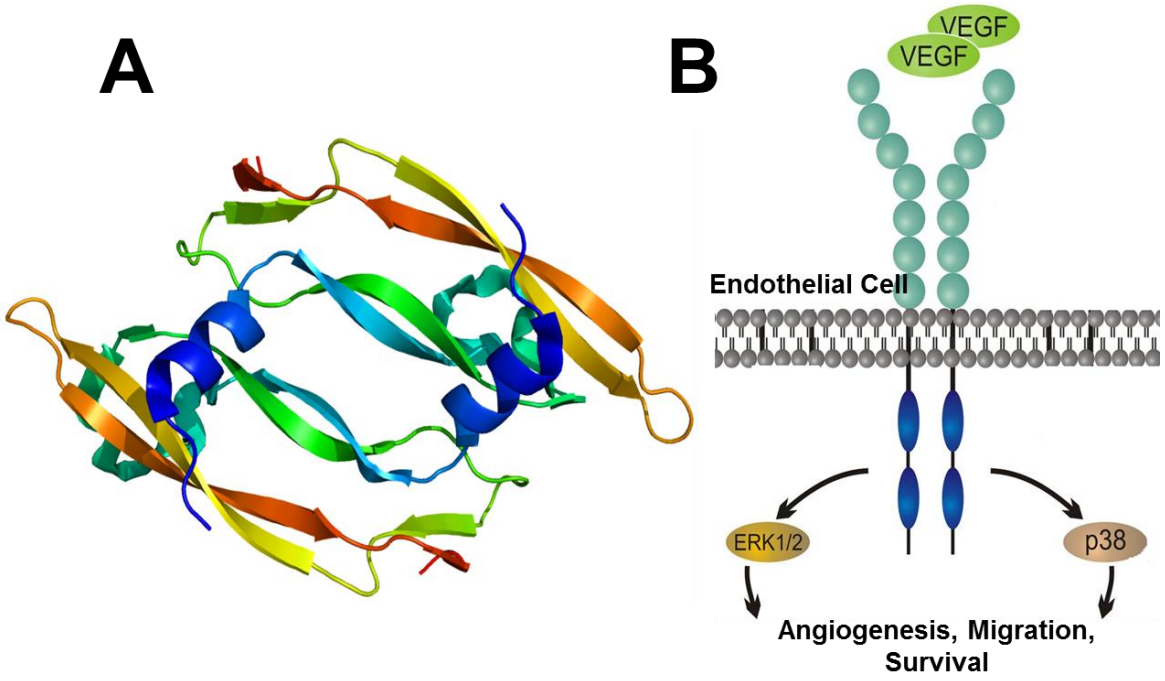


Figure 2: Ribbon Representation of VEGF Structure and its Receptor-Binding Domain
(A) Secondary Structure of VEGF homo-dimer comprised of beta sheets and alpha chains, **(B)** Relationship between VEGF and VEGF receptors initiating angiogenesis via different downstream pathways (Muller et al., 1999)

5.2. bFGF

bFGF an 18kDa protein also known as FGF-2 is a member of the heparin binding growth factor family (Presta et al., 1994). It was the first pro-angiogenic molecule to be identified, and is secreted by human adipocytes and induces the proliferation of endothelial cells, smooth muscle cells and chondrocytes (Cross and Claesson-Welsh, 2001). In normal tissue, bFGF is present in basement membranes, the sub-endothelial extracellular matrix of blood vessels, bound to heparin sulphate and stays membrane bound until released by heparinises and proteases.

The FGF family comprises of 22 members, FGF-1 to FGF-22, which variously bind to seven different FGF receptor isoforms, namely FGFR1b; FGFR1c; FGFR2b; FGFR2c; FGFR3b; FGFR3c and FGFR4. Among the 22 members of the FGF family, only a few have been identified to exert angiogenic properties *in vitro* and *in vivo*, and these include acidic FGF and bFGF (Johnson et al., 1993). Endothelial cells of different origin express FGFR1 and FGFR2, whereas the expression of FGFR3 and FGFR4 have never been reported (Presta et al., 2005). The bFGF molecule exists as a homodimer containing alpha chains and beta sheets (Figure 3A).

bFGF binds to its tyrosine kinase receptor, FGFR-1 inducing dimerization of the receptor and thus modulating their effects via downstream signal transduction pathways (Yang et al., 2015) (Rak and Weitz, 2003). The binding of bFGF to its receptor is stabilised by heparin, facilitating the formation of a complex of two FGF molecules and two receptors (Klagsbrun and Baird, 1991). This dimerization leads to auto-phosphorylation, which in turn initiates endothelial cell migration, differentiation, proliferation and the formation of new blood vessels (Figure 3B)(Cross and Claesson-Welsh, 2001).

In the absence of heparin, bFGF binds to its receptor with low affinity and thus affects the GF's angiogenic potential because it is hypothesized that during wound healing of normal tissues, the action of heparin degrading enzymes present in the ECM activate bFGF, thus mediating angiogenesis (Sellke et al., 1996).

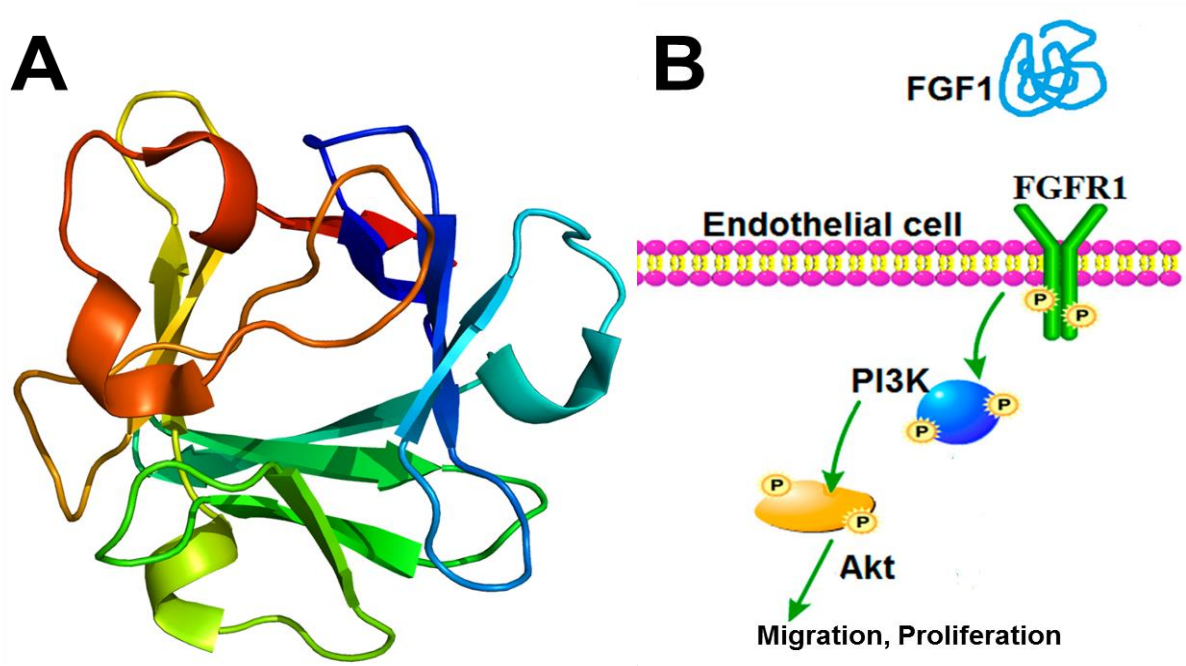


Figure 3: Ribbon Representation of bFGF Structure and its Receptor-Binding Domain **(A)** Secondary Structure of bFGF homodimer comprised of beta sheets and alpha chains, **(B)** Relationship between bFGF and bFGF receptors initiating angiogenesis via different downstream pathways (Cross, 2001).

5.3. Therapeutic Angiogenesis

Initial attempts to achieve therapeutic angiogenesis through delivery of VEGF and bFGF were carried out through direct injection of a bolus of single GFs. These early clinical and pre-clinical trials had mixed results possibly due in part, to their lack of placebos (Isner et al., 1996) (Schumacher et al., 1998). Certainly concerns raised by this initial approach were the possibility of toxic consequences of a large bolus of GF, with the potential for edema and hypotension with VEGF (Hariawala et al., 1996) (Horowitz et al., 1997). Furthermore it is always desirable to mimic the physiological situation where pro-angiogenic factors are released in a sustained manner from a localised region such as happens in wound healing (Zisch et al., 2003b). The need for localised and sustained delivery has been demonstrated by many including our laboratory. Perhaps the most compelling evidence comes from the transgenic mouse model where VEGF production could be conditionally switched on and off in the heart and liver of adult mice, and vessels lasted for months after VEGF production was terminated (Dor et al., 2002). Importantly, it was shown that VEGF had to be delivered

for longer than 2 weeks for this stability to be achieved (Dor et al., 2002) (Davies et al., 2008) (Richardson et al., 2001). The localized micro environmental dosage of GF delivered was also demonstrated to be critical through the use of myoblasts that expressed precise levels of VEGF (von Degenfeld et al., 2006). Here, localized delivery between a low and a high threshold was required to generate stable vessels without hemangioma formation.

Thus a drive in the field of therapeutic angiogenesis has been the controlled delivery of pro-angiogenic factors in a localized manner as might be expected from the ECM. Hydrogels are certainly an optimal candidate for this approach because of their biological and structural similarity to the ECM.

6. Hydrogels

Hydrogels have several desirable characteristics that render them suitable as tissue regenerative biomaterials, chief amongst which is their ability to provide a highly swollen, three-dimensional environment that mimics the ECM and allows the diffusion of nutrients, growth factors and metabolites (Zhu, 2010). Indeed, hydrogels are defined as three-dimensional polymeric networks swollen by water, the chief constituent of the gel system (Zisch et al., 2003b). A further advantage of hydrogels is that they can frequently be delivered in an injectable format allowing them to be administered to a site in a minimally invasive manner (Nelson et al., 2011, Kofidis et al., 2005). Hydrogel precursors ideally should have the potential to gelate under mild conditions due to physical changes such as pH, temperature, ionic concentration and chemical reactions like Michael addition and Schiff base formation (Lee and Mooney, 2001). It is highly important that hydrogels that are utilised for tissue regeneration such as generation of vascular tissue be compatible with the site where they are to be administered (Li et al., 2012). Injectable hydrogels need to satisfy several requirements. They should be made of biocompatible materials and biodegrade into biological products (Bensaıd et al., 2003, Wang et al., 2010). Their viscosity should be sufficiently low before gelation, allowing not only the dispersion of incorporated cells and proteins, but also their insudation of solid tissue when injected. Hydrogels must have rapid enough gelation rates after *in vivo* injection, such that they are not dispersed in the circulatory system (Wang et al., 2010). Optimal hydrogels should maintain a relatively stable environment

and exhibit a suitable durability to hold cells and proteins and avoid initial burst effects (Mooney and Mikos, 1999) (Marler et al., 1998). It is also paramount that hydrogels present an adequate percentage of porosity to allow space for cell activity and the exchange of nutrients and oxygen (Drury and Mooney, 2003) or allow for cell directed invasion of the hydrogel in a manner similar to that of cells in the ECM (Lutolf, 2009). There are two major types of hydrogels, natural and synthetic hydrogels, classified according to their origin.

6.1. Natural Hydrogels

Natural hydrogels, as implied by their name, are derived from biological polymer-based materials, such as fibrin, alginate and chitosan. Their inherent biocompatibility and ease of production ensured that these types of hydrogels were amongst the first to be utilised as tissue regenerative scaffolds (Nelson et al., 2011, Rufaihah and Seliktar, 2016). Alginate hydrogels result from ionic interactions between linear, unbranched copolymers that contain (1,4)-linked b-D-mannuronic acid, and a-L-guluronic acid residues. Alginate has been investigated as a delivery vehicle for VEGF and PDGF-BB in a rat MI model. In this study, PDGF released more controllably, and the relatively sequential delivery generated mature vessels in the infarcted heart (Hao et al., 2007). VEGF and bFGF delivered in alginate subcutaneous discs in SCID mice elicited a more pronounced angiogenic response.

Chitosan, a linear polysaccharide consisting of N-acetyl-D-glucosamine units is derived from chitin via partial deacetylation. Photo-crosslinkable and water-soluble chitosan derivatives have been synthesized to make chitosan suitable for hydrogel formation. In a previous experiment, bFGF-containing chitosan was photo cross-linked on the surface of infarcted rabbit hearts and resulted in functional improvement due to increased vascularisation (Fujita et al., 2004). VEGF was also similarly found to be pro-angiogenic when delivered from chitosan in a wound-healing model in diabetic db/db mice (Obara et al., 2005).

Another natural hydrogel frequently utilised is fibrin. This hydrogel has been used to deliver VEGF and bFGF with potentially promising angiogenic responses in both the clinical and pre-clinical setting (Kipshidze et al., 2003, Shireman et al., 1999). It was

previously analysed as a cell delivery vehicle, and shown to elicit functional improvement and increased angiogenesis as a stand-alone therapy in a rat MI model. However, an issue with such direct loading of fibrin, namely burst release and poor sustained release was apparent and this is potentially expected for all these types of relatively porous natural hydrogels (Tabata, 2000). Certainly this issue is a concern with VEGF. The delivery of high doses, through injections or systemic delivery of VEGF have previously shown low efficacy, because the majority of the protein is either rapidly cleared from the target site or reaches it in insufficient quantities. Furthermore, excess amounts of VEGF can cause severe vascular leakage and hypotension (Lee et al., 2000).

A significantly utilised approach to increase retention of growth factors in such hydrogels, and thereby reduce the burst release effect and sustain delivery has been the incorporation of heparin (see below for a detailed description of this proteoglycan) (Sakiyama-Elbert, 2014). This proteoglycan has a strong affinity for a wide range of growth factors. Lopez *et al* showed that large doses of bFGF (10 and 100 μg) were injected into the myocardium of infarcted pigs after incorporation into alginate in the form of microspheres conjugated with heparin. A pronounced angiogenic response that stimulated collateral formation blood vessels was observed for both groups, resulting in functional improvement (Lopez et al., 1997). A follow up clinical trial with the same dosages in coronary artery bypass grafting (CABG) patients observed a trend towards better functional recovery in the higher dose arm (Laham et al., 1999). Chitosan was also mixed with heparin, which served the dual purpose of forming both an injectable format of chitosan (through electrostatic interaction with the negatively charged GAG) and control the release of bFGF in subcutaneous mouse and rat models to generate a pro-angiogenic response (Fujita et al., 2004). An elegant approach to heparinise fibrin was employed by the Hubbell group whereby a heparin binding peptide was crosslinked into fibrin with Factor XIII allowing for the immobilisation of the heparin and subsequent binding of bFGF (Sakiyama-Elbert and Hubbell, 2000) (Ruvinov et al., 2010). However, this system was not directly used for stimulating angiogenesis. In a more standard chemical crosslinking approach, bFGF from heparin conjugated fibrin was shown to generate neovascularisation in the ischaemic hind limb of mice whilst non-heparinised fibrin did not (Yang et al., 2010a).

Although natural hydrogels have been determined to possess many advantages useful for potential regenerative medicine therapies, they also have limitations in handling and engineering, and a potential for immunogenicity {Mano, 2007 #55}. For these reasons, a wide range of synthetic hydrogels has been explored in the context of therapeutic angiogenesis.

6.2. Synthetic Hydrogels

As indicated above, synthetic hydrogels are desirable due to the control over their engineering, allowing the precise introduction of required characteristics. These include polymers of poly-acrylic acid (PAA), polyethylene glycol (PEG), its co-polymers and poly-vinyl alcohol (PVA). Some specific advantages that synthetic hydrogels possess are photo-polymerization abilities, adjustable chemical and mechanical properties, controlled incorporation of bio-functions and control over their transport properties (Drury and Mooney, 2003) (Zhu, 2010). Because of their ability to be tailored chemically and mechanically, synthetic hydrogels can be sub-divided into thermos-sensitive, photosensitive, pH responsive and ion induced hydrogels. When a hydrogel's polymer chains are held together by irreversible covalent chemical interactions, the network is relatively strong and stable (Jeon et al., 2011) (Bracher et al., 2013). However, the incorporation of reversible molecular interactions allows the network to be less robust and is advantageous in being a closer mimic of the degradable ECM (Bryant and Anseth, 2003). Degradation of a synthetic hydrogel can be designed by introducing either proteolytic or hydrolytic moieties into the polymer network. Bioactivity can be designed into the hydrogel by combining growth factors and cell specific molecules onto the hydrogel backbone (Seliktar, 2012).

In one approach, a hybrid of natural and synthetic hydrogels was developed where fibrin was covalently conjugated with PEG to increase its mechanical durability and used to deliver VEGF to infarcted rat hearts. The VEGF loaded injectable hydrogels improved function and increased vascularization in the infarcted region (von Degenfeld et al., 2006). The polyesters of lactide and glycolide (PLGA) have also shown use in delivery of GFs though not in an injectable format. These well tolerated polymers have been used in foamed porous hydrogel discs to deliver VEGF and PDGF-BB in both rat subcutaneous and mouse ischaemic hind limb models (Richardson et al., 2001). Here,

PDGF-BB containing PLGA microspheres were embedded in the porous VEGF bearing scaffolds thus delaying release of PDGF-BB relative to VEGF and the staggered release was found to generate stable vessels.

PEG has, of course, not only been used as a cross-linker of fibrin as described above. The polymer is of wide interest for regenerative medicine approaches because of its inertness, and very tuneable mechanical properties (Oliviero et al., 2012). Our laboratory has shown its utility as a biomechanical support in infarcted rat hearts (Kadner et al., 2012) (Dobner et al., 2009) and also through its ability to deliver cardio-protective growth factors that were sterically entrapped as micro-coacervates (Johnson et al., 2015). Thus this polymer is explored in greater detail below.

7. Polyethylene Glycol (PEG) Hydrogel

PEG-based hydrogel is a polysaccharide synthesized and used for its mechanical properties. Hydrogels formed from this polymer are listed among the most inert synthetic biomaterials, have very low reactivity toward tissue, and are considered to possess high biocompatibility {Santin, 2012 #57}. Additionally the versatility of the chemistry that can be used to synthesize PEG hydrogel monomers has allowed for the design of numerous intelligent gel systems using this molecule (Zieris et al., 2010). PEG hydrogels can be polymerised from a linear monomer, or more complex 4-arm or 8-arm structures (Figure 4A). A wide range of chemistry is used to form PEG hydrogels and their details are beyond the scope of this review. In brief, they include free radical polymerisation of PEG acrylates, condensation, click chemistry, chemical ligation, enzymatic reaction and Michael-type addition reactions (Figure 4B) (Zhu, 2010). The latter has shown great promise as an approach towards generating intelligent ECM mimic scaffolds and is the chemistry concentrated on by our group.
The use of the Michael-type addition reaction to polymerise a PEG hydrogel is desirable as it can occur at physiological pH without requiring organic solvents and does not form damaging free radicals (Lutolf et al., 2001) (Zhu, 2010). In brief, a Michael addition involves the formation of a chemical bond between a nucleophile, which donates a pair of electrons and is referred to as the Michael donor, and an electrophilic olefin, the Michael acceptor (an unsaturated chemical compound that

contains one or more carbon-to-carbon double bonds). In the system developed by Lutolf and Hubbell, crosslinking is based on a Michael-type addition reaction between thiols on the cross-linker molecule and acrylate or vinyl sulfone groups on the termini of the PEG monomers (Lutolf et al., 2001). This allows for the crosslinking of a PEG hydrogel with peptides containing two cysteine moieties and also the derivitisation of the hydrogel with bioactive peptides (Lutolf and Hubbell, 2003). This is beneficial as it allows for the creation of synthetic ECM mimics in that the PEG hydrogel is enzymatically degradable and can also have cell adhesive peptides attached (Lutolf et al., 2001). The latter is critical as PEGs have very low cell adherence and thus the combination of this type of hydrogel with bioactive peptides has proven to be a necessity for tissue engineering {Censi, 2012 #58}.

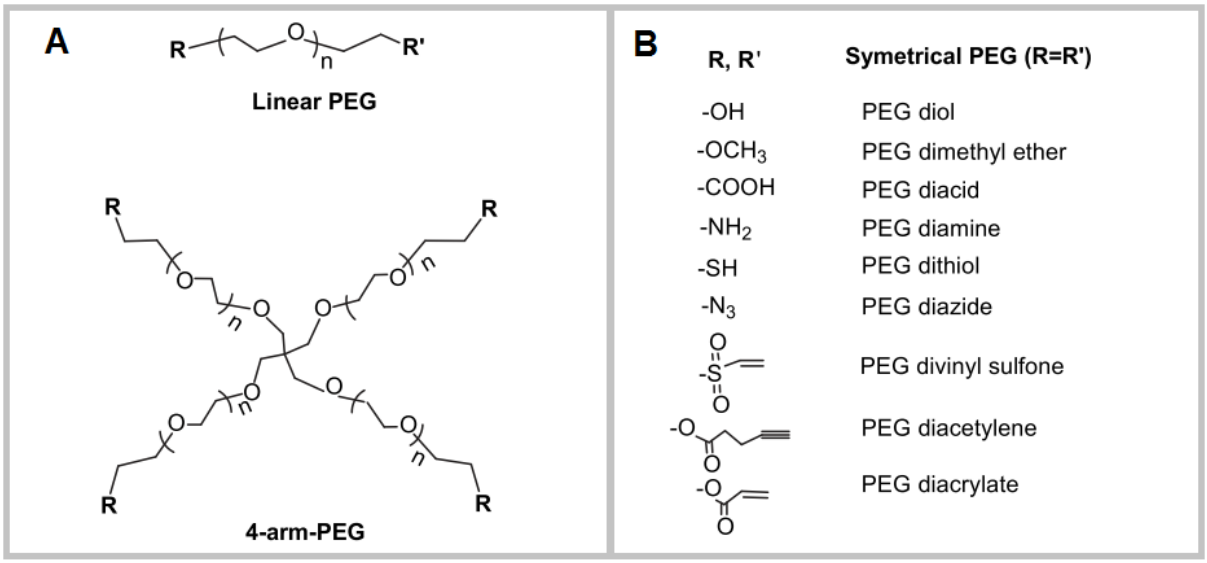


Figure 4: Structure of Polyethylene Glycol and its Functional Group Derivatives
Polyethylene glycol constructs in **A**. Linear for or branched structures (multi-arm form). **B** The end groups of PEG can be converted into the above symmetrical or asymmetrical functional groups that allow for different pathways to polymerisation (Zhu, 2010).

Bioactive peptides have been discovered to play a vital role in the function of ECM proteins, influencing critical aspects such as cell adhesion and migration {Ruoslahti, 1996 #59}. The tripeptide RGD, present in all major extracellular matrix (ECM) proteins (fibronectin, collagen, laminin), has been the most intensively investigated cell-binding bioactive peptide sequence.

The ability to engineer this type of system allows for additions of multiple bioactive peptides. Our group has previously shown that combining the laminin derived peptide YIGSR with RGD preferentially enhanced migration of endothelial cells on PEG hydrogel surfaces relative to smooth muscle cells {Fittkau, 2005 #60}. Thus it is clear that this type of Michael addition based PEG hydrogel system can allow introduction of components and functions of the ECM in a methodical fashion.

Another aspect mentioned above is the potential to introduce enzymatically degradable peptide cross-linkers to allow for cell driven degradation and invasion of the hydrogel (Furth et al., 2007)(Figure 5). As the porosity or mesh size of the hydrogels formed from branched PEG monomers in the 10-40 kDa, molecular weight range is in the 10-30 nm range, cellular movement in 3D within the hydrogels is only possible as a consequence of cell mediated proteolysis (Lutolf et al., 2001). In the first demonstration of controlled cell invasion, PEG hydrogels were formed from 4-arm 20kDa vinyl sulfone derivatised PEG monomers (PEG-VS) and cross-linked with matrix metalloproteinase (MMP) sensitive peptides. Encapsulated fibroblasts were observed to spread and migrate within these hydrogels. The importance of MMP activity was demonstrated by either cross-linking hydrogels with MMP-insensitive peptides or inhibition of MMP activity, both of which resulted in termination of cellular movement. Cellular invasion into hydrogels can therefore be controlled by altering the enzyme specificity of the cross-linking peptides (Chun et al., 2004, Bracher et al., 2013). Fibroblasts and SMC both equally invaded PEG hydrogels cross-linked with a sequence that can be cleaved by a range of MMPs, but when an MMP-14 sensitive peptide sequence was used, a strong SMC specific invasion was observed (Bracher et al., 2013). This once again demonstrates the engineerability of these hydrogels. Our laboratory further extended this work to regulate tissue invasion in a subcutaneous rodent model assay (Goetsch et al., 2015). Controlling the population of a scaffold and its removal by host cells is desirable to allow for an appropriately sustained period of release of angiogenic growth factors to generate a stabilized vasculature, or to allow for the adequate deposition of matrix proteins by cells when reconstructing structural tissues (Bahney et al., 2011). The formation of hydrogels with varying degrees of enzymatic degradability through altering the ratios of 2 differentially degradable cross-linker peptides allowed for control of tissue ingrowth (Goetsch et al., 2015). Importantly, this regulated tissue invasion without varying the biomechanical parameters such as

stiffness of the hydrogels. Engineered PEG hydrogels containing a combination of RGDSP peptide for cell adhesion, and MMP specific cross-linkers were examined as cell ingrowth matrices for bone regeneration by the delivery of recombinant human bone morphogenetic protein-2 (rhBMP-2) to the sites of defect in rat crania. This stimulated fibroblast migration, which remodeled into bony tissue within five weeks. In a related study by our group and that of Hubbell, both $VEGF_{165}$ and an engineered $VEGF_{121}$ that could be covalently coupled into the enzymatically degradable hydrogels were found to stimulate *in vitro* angiogenesis in a chick chorioallantoic membrane assay. (Lutolf and Hubbell, 2003),

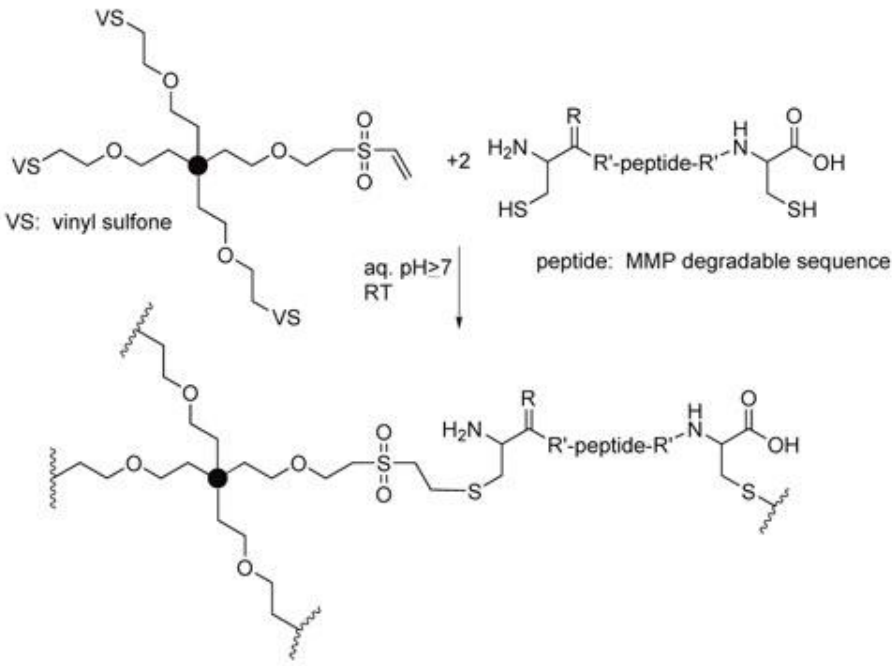


Figure 5: Polymerization of Enzymatically Degradable PEG-Vinyl Sulfone (VS) Based Hydrogels. Michael addition of cysteine containing peptide to the VS end groups (Kasko, 2013)

In delivery of these types of hydrogel(s) to infarcted hearts, our laboratory showed that non-degradable 8-arm PEG vinyl sulfone hydrogels (PEG-VS) coupled by non-degradable dithiol positively influenced the retardation of left ventricular remodelling and dilation (Dobner et al., 2009). However, a possible limitation due to the non-degradability of the formulation used was an ongoing macrophage-based, foreign body response. In a follow up experiment investigating timing of hydrogel delivery after MI, MMP degradable hydrogels (8-arm PEG-VS coupled by an MMP degradable

peptide) showed positive outcomes in the reduction of an inflammatory response to the hydrogel, improved scar thickness and function (Kadner et al., 2012).

As seen from the above, this type of PEG hydrogel system has great engineerability and potential as an injectable cardio therapeutic vehicle. These injectable hydrogels allow for the construction of engineered ECM mimics by introduction of proteolytic degradability and bioactive peptides. However, a key component of the ECM that is not present in these hydrogels is the glycosaminoglycans (GAGs). The introduction of such a moiety, in particular heparin, would render the PEG gels more ECM-like and also greatly expand their angiogenic factor delivery capabilities.

8. Heparin

Heparin is a glycosaminoglycan that is biosynthesized in the Golgi compartment. Its average molecular weight is from 4 to 60kDa but most commercial heparin preparations are between 12 and 15kDa (Khorana et al., 2003). The heparin polymeric chain is composed of repeating disaccharide unit of D-glucosamine and uronic acid linked by 1-4 inter-glycosidic bond (Rabenstein, 2002). Heparin as described above is capable of interacting with a wide range of growth factors that contain heparin binding motifs. Interactions between heparin and growth factors occur via electrostatic forces of attraction between the –N and –O sulphated groups of the heparin (high negative charges) and the lysine and arginine groups in the growth factors (high positive charges) (Oliviero et al., 2012). The binding of heparin to growth factors has also been reported to prevent denaturation and digestion by proteinases {Mano, 2007 #55}. The commonly studied angiogenic growth factors VEGF and bFGF express a high affinity with heparin, facilitating GF interaction with bFGF's receptor and promoting receptor activation, thus having potential in its use in angiogenesis experiments {Tae, 2007 #62}. Heparin has been demonstrated by numerous studies to control the release of VEGF and bFGF *in vivo* resulting in increased neovascularisation of implants.

Our group has recently developed a controlled approach for the functionalization of heparin hydroxyl groups with acrylate moieties (van Rensburg et al., 2016). Heparin was acrylated on its hydroxyl groups through reaction with acryloyl chloride at high pH.

This novel formulation of heparin allows for coupling of the acrylated heparin into the PEG-VS hydrogels through thiol containing crosslinkers via a Michael addition reaction.

This chemical modification therefore allows for the relatively facile generation of an ECM mimic by covalently coupling heparin into the enzymatically degradable PEG-VS hydrogels that are derivatised with cell binding peptides. It is envisaged that this potentially more realistic ECM mimic will be a pro-angiogenic vehicle when combined with the angiogenic GFs, VEGF and bFGF

9. Aims

It is clear from the above that heparin has shown potential to sustain growth factor release and stimulate angiogenesis. Therefore the aims of this study are:

1. To increase the ECM mimicry of the enzymatically degradable PEG hydrogel system used by our group through the conjugation of heparin. This system has not previously been so modified.

2. Assess the influence of heparin conjugation on growth factor uptake by both polymerised hydrogels and hydrogels in a pre-polymerisation state. The former will inform on the ability of hydrogels to enhance the paracrine effect of encapsulated stem cells and the latter on the potential of these hydrogels as injectable growth factor vehicles.

3. Assess the controlled release of the growth factors and determine the bioactivity of the released factors.

4. Assess the pro-angiogenic potential of these heparinised hydrogels in an *in vivo* subcutaneous rat model for scaffold neovascularisation.

Materials and Methods

10. Preparation of 8-Arm Polyethylene Glycol Vinyl Sulfone (PEG-VS) and PEG Acrylate (PEG-AC) Hydrogels

As previously described by our laboratory (Lutolf and Hubbell, 2003), the preparation of 20kDa 8-arm PEG-VS and PEG-AC was done by the derivatisation of PEG-OH. In brief, PEG-OH was dissolved in dichloromethane (DCM) at a concentration of 1.67% w/v, followed by Sodium hydride (NaH) to deprotonate the solution and excess NaH was further quenched by 3.5ml of acetic acid (excess acid until visual colour change is seen). For preparation of PEG-VS, vinyl sulfone moieties (Aldrich, Buchs, Switzerland) were then added in excess at a 50 to 100-fold molar excess. The solution was then added into cold diethyl ether to precipitate PEG. The 20kDa 8-arm PEG-AC was synthesized in dry DCM by adding acryloyl chloride to PEG-OH in presence of Triethylamine (TEA) in a dry working environment (Sakiyama-Elbert and Hubbell, 2001) Both precipitated PEG-VS and PEG-AC were vacuum dried (RT, 750 mmHg, overnight), purified by dialysis against deionized water (cut off value = 1 kDa, 24 hr) and freeze dried. Acrylated heparin (Hep-AC) was synthesized as follows: a heparin solution (0.02 g/ml deionized water (DI)) was stirred (30 min, 0 °C, pH = 9.5, 0.5 ml 0.1 M NaOH), acryloyl chloride (2.5 ml, 20 mole eq of OH groups, 2.5 ml/h) added using a syringe pump, and reacted for an additional hour (< 5 °C, pH = 8-9, 10 ml 1-5 M NaOH). After vacuum filtration, Hep-AC was isolated by precipitation (ethanol, 0 °C, 10 x excess volume) and then processed as for the precipitated PEG (van Rensburg et al., 2016). All these procedures were performed by the group of Assoc. Prof. Deon Bezuidenhout (Cardiovascular Research Unit, UCT).

Hydrogels were polymerized via a Michael-type conjugate addition between equimolar ratios of vinyl sulfone moieties of PEG-VS and thiols of cysteine residues present in MMP enzyme recognition peptide cross-linkers. A 1733 Da MMP-1 degradable enzyme specific peptide sequence, GCREGPQGIWGQERCG (GenScript USA Inc., Piscataway, NJ) was used to form 4% (mass/volume ratio) hydrogels. 1.5% (mass/mass) Hep-AC was incorporated into PEG to form part of the polymer. PEG was incubated with a 1025 Da RGD adhesion peptide (GCGYGRGDSPG) with a molar ratio of 12.5:1 PEG-VS (0.2 mg/120µl iso-PBS) for 20 min at 37°C to allow for cellular interaction, followed by the addition of Hep-Ac molecule and MMP-1 enzyme

recognition peptide at respective values indicated in table 1. Hydrogels were brought up to total volume using iso-osmotic phosphate buffered saline (Iso-PBS, pH 7.5). Iso-osmotic phosphate buffer saline (iso-PBS) used for preparation of all hydrogels was prepared by mixing 13 ml $NaH_2PO_4.1H2O$ solution with 87 ml $Na_2HPO_4.12H2O$ solution, to which 100 ml of NaCl was added. The 4% 8-arm PEG hydrogels were prepared as in Table 1:

Table 1: Constituents for 4% PEG Hydrogel Preparation

Constituents	PEG	PEG-Hep 1.5%
10% PEG (10mg/100µl)	20 µl	19.7 µl
1.5% Hep-Ac (1.5 mg/100mg)	-	2 µl
MMP-1 Peptide (0.024 mol/L)	16.7 µl	16.7 µl
Iso-PBS	13.3 µl	11.3 µl
VEGF/bFGF (1 µg/µl)	Relative to Gel Type	Relative to Gel Type
Total	**50 µl**	**50 µl**

10.1. Growth Factors

Recombinant human bFGF (154 a.a. catalogue # 100-18b) and recombinant human VEGF (catalogue # 100-39) were purchased from Peprotech (New Jersey, USA) and reconstituted according to their respective protocols.

10.2. Preparation of GF Soaked PEG Hydrogels

A 4% 8-arm PEG-VS hydrogel stock volume was prepared as described in Table 1, excluding the incorporation of GF. Hydrogels (10µl) were carefully pipetted onto sterile parafilm layed in a non-tissue culture petri dish and allowed to gel in a tissue culture incubator (1 hr, 37°C). Droplets of water were placed around the hydrogel to avoid shrinkage. Once set, hydrogels were individually immersed into sterile Eppendorfs containing iso-PBS (500 µl, 2 hr) to allow for swelling and thereafter, transferred into

250 µl 1% Bovine Serum Albumin (BSA-PBS) buffer solution containing either 1 µg VEGF, bFGF or both combined and shaken for 24 hrs at 37°C. A wash procedure followed where hydrogels were moved to 500 µl 1% BSA-PBS solution and washed for 1hr. This was repeated twice to remove excess growth factor washed out after gelation. Gels were subsequently immersed in buffer (500 µl iso-PBS, 1% BSA, 0.02% sodium azide) at 37°C and eluates were collected and replaced by fresh buffer every 3 days for 12 days. All washes and eluates removed were stored at -20°C for further analysis.

10.3. Preparation of PEG Hydrogels with Growth Factor added Pre-Polymerisation

Incorporation of growth factor into hydrogel prior to polymerisation was carried out according to table 1 above. Similarly to Section 10, all hydrogel stock solutions were prepared beforehand, and differed with growth factors incorporation before gelation process. The respective growth factors VEGF and bFGF (1 µl, 1 µg/µl) were added to the hydrogel mixture before the addition of MMP-1 enzyme specific peptide. This mixture was vortexed, and hydrogels were carefully pipetted onto a parafilm coated petri-dish, and allowed to gel in a tissue culture incubator (1 hr, 37°C). Droplets of water were placed around the hydrogels and once set, hydrogels were individually immersed into a sterile Eppendorf containing 1% BSA-PBS solution and washed (500 µl, 1 hr). This was repeated twice to remove excess GF unbound during gelation process. Gels were subsequently immersed in buffer (500 ul iso-PBS, 1% BSA, 0.02% sodium azide) at 37°C and eluates were collected and replaced by fresh buffer every 3 days for 28 days. All washes and eluates removed were stored at -20°C for further analysis.

10.4. Preparation of hydrolytically/enzymatically degradable PEG hydrogels

A novel formulation of PEG hydrogels was utilised to potentially control tissue invasion into PEG-Hep hydrogels and increase growth factor release. PEG hydrogels were prepared as explained in section 1, and respective ratios of working concentrations were prepared according to Table 2 below:

Table 2: Constituents for PEG-VS and PEG-AC Hydrogel Degradation Assay

	Hydrogel Ratio Constituents (PEG-VS : PEG-AC) in µl				
	100% VS	**75% : 25%**	**50% : 50%**	**25% : 75%**	**100% AC**
10% PEG-AC (10mg/100µl)	-	30.78	61.55	92.33	123.1
10% PEG-VS (10mg/100µl)	123.1	92.33	61.55	30.78	-
1.5% Hep-Ac (1.5mg/100mg)	10	10	10	10	10
MMP-1 Peptide (0.024 mol/L)	82.53	82.53	82.53	82.53	82.53
Iso-PBS	34.37	34.37	34.37	34.37	34.37
Total	250	250	250	250	250

VEGF and bFGF were also added to these hybrid hydrogels in the same manner as detailed in sec 9.2.

11. GF Elution Profile Analysis via Enzyme-linked immunosorbent assays (ELISA)

The concentration of VEGF and bFGF released from the PEG/PEG-Hep hydrogels over time was quantified using enzyme-linked immunosorbent sandwich assay (ELISA) experiments. DuoSet® ELISA Development System kits for human VEGF and bFGF were obtained from R&D Systems (Minneapolis, USA). ELISA PBS (pH 7.2 – 7.4) was used to dilute all antibody and 1% BSA diluent reagents. The capture antibody (120 µg/ml) was diluted to the working concentration (1:120 dilution, 1 µg/ml)

in PBS and 100 µl of this was added to each well in a 96-well plate (Nunc, Denmark), covered in parafilm and incubated at room temperature overnight. Thereafter the 96-well plate was washed with 0.05% Tween20 in ELISA PBS (pH = 7.2-7.4, 400 µl, 4 times) followed by a blocking step with 1% BSA-PBS (300 µl, RT, 1 hr). All eluates and wash samples eluate collected over time were diluted to working concentrations relevant to growth factor standard curve. Eluate samples were diluted with diluent reagent loaded individually and incubated (1:30 dilution, 100 µl/well, 2 hr). The wash step was repeated and biotinylated detection antibody was added (1:60 dilution, 100 µl/well, 1 hr) followed by streptavidin conjugated to horseradish-peroxidase (1:60 dilution, 100 µl/well, 20 min). A hydrogen peroxide and Tetra-methyl-benzidine substrate solution (1:1 working concentration, 100 µl/well, 5 min) was then added, monitored for colourimetric development and stopped with 2N H_2SO_4 (2 M, 50 µl/well), monitoring the colourimetric development, followed by the addition of 50 µl of stop solution (2 N H2SO4)(Figure 6A).

Optical density readings were measured using an iMark microplate reader (BioRad, California, USA) and taken at 450 nm, with background correction at 570 nm. Growth factor standard solutions were made and treated in a similar manner (0.0313–2ng/ml) to obtain a standard curve (Figure 6B). The growth factor eluate concentrations (ng/ml) were calculated using the curve and cumulative controlled release curves were established. The quantity growth factor bound was calculated by subtraction of all growth factor contained in washes from the amount loaded. For the soaked hydrogels, the amount remaining in the loading solution was also subtracted.

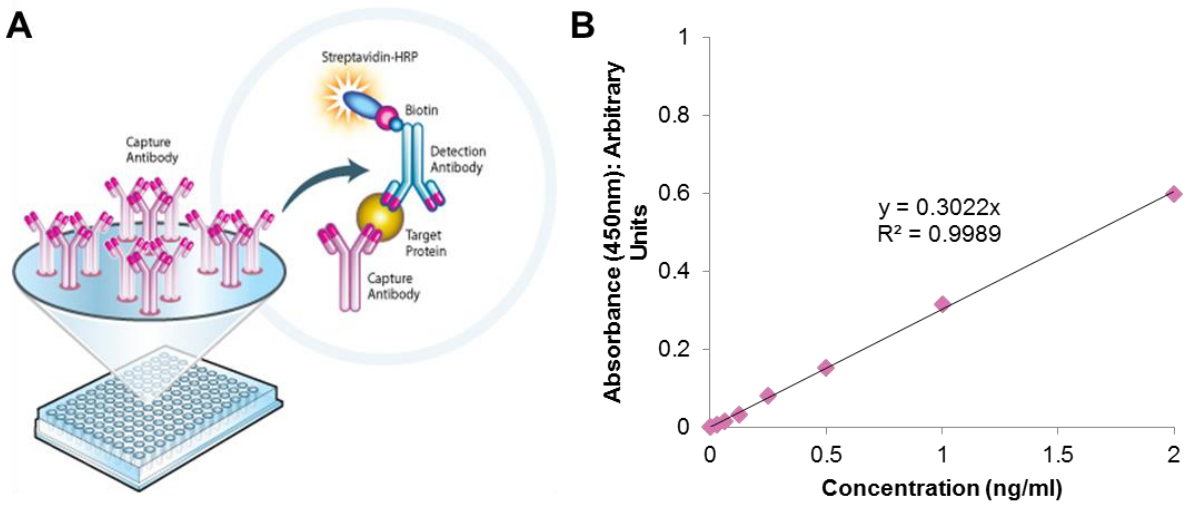


Figure 6: Quantification of GF Release from Hydrogels using ELISA. (A) Illustration of sandwich ELISA process, (B) Standard curve generated from serial dilution of GF for calculation of relapsed concentrations.

12. Cell Culture

Human saphenous vein endothelial cells (HSVEC) and human dermal fibroblasts (HdFB) were cultured in MCDB-131 culture media (Sigma-Aldrich M8537, USA), constituted with 1% penicillin/streptomycin (Sigma, USA), 1.176 g/L $NaHCO_3$ and 10% fetal bovine serum (FBS, Biochrom GmbH, Germany) in a 37ºC incubator (Hera Cell) with 5% CO_2. Media was changed every second day.

12.1. Cell Passaging

Cells were grown to 80–90% confluence and then passaged from 75 cm2 (T75) flasks (Corning, New York, USA). Cells were rinsed with sterile Phosphate Buffer Saline (PBS) and then lifted using 2 ml Trypsin (Biochrom GmbH, Germany). Trpsynisation process was carried out for 3 minutes, followed by inactivation using 2 ml 20% FBS MCDB media. The cell volume was pipetted into a 15 ml Falcon centrifuge tube and centrifuged at 3000 g for 5 minutes. Supernatant was discarded after centrifugation and cell pellet was re-suspended in 1 ml culture medium. Cells in pellet were counted by mixing 20µl of the cell suspension with an equal volume of Trypan Blue in a sterile Eppendorf tube. 10µl was then loaded onto the haemocytometer (Superior, Germany) and a cells were counted using a light microscope (Olympus, Wirsam Scientific, South Africa). HSVEC were used between passage 3-5 and HdFB between passage 6-10 for all experiments.

12.2. Cell Freezing

Cells were trypsinised and counted as mentioned above (Section 12.1). Freezing media containing $5x10^3$ cells was added to cryotube vials (Thermo Scientific, Denmark) containing 500 µl of 15% dimethyl sulphoxide (DMSO)(Sigma-Aldrich, Missouri, USA) culture medium . A slow freezing method was done, where cryotubes were placed on ice immediately after addition of the freezing media and then placed in a -80 °C freezer overnight, after which cells were transferred to liquid nitrogen tank for long term storage.

13. XTT Bioactivity Metabolic Assay

To determine the bioactivity of growth factors released from PEG/PEG-Hep hydrogel constructs, XTT metabolic assay (Cell Signaling Technology, #9095) was conducted on HSVEC and HdFB cells treated with VEGF and BFGF eluted from respective hydrogels. The XTT assay is a colorimetric assay for the non-radioactive quantification of cellular proliferation and viability, which analyses the number of viable cells from their ability to cleave tetrazolium salts added to the culture medium. Cells that are metabolically active will reduce the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl amino)carbonyl] – 2H– tetrazolium hydroxide (tetrazolium XTT salt) to formazan dye (orange in colour) (Huyck et al., 2012).

A XTT Cell Viability Assay kit was obtained from Cell Signalling Technologies (Massachusetts, USA). HSVEC (P3) and HdFB cells (P5) were trypsinised and 5000 cells were seeded per well in a 96-well plate, with each well containing 100 µl of culture medium. After 24 hours HSVEC Cells and HdFB cells were treated with 2% FBS MCDB media containing 5 ng/ml VEGF and serum free MCDB media containing 5 ng/ml bFGF respectively. Cells treated with growth factor-free media were used as a control. Cells were incubated at 37°C (5% CO_2) and the cell viability was tested using XTT assay after 24, 48 and 72 hours. After 48 hours the medium was removed and fresh media was added for each respective cell type. The electron coupling reagent, phenazine methosulfate (X4751) provided was mixed with the XTT reagent in a 1:50 ratio, and 50 µl of the solution was then added per well. The plate was incubated (37°C, 1 hr) followed by optical density readings at 450nm using an iMark microplate reader, with background correction at 655nm.

14. *In vivo* Surgical Procedures

All animal experiments involving surgical procedures were approved by the Animal Research and Ethics Committee of UCT and complied with the Guide for the Care and Use of Laboratory Animals (protocol number: HSFAEC 014/016). Male Wistar rats were obtained from the UCT Animal Unit and weighed to establish baseline weights for post-operative welfare monitoring. All experiments were carried out under aseptic conditions.

14.1. Preparation of Porous Polyurethane Discs

In preparation for subcutaneous assay, porous polyurethane (PU) discs were produced as described by Bezuidenhout et al. with a diameter of 5.4 mm and a thickness of 1 mm with high porosity (82% porosity, 157 ± 1 μm diameter pores) (Bezuidenhout et al., 2002). Discs were sterilized by sonication in a 70% ethanol solution for 30 min followed by incubation at RT for 24 hrs. The discs were then air dried in the tissue culture laminar flow hood. Various PEG hydrogels groups containing the bFGF and/or VEGF were prepared as described above (section 8.2) and RGD peptide was included to promote cellular attachment within the hydrogels. Unpolymerised PEG hydrogel constituents (25 ul per disc) were mixed and aspirated into the PU discs by squeezing the disc consistently (5 times per disc) with a 1 ml syringe plunger (Codan Medical Apl., Rodby, DK) to remove trapped air and efficiently load the hydrogel. Hydrogel filled PU discs were incubated under sterile tissue culture conditions to allow for gelation (37°C, 5% CO_2, 2 hr).

14.2. *In vivo* Subcutaneous Implants

Implants were carried out in male Wistar rats as described by Goetsch *et al* (Goetsch et al., 2015). Animals were anaesthetized (1.5% isoflourane) and shaven, and 1 cm longitudinal incisions were made subcutaneously on the dorsal midline and a pocket for each disc was aseptically secured via dissection (Figure 7). The discs were implanted, within each rat (n = 6) with 6 subcutaneous implants per rat. The different hydrogel groups were randomized per rat and the surgeon was blinded to the groups. The skin incisions were closed with single 4–0 prolene sutures. After 14 and 28 days of implantation, the rats were euthanased by inhalation of halothane and discs were explanted with their surrounding capsules, cut into semi-cylindrical sections, and processed for immunohistology.

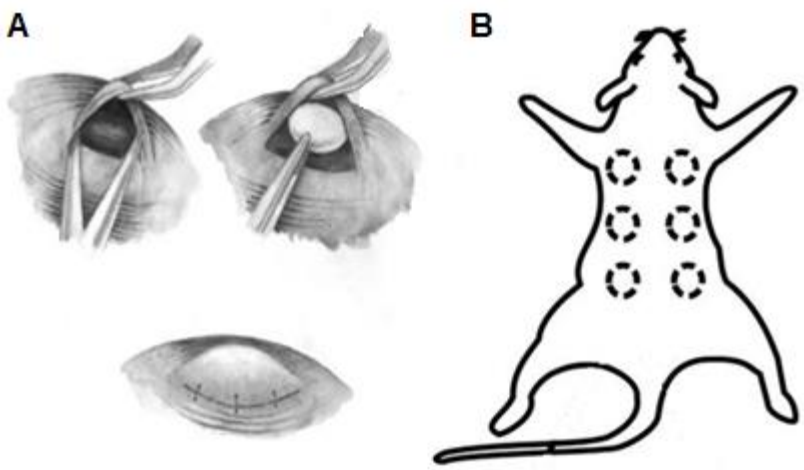


Figure 7: Illustration of Subcutaneous Implantation of Hydrogel Filled PU Discs. (A) Incision of 1 cm pockets and disc implantation, (B) Discs placed in dorsal line, 6 discs per rat

14.3. Histology

PU discs were explanted and fixed in either paraformaldehyde (Saarchem, Kruegersdorp, South Africa), 2.3 mM zinc acetate (Merck KGA, Darmstadt, Germany), 3.7 mM zinc chloride (Sigma-Aldrich, Steinheim, Germany) in 0.1 M Tris. HCl pH 6.75 (Sigma-Aldrich, Steinheim, Germany) or zinc fixative (2.8 mM calcium acetate (Merck KGA, Darmstadt, Germany) for 24 hr (BD Biosciences, California, USA). Fixing was repeated for another 24 hrs. All discs were processed using graded alcohol and embedded in paraffin wax. Sectioning of PU samples was performed using a Microtome. The 5 µm sections prepared on microscope slides were stained with haemotoxylin and eosin (H&E) stained in order to identify cellular invasion. CD31 staining was also conducted to identify blood vessels in zinc fixed sections. For detection of tissue invasion, formaldehyde-fixed sections were incubated in Haematoxylin for 5 mins. After a 5 min wash in running tap water, the slides were placed in Eosin for 30 seconds, dipped in distilled water, dehydrated through the alcohols, before being mounted with Entellan (Merck, Darmstadt, Germany). Vessels were detected by incubation with a 1:25 dilution of mouse anti-rat CD31 primary antibody (RDI Research Diagnostics Inc., Flanders, NJ, USA) in 1% bovine serum albumin (BSA, Jacksons Immunoresearch, West Grove, PA, USA)/PBS for 2 hours at room temperature (RT). Sections were rinsed twice for 10 minutes each with 0.1 %

tween in PBS prior to using the alkaline phosphatase Biocare Medical kit. Sections were incubated with the Rodent body for 30 minutes, followed by two 10 minute washes with TBS. The Mouse on Rat alkaline phosphatase polymer was subsequently added for 30 min, followed by 2 washes with 0.1 % tween in PBS. Finally, sections were incubated with the substrate BCIP/NBT DAKO (DAKO, A/S, Glostrup, Denmark) until colour development was complete. After a rinse with running tap water, the sections were dehydrated, cleared in 2,2,4 trimethylpentane, and mounted with Entellan (Merck, Darmstadt, Germany). Samples were observed and images captured with a Nikon Eclipse 90i microscope with Digital Camera DXM-1200C (Nikon Corporation, Tokyo, Japan) and micrographs covering an entire cross section of the disc were stitched together by automatic scanning (NIS Elements software). The entire cross-section was then used for quantitative image analysis. Image analysis for vessels was performed by training the Visiopharm Integrated Systems software (Visiopharm A/S, Denmark) to automatically detect vessel structures. Tissue invasion was done manually using Visiopharm Integrated Systems to delineate areas of invasion. In all analyses, the researcher was blinded.

15. Statistical Analysis

Animal studies were randomized and blinded. Comparison between the groups was made by means of 1-way ANOVA (STATISTICA), followed by a two-tailed Student T-test. Statistical significance was defined at $p<0.05$. All data are expressed as means ± SEM.

Results and Discussion

16. VEGF and bFGF Hydrogel Binding Potential and Sustained Release of heparinised PEG hydrogels – Modelling the Paracrine Effect

As detailed above, there is considerable evidence that paracrine effects, mediated by factors released by adult stem cells, play an essential role in the reparative process observed after stem cell injection into infarcted hearts. These cells produce and secrete a variety of cytokines, chemokines, and growth factors that may potentially be involved in cardiac repair (Gnecchi et al., 2008). Paracrine factors influence adjacent cells and exert their actions through different mechanisms to promote myocardial protection, cardiac metabolism, regeneration and neovascularization. It is likely that the paracrine factors are released in a temporal manner exerting different effects depending on the environment after injury.

Injectable hydrogels have been used to entrap and localise delivery of stem cells to the heart (See Introduction section 3 – Injectable Hydrogels). They can also potentially be further used to enhance the therapeutic effect by capturing and sustaining the release of paracrine factors. In this initial study, the capacity of covalently binding heparin to a PEG hydrogel to enhance the potential of the PEG hydrogel to bind and control the release of growth factors was assessed. If this hydrogel was used to deliver cells in an injectable format into the infarcted hearts, the cells would by necessity be secreting paracrine factors into a fully polymerised hydrogel. Thus a model was set up that mimics this scenario. Hydrogels (covalently conjugated with either 0, 1.5 or 30% heparin m/m polymer) were preformed, allowed to fully polymerise (2 hours) and then swollen in PBS at 37°C overnight. These fully swollen hydrogels were then incubated in GF containing solutions for 24 hrs. After rinsing, the mass of bound GF's was calculated and their controlled release assayed.

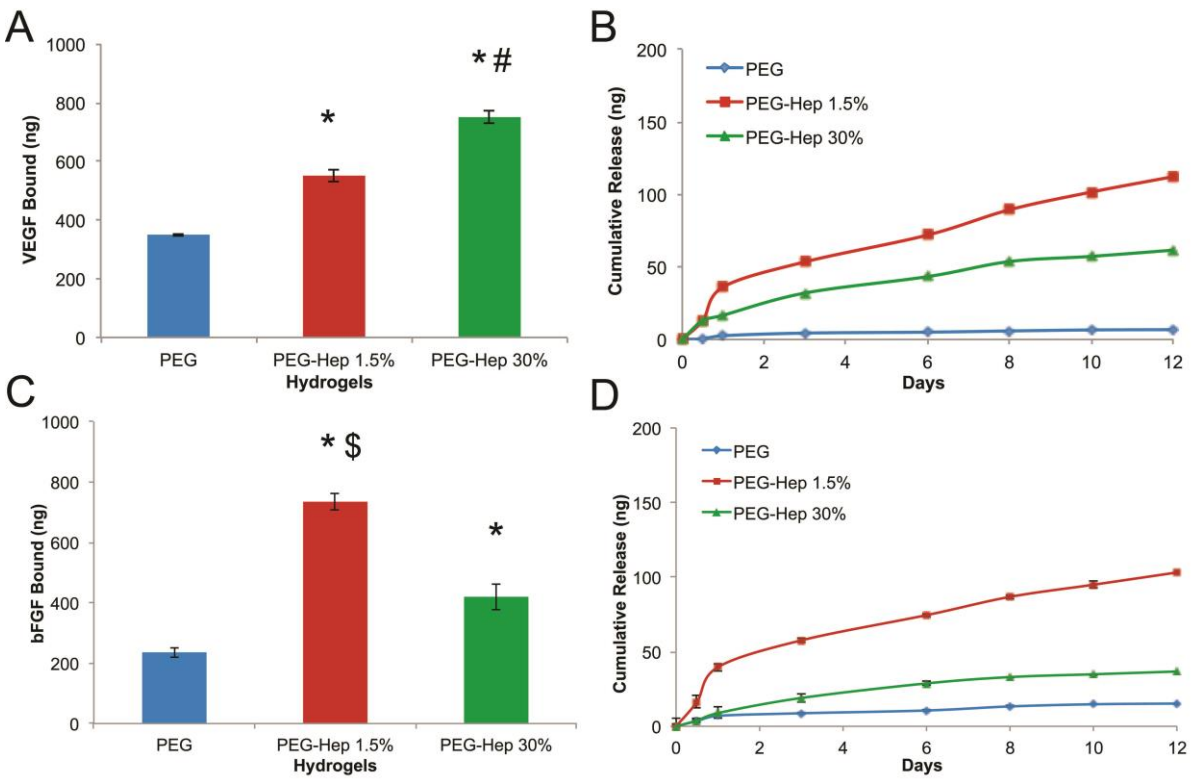


Figure 8: Characterization of Interaction of VEGF and bFGF with PEG and PEG-Hep Hydrogels. (A) Total binding of VEGF to PEG, PEG-Hep 1.5%, and PEG-Hep 30%, (B) Cumulative release of VEGF from the respective hydrogels over a 12 day period, (C) Binding capacity of bFGF to PEG, PEG-Hep 1.5%, and PEG-Hep 30%, (D) Cumulative release of bFGF from the respective hydrogels over a 12 day period. (n=4) * Indicates $p < 0.001$ in significance to PEG, $ $p < 0.001$ in comparison to PEG-Hep 30% and # $p < 0.001$ in comparison to PEG-Hep 1.5%

To evaluate the VEGF binding capacity via the paracrine mimic method, VEGF binding potential to PEG, PEG-Hep 1.5% and PEG-Hep 30% was measured *in vitro*. In Figure 8A, the VEGF binding results showed 350ng ± 4.2ng bound to PEG (35% bound), 551ng ± 20.19ng bound to PEG-Hep 1.5%, (55.1% bound) and 750ng ± 20.72ng bound to PEG-Hep 30% (75% bound). PEG-Hep 1.5% bound 201ng more VEGF (57% increase) in comparison to PEG hydrogel. PEG-Hep 30% bound 400ng more VEGF (2-fold increase) compared to PEG. PEG-Hep 30% also bound 199ng more than PEG-Hep 1.5%. There were therefore substantial and significant increases between PEG and PEG-Hep 1.5% ($p < 0.001$) and between PEG-Hep 1.5% and PEG-Hep 30% vs. PEG ($p<0.001$).

Figure 8B shows the sustained cumulative release of VEGF from the respective hydrogels over a period of 12 days. PEG hydrogel released the least amount of VEGF, showing an initial burst release of 2.6ng in the first day, followed by a further 0.37ng released per day over the remaining 11 days, with only 6.7ng released in total. PEG-Hep 1.5% showed a burst release of 36.19ng on day 1, with consistent release over the 12 day period, with an average of 6.9ng released per day after the initial burst (all per day release rates are given for the period post burst release). 112ng of VEGF was released in total. This hydrogel showed a 16-fold increase in release rate compared to PEG hydrogel. PEG-Hep 30% showed a burst release of 16.75ng in the first day, followed by an average release of 4.05ng per day. 61.58ng of VEGF was released in total, with a 10-fold increase in comparison to PEG hydrogel.

Figure 8C indicates that 235.9ng ± 16.2ng (23.5% bound), 734.61ng ± 26.99ng (73.4% bound) and 419.83ng ± 43.2ng (41.9% bound) of bFGF bound to PEG, PEG-Hep 1.5% and PEG-Hep 30% respectively. An amount of 499ng more bFGF was bound to PEG-Hep 1.5%, showing a 2-fold increase over that bound to PEG alone. BFGF bound 184ng more (78%) in PEG-Hep 30% compared to PEG hydrogel. A 315ng increase (75%) in bFGF binding ability was also observed in PEG-Hep 1.5% compared to PEG-Hep 30%. ($p<0.001$). It is therefore clear that heparinized hydrogels had a significant increase in bFGF binding potential. In this case, the increase in heparin concentration to 30% led to a decrease in bFGF binding potential.

The sustained cumulative release of bFGF from the respective hydrogels is seen in Figure 8D. PEG hydrogel showed a burst release of 6.98ng in the first day, followed by a rate of 0.74ng per day and a total of 15.24ng released in 12 days. PEG-Hep 1.5% showed a burst release of 39.62ng on day 1, with a stable average release of 5.7ng per day over the 12 day period, and 103.17ng released in total. PEG-Hep 30% had a burst release of 8.85ng of bFGF in the first day, followed by a release rate of 2.54ng with 36.76ng of bFGF released in total. The PEG-Hep 1.5% hydrogel resulted in the highest bFGF release rate over 12 days. This hydrogel had a 9-fold increase, and PEG-Hep 30%, a 2-fold increase compared to PEG hydrogel.

A further experiment was set up to analyse the binding and release potential of PEG and PEG-Hep hydrogels for VEGF and bFGF when the GFs were loaded in combination with one another.

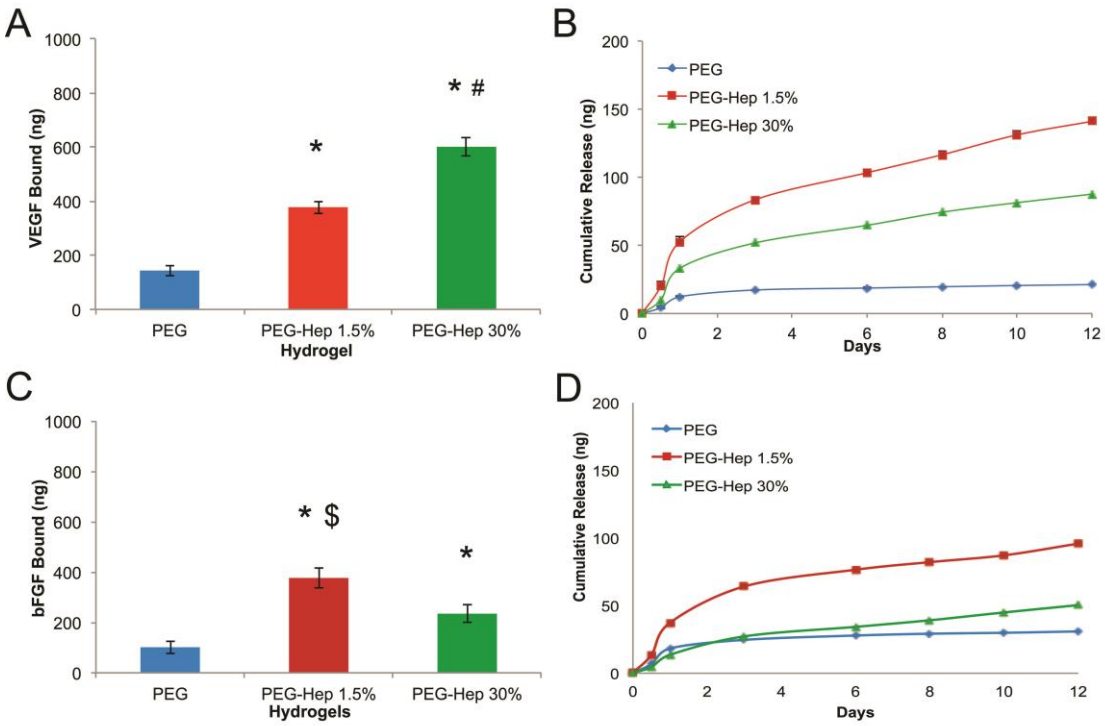


Figure 9: Characterization of Interaction of VEGF and bFGF loaded in combination, with PEG and PEG-Hep Hydrogels. (A) Total binding of VEGF to PEG, PEG-Hep 1.5%, and PEG-Hep 30%, (B) Cumulative release of VEGF from the respective hydrogels over a 12 day period, (C) Binding capacity of bFGF to PEG, PEG-Hep 1.5%, and PEG-Hep 30%, (D) Cumulative release of bFGF from the respective hydrogels over a 12 day period. (n=4). * Indicates $p < 0.001$ in significance to PEG, $ $p < 0.001$ in comparison to PEG-Hep 30% and # $p < 0.001$ in comparison to PEG-Hep 1.5%.

The binding of VEGF and bFGF to the different hydrogels when loaded in combination (Figure 9A and C) indicated a similar trend when loaded singly (Figure 8A and C). Figure 9A showed 143.38ng ± 19.03ng (14.3% bound), 378.14 ± 22.91ng (37.8% bound), and 601.45 ± 35.5ng (60.1% bound) of VEGF was bound to PEG, PEG-Hep 1.5% and PEG-Hep 30% respectively. A 1.6-fold increase was seen in PEG-Hep 1.5% compared to PEG hydrogel ($p<0.001$), with 235ng more VEGF bound to heparin molecules. A 3.2-fold increase was seen in PEG-Hep 30% compared to PEG hydrogel, with 458ng VEGF bound to the heparin. ($p<0.001$). Here again, heparin concentration significantly increased the binding of VEGF.

A sustained release of VEGF was observed from the hydrogels. PEG released 21.21ng of VEGF in 12 days, with a burst release of 12.2ng in the first day and a daily rate of 0.81ng. PEG-Hep 1.5% showed a constant release of VEGF over 12 day, with

a burst release of 52.65ng on day 1, followed by an average rate of 8.09ng per day and 141.03ng released in total. PEG-Hep 30% released 33.2ng in a burst on the first day, followed by a release of 4.9ng per day, with a total of 87.59ng released in 12 days. The presence of heparin in PEG hydrogel significantly increased the rate of growth factors release, showing a 7-fold increase with PEG-Hep 1.5%, and a 4-fold increase with PEG-Hep 30%.

The binding potential of bFGF was also analysed, and figure 9C showed 101.65ng ± 24.5ng (10.1% bound), 376.84 ± 39.14ng (37.6% bound), and 235 ± 34.34ng (23.5% bound) was bound to PEG, PEG-Hep 1.5% and PEG-Hep 30% respectively. PEG-Hep 1.5% had 275ng more of bFGF bound (2.7-fold increase), and this was bound to the heparin incorporated in the hydrogel. PEG-Hep 30% bound 134ng more bFGF (2-fold increase) due to heparin compared PEG respectively. The presence of heparin in these hydrogels increases their binding potential, showing a clear significant increase in PEG-Hep 1.5% compared to PEG ($p<0.001$), and significant increase in bound BFGF on PEG-Hep 1.5% compared to PEG-Hep 30% ($p<0.001$).

In figure 9D, sustained bFGF release was analysed and PEG released 30.91ng in 12 days. This was observed as the highest amount of growth factor released from PEG, in comparison with all other PEG hydrogel release profiles assayed. In this hydrogel, 18.31ng was released due to burst effect in the first day, followed by an average of 1.09ng released per day. PEG-Hep 1.5% hydrogel showed a burst release of 37.35ng in the first day, followed by a steady release of 5.27ng of bFGF per day with a total of 95.84ng released in 12 days. PEG-Hep 30% showed a burst release of 13.67ng in the first day, followed by 3.06ng released per day, and a total of 50.39ng of bFGF released in 12days. PEG-Hep 1.5% had a 3-fold increase in bFGF release rate, and PEG-Hep 30% showed a 60% increase in bFGF release rate compared to PEG hydrogel.

The observation of the amounts of bFGF bound and released was substantially lower compared to VEGF amounts, but still significant in the model described above. Though it has been demonstrated by our laboratory in collaboration with the inventors, Hubbell group, of this proteolytically degradable PEG hydrogel system that the VS moieties on the PEG are able to covalently bind growth factors through exposed sulfhydryls on their cysteine molecules (Zisch et al., 2003a), it was expected that in the scenario described above that all VS would have been reacted with the sulfhydryls contained in the crosslinking peptide sequence during polymerisation. This does not appear to be the case and it seems that possibly the non-reaction of a small percentage of VS

during polymerisation is at a sufficient level to allow for the capture of some of the growth factors. This capture is most probably enhanced by the 24hr incubation period in the growth factor solution. However as shown above and discussed below, heparin substantially increased growth factor capture and greatly elevated the release rates of the two growth factors examined here.

VEGF and bFGF showed similar trends in binding and sustainable release for both the single and combined loading hydrogel profiles. Table 3 shows the amount of growth factor bound to heparin molecules in each growth factor binding analysis. VEGF bound in similar amounts to PEG-Hep 1.5% in both the single growth factor experiment, and when loaded in combination with bFGF. A similar trend was seen with PEG-Hep 30%. The binding of bFGF to PEG-Hep 1.5% and PEG-Hep 30% between single and combination growth factor profiles was also increased by heparin, but the amount of bFGF was less in the combination study, particularly for the PEG-Hep 1.5% (Table 3). An anomaly is, that though PEG-hep 30% hydrogels bound the highest amount of VEGF as might be expected, this was not observed for bFGF. This unexpected result was observed in both the single and combination studies and thus is reproducible. It is not clear why the increased heparin concentration did not bind additional bFGF molecules and this observation requires further investigation. It seems unlikely that the affinity of heparin for the two growth factors might play a role here as bFGF has been shown with surface plasmon resonance analysis to have an approximately 8 fold higher affinity for heparin than VEGF (Ashikari-Hada et al., 2004).

Table 3: Amount of Growth Factor Bound to the Heparin Moiety

	Single		Combination	
	VEGF	bFGF	VEGF	bFGF
PEG-Hep 1.5%	201ng	499ng	235ng	275ng
PEG-Hep 30%	400ng	184ng	458ng	135ng

The release rates for the heparinised gels is, in almost all cases, substantially elevated above that of PEG hydrogels. In this area, the behaviour of the two different heparin hydrogels is as expected. For both VEGF and bFGF, PEG-Hep 30% hydrogels

had a lower cumulative release rate in comparison to PEG-Hep 1.5%. Although it might have been predicted, at least for VEGF, that PEG-Hep 30% would have a higher release rate due to more VEGF bound to the hydrogel, a probable reason for the observed low release rate is that high levels of heparin significantly increase the hydrogel's overall avidity for GF, thus reducing the ability of the VEGF to escape the hydrogel. The especially pronounced effect of increased heparin concentration on bFGF release rates might, in this instance, reflect the higher affinity of heparin for this GF. This phenomenon has also been observed by Pike et al for VEGF and bFGF release from heparinised hyaluronic hydrogels (Pike et al., 2006). Thus it is clearly important to titrate the heparin content in hydrogels for optimal binding and release rates. In the studies above, it is clear that PEG-Hep 1.5% is the optimal concentration of the two heparin gels tested here and later work in this project focused on the PEG-Hep 1.5% hydrogels. As stated above, the ultimate aim of this portion of the project was to determine the suitability of these hydrogels as an injectable hydrogel for cardiac delivery of MSC to infarcted hearts. The data presented above resulted in the choice of PEG-Hep 1.5% for such a study carried out by our laboratory and collaborators at the University of Pavia, Italy. The hydrogel formulation did significantly enhance the capture and therapeutic efficacy of injected MSC in a rat infarction model (the associated manuscript is presently undergoing review).

17. Characterisation of VEGF / bFGF loaded in an injectable appropriate manner

Though the above experiments demonstrated the suitability of heparinised PEG hydrogels to serve as a scaffold for capturing stem cell secreted paracrine factors after hydrogel polymerisation, this type of hydrogel also has the potential to deliver growth factors in a controlled release manner after cardiac injection. In this type of scenario, growth factors would need to be contained in the pre-polymerised solution prior to injection and subsequent *in vivo* polymerisation. Thus further experiments were carried out, whereby hydrogels were formed in that exact manner with VEGF and bFGF contained in the pre-gel solution. As PEG-Hep 1.5% had been shown to behave optimally of the two heparin concentrations used above, it was assessed for its potential to bind and control the release of the growth factors over a further extended period of 28 days. It has previously been demonstrated by our laboratory that growth

factors require a sustained delivery for the generation of stable vessels (Davies et al., 2008).

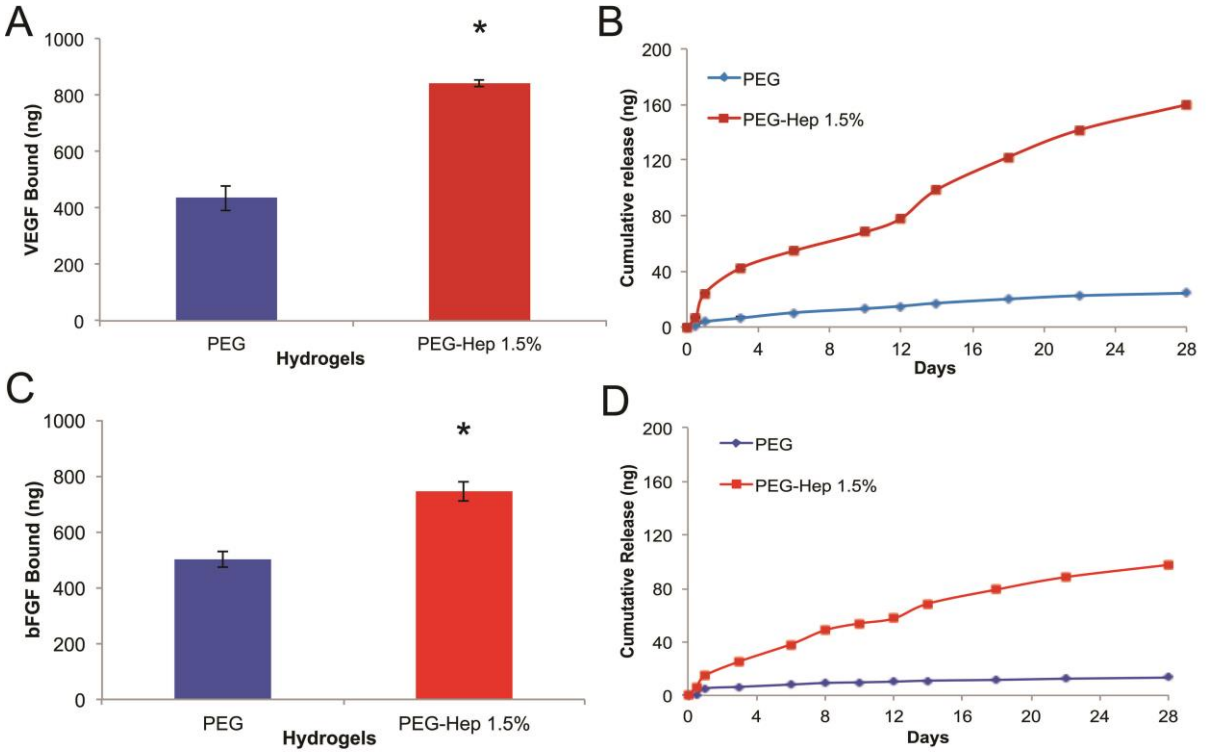


Figure 10: Characterization of Interaction of VEGF and bFGF mixed in PEG and PEG-Hep 1.5% Hydrogels. (A) Total binding of VEGF to PEG and PEG-Hep 1.5% (B) Cumulative release of VEGF from the respective hydrogels over a 28 day period, (C) Total binding of bFGF to PEG and PEG-Hep 1.5% (D) Cumulative release of bFGF from the respective hydrogels over a 28 day period. (n=4) * = $p<0.001$ in (A) and (C).

VEGF binding capacity was assayed after polymerisation of the hydrogel solutions with growth factor present. After washing, as for the previous binding experiments, 435.79ng ± 44.58ng VEGF was bound to PEG (43.5% bound) and 842.17ng ± 14.10ng bound to PEG-Hep 1.5% (84.2% bound) (Figure 10A). A substantial increase in VEGF binding was observed in PEG-Hep 1.5% compared to PEG alone, where PEG-Hep 1.5% bound a 406ng more VEGF (2-fold increase) in comparison to PEG hydrogel ($p<0.001$). Figure 10B shows the sustained release of VEGF from the respective hydrogels over a 28-day period. PEG hydrogel released a small amount of VEGF, showing an initial burst release of 3.8ng in the first day, followed by a further 0.73ng released per day over the remaining 27 days, with only 24.34ng released in total. PEG-

Hep 1.5% showed a burst release of 24.25ng on day 1, with consistent release over the 28-day period, showing an average of 5ng released per day. 160ng of VEGF was released in total. This hydrogel showed a 7-fold increase in release rate compared to PEG hydrogel. PEG-Hep 1.5% released approximately 33% of VEGF bound to the heparin in the hydrogel over the 28-day period. Figure 10C indicates that 501.34ng ± 30.09ng (50.1% bound), and 745.19ng ± 36.7ng (74.5% bound) of bFGF bound to PEG and PEG-Hep 1.5% respectively. An amount of 234.85ng of bFGF was bound to the heparin molecules in the PEG-Hep 1.5% hydrogel, showing a 47% increase in binding potential, compared to PEG alone ($p<0.001$).

Figure 10D shows sustained release of bFGF from PEG and PEG-Hep 1.5% over 28 days. PEG hydrogel released 5.2ng in the first day, followed by a further 0.34ng released per day over 27 days, with only 14.47ng released in total. PEG-Hep 1.5% showed a burst release of 14.82ng on day 1, with consistent release over the 28 day period, showing an average of 3ng released per day. 97.81ng of bFGF was released in total. This hydrogel also showed a 9-fold increase in bFGF release rate compared to PEG hydrogel. PEG-Hep 1.5% released 34% of bFGF bound to the heparin in the hydrogel over 28 days. Thus, in this injectable growth factor delivery model, heparinisation of the PEG again significantly increased bound growth factor and also substantially elevated growth factor release levels. Interestingly, the amount of growth factor bound to the non-heparinised PEG did not differ as much as might have been predicted as a consequence of the raised levels of vinyl sulfone moieties temporarily available during polymerisation. If the binding for the single growth factors to a pre-polymerised PEG hydrogel (Figure 10) are compared to those in Figure 8, 25% more VEGF and 2-fold more bFGF was bound when growth factors were pre-mixed. This suggests that both the reaction rate of the thiolated peptide cross-linker as being much higher due to its hugely higher concentration relative to the growth factors (2000 and 4000 fold for bFGF and VEGF respectively), and the reduced time available for crosslinking in the pre-mix scenario (1 hr vs. 24 hr for the paracrine model), greatly limited binding of the growth factors to the VS moieties during polymerisation.

**Soak method: Polymerised Hydrogel + Growth factor mixture = Growth factor captured in Hydrogel*

**Mix method: Pre-polymerised hydrogel + GF = growth factor incorporated Hydrogel Polymerisation*

18. Bioactivity of Hydrogel Released VEGF and BFGF

As the growth factors were released over an extended period of time (28 days) at 37°C, the bioactivity of the growth factor fraction released at the 28 day time point was determined. This would allow for the potential of the heparinised PEG hydrogels to retain and control the release of growth factors in a manner that was suitable for generating neovascularisation. VEGF and bFGF released from PEG-Hep 1.5% hydrogels at 28 days were assayed for their influence on the proliferation of human saphenous vein endothelial cells (HSVEC) and human dermal fibroblasts (HdFB) respectively. First, a dose response assay was set up to identify the optimal growth factor concentration to test on the different cell lines. Bioactivity of 28 day eluted growth factors was then analysed.

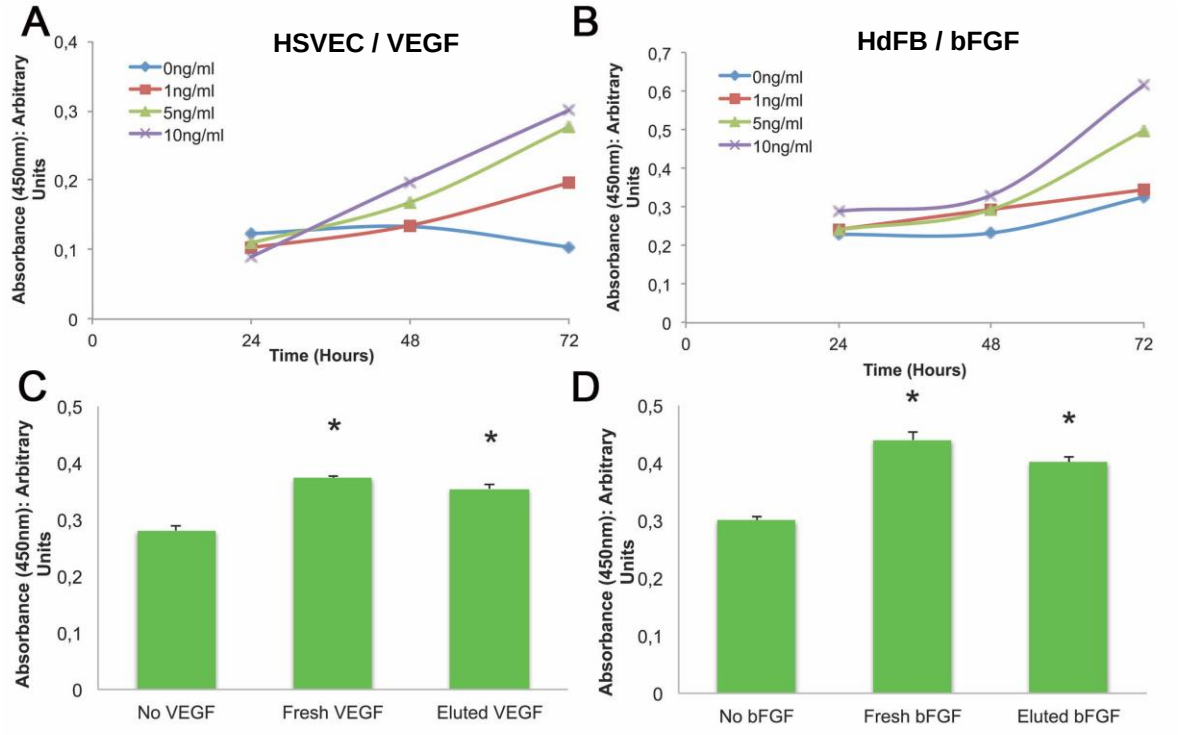


Figure 11: Dose Response Tested VEGF and bFGF Released from PEG-Hydrogel Promote Cell Proliferation. The XTT based absorbance was measured for VEGF stimulated HSVEC (A and C) and bFGF stimulated HdFB cells, (B and D). A dosage response was established for VEGF (A) and bFGF (B). The response at 72 hr for fresh and 28 day eluted VEGF (C) and bFGF (D) was then determined (n=3) * =p<0.01 relative to no VEGF control.

As observed in dose response assays shown in figure 11, increases in VEGF and bFGF concentrations led, as expected, to increases in HSVEC and HdFB proliferation

respectively. The 5ng/ml concentration was chosen to further analyse growth factor bioactivity eluted from the hydrogels, because this was the lowest concentration that showed a pronounced effect for proliferation of both cell types, particularly after 72 hr culture. In Figure 11C, a similar trend was seen in increased XTT based absorbance for HSVEC treated with fresh versus eluted VEGF. Fresh VEGF showed an increase in absorbance readings from 0.28 in untreated cells to 0.37 units (33% increased metabolic activity), and eluted VEGF increased to 0.35 units (26% increase in metabolic activity) after treatment for 72 hr ($p<0.01$). No significant difference was noted between fresh VEGF and eluted VEGF, indicating maintained bioactivity. The bioactivity profile in Figure 11D showed that eluted bFGF increased absorbance from 0.3 to 0.402 units (34% increase in metabolic activity) in comparison to untreated cells and fresh bFGF showed an increase from 0.3 to 0.44 units (47% increase in metabolic activity). Both fresh and eluted bFGF significantly increased in cellular metabolic activity ($p<0.01$), and although differences were observed between fresh and eluted bFGF, there was no significant difference, thus indicating maintained bioactivity. Thus both VEGF and bFGF retained full bioactivity over the 28-day elution period, suggesting that these hydrogels could elicit an angiogenic response when implanted subcutaneously. The observation that the growth factors are stable over this period is supported by other work carried out by our laboratory, where VEGF contained in an osmotic pump implanted in a rat was shown to be fully active after 42 days (Davies et al., 2008).

The above work is the first, to our knowledge, describing the capture and controlled release from an injectable synthetic ECM in the form of a heparinised PEG hydrogel cross-linked with proteolytically degradable peptides. However, due to heparin's affinity for a broad range of growth factors, heparin has been used widely to covalently derivatise other natural and synthetic hydrogel polymers. The discussion here will focus on the more injectable formats of heparin derivatised biomaterials though heparin has been used to enhance the growth factors' binding and release potential of a wide variety of pre-formed polymeric scaffolds such as polyurethane (Davies et al., 2011) collagen (Wissink et al., 2000) and poly(L-lactide-co-glycolic acid) (Jeon et al., 2006).

In past experiments, after activating the carboxylic acids on heparin with N-hydroxysuccinimide (NHS) and 1-ethyl-3-3-(3-dimethylaminopropyl)-carbodiimide

hydrochloride (EDC)(Yang et al., 2010b), the activated heparin was reacted with amines on fibrinogen. This heparinised fibrin was mixed with natural fibrin before polymerisation with thrombin. The derivatised natural hydrogel was found to release bFGF at a steady rate of about 3% per day at a rate roughly similar to that assayed in the above controlled release studies. This natural hydrogel has promise as injectable formulation due to the FDA approval of unmodified fibrinogen. Another naturally occurring polymer that has been used extensively by the Peattie group to form heparinised GF release hydrogels is hyaluronic acid (Peattie et al., 2004) (Pike et al., 2006) (Elia et al., 2010). Here, thiolated heparin was reacted with thiolated hyaluronic acid via a crosslinking PEG-diacrylate. The hydrogels were shown to release both bFGF and VEGF in the presence of exogenous heparin but, as also observed in our study, release rates were very much curtailed with increasing amounts of heparin (Pike et al., 2006). Indeed, for both growth factors, it was found necessary to add thiolated gelatin to increase release rates. The exact reason for this was unclear, but it was speculated that the shorter chain lengths of the gelatin molecules compared to those of the hyaluronic acid chains, might have reduced steric hindrance to the eluting growth factors. It is noteworthy that the potential crosslinking of the growth factors to the PEG diacrylate via their cysteines was postulated for these hyaluronic acid hydrogels (AC and VS both form covalent links with sulhydryls through Michael addition reactions, the former forms a hydrolytically degradable bond and the latter a stable covalent link). Both these hydrogel types have promise due to their innate biocompatibility but potentially lack the engineerability achievable with the synthetic ECM PEG analog analysed in the preceding sections. Additionally, they are less well chemically defined as a result of their purification from biological sources (Lutolf et al., 2001).

Several studies have examined the potential of hydrogels formed via the use of modified heparin and PEG chains. In one instance, heparin was thiolated and then cross-linked with a PEG diacrylate (Choi et al., 2008). The release of human growth hormone was compared between pure PEG hydrogels and the heparin cross-linked hydrogels. Unlike our hydrogels with their defined proteolytically degradable peptide crosslinkers, these would rely solely on hydrolytic degradability. Heparin was found to sustain the release of growth hormone from the PEG hydrogels. Interestingly, though a high percentage of polymers were used to form these hydrogels (10-15% vs the 4% used in for our hydrogels), no evidence for covalent binding to the PEG backbone was

observed. This is in contrast with that observed for our hydrogels and that detailed above for the hydrogels from the Peattie group (Pike et al., 2006) (Peattie et al., 2004). The reason for this difference is not clear but it may reflect the particular protein used, namely human growth hormone. In a different approach, thiol binding groups were added to heparin, namely maleimide, and the maleimide heparin was then reacted with thiolated PEG (Nie et al., 2007). These hydrogels might be expected to be minimally degradable due the stable link formed between the maleimide and the thiols. Here again, higher heparin concentrations resulted in slower release rates of bFGF but also an elegant study was carried out where the polymer percentage was varied. This showed that even if the ratio of heparin was kept low, a higher concentration of the two polymers caused a decrease in release. Again it was speculated that this might be due to covalent links between the bFGF and the maleimide of the heparin.

Perhaps the most similar system to ours is that described in a series of studies from the Werner group (Tsurkan et al., 2010) (Zieris et al., 2010). These hydrogels are star PEG based (4 arm relative to our 8 arm PEGs) and the PEGs are reacted via terminal amines with EDC/NHS treated heparin. In certain instances, an MMP degradable peptide was pre-coupled to the star PEG prior to reaction with the modified heparin (Prokoph et al., 2012). The cell binding peptide RGD was also coupled to the heparin prior to hydrogel polymerisation (Zieris et al., 2010) (Prokoph et al., 2012) (RGD was also used in our hydrogel system for all *in vivo* studies below). Although this system allows for the same engineerability as our system, the chemistry is less specific and it might be expected that proteins incubated with the polymers whilst gelation was occurring could be extensively cross-linked to the heparin via free amines. Amines are present in much higher amounts in proteins relative to thiols (Wu et al., 2015). It may be for this reason that release studies were carried out for this EDC/NHS based system only in a manner analogous to the paracrine model described above, where hydrogels are pre-formed for 24hr and then soaked in growth factor solution. A variety of growth factors such as bFGF, VEGF and SDF-1 have been assessed for control of their release. These hydrogels are characterised by a relatively large burst release in the first few days followed by a fairly flat release rate. This may result from covalent coupling of the protein to the hydrogel. These hydrogels were also able to bind both bFGF and VEGF together in a similar fashion to our hydrogels. Thus it is apparent that the heparinised hydrogels analysed in this project behave similarly to those reported for other systems and due to their controlled chemistries, have some potential

advantages to those discussed above. Therefore their ability to stimulate angiogenesis in a subcutaneous rat model was examined below.

19. Neovascularisation of proteolytically degradable hydrogels releasing bFGF and VEGF

VEGF and bFGF containing PEG-Hep 1.5% hydrogels were prepared singly and in combination and then polymerised within porous polyurethane (PU) discs (Figure 12). These types of PU discs have been utilised extensively by our laboratory as scaffolds for localising neovascularisation in a rat subcutaneous angiogenesis model (Davies et al., 2008) (Bezuidenhout et al., 2002). The discs allow for clear delineation of the target area for potential neovascularisation after explantation. Their substantial porosity allows for easy and uninhibited ingress of tissue.

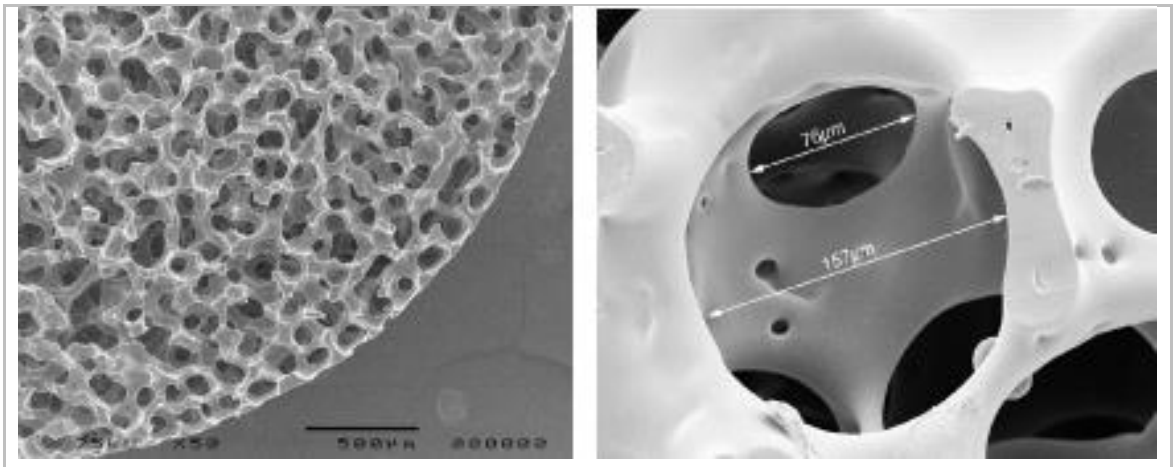


Figure 12: Scanning Electron Microscopic (SEM) Pictures of Porous Polyurethane (PU) Discs. PU discs with a diameter of 6 mm and a thickness of 1mm (50x magnification). (B): Discs show well-defined open porosity of 82%, a pore size of 157±1µm and interconnected windows of 76±1µm (SEM, 500x magnification).

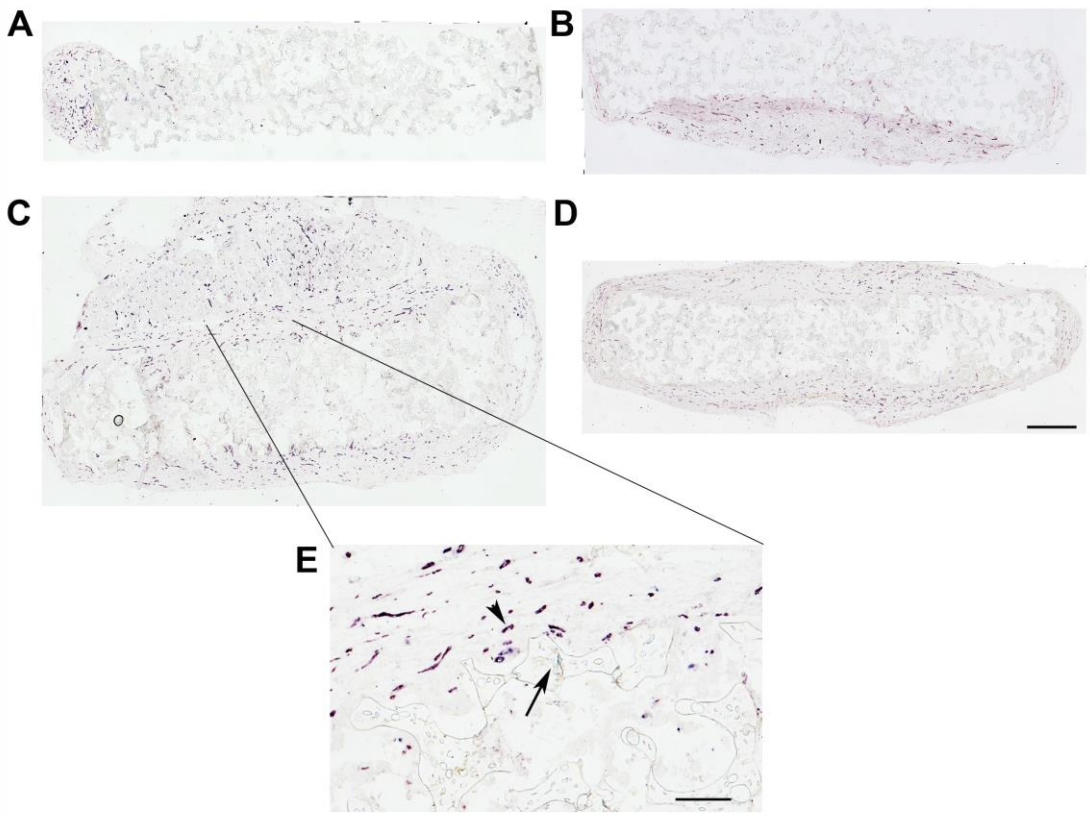


Figure 13: CD31 Immunohistology of PU Discs Explanted at 28 Days. A. PEG-Hep 1.5%; B PEG-Hep 1.5% VEGF; C PEG-Hep 1.5% bFGF D PEG-Hep 1.5% bFGF/VEGF (Size bar D 500 µm) E. High magnification: Arrowhead indicating vessels; arrow indicating PU and beginning of disc (Size bar 100 µm)

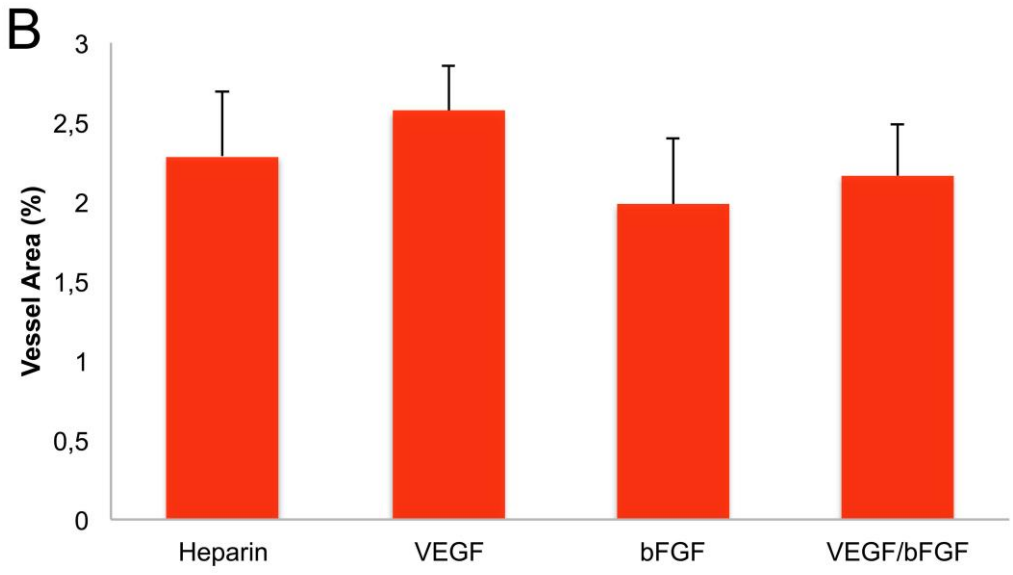
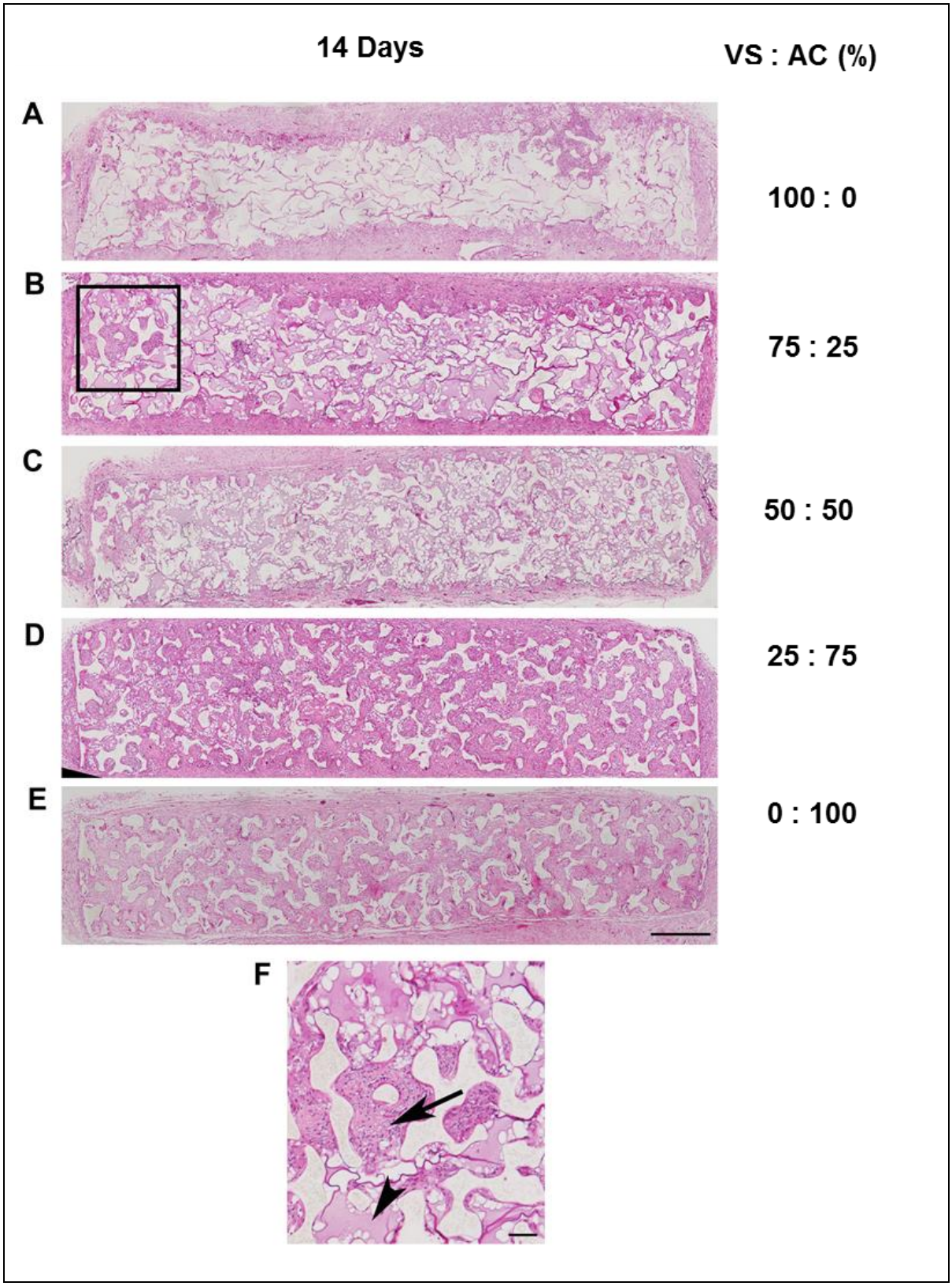


Figure 14: Capsule Vessel Density Quantified in PEG-Hep 1.5% Hydrogels Mixed with VEGF or bFGF, and IHC Stain of Explanted PU Discs with CD31. (n=6)

In figure 13 above, explanted PU discs were stained with CD31 to identify vessel ingrowth induced by the sustainable release of VEGF and BFGF into the hydrogel, showing their potential to induce an angiogenic effect. As seen in figure 13 above, hydrogel filled PU discs showed very little evidence of vessel ingrowth after explantation at 28 days. In our laboratory, the analysis of vessel ingrowth into the scaffold has been the standard process because of its well-defined area for quantification of neovascularisation of the hydrogel (Davies et al., 2011). This measure of vascularization was not possible for this particular experiment, and an attempt was made to analyse the influence of released GF on neovascularization in the surrounding capsule. Capsule sizes showed substantial variability within the different groups, and no significant difference in area of vascularisation was observed. Though VEGF and bFGF were released at apparently reasonable rates from PEG-Hep 1.5%, this did not induce a significant effect on the capsule vessel density (Figure 14). It is not clear whether the varying encapsulation of the discs influenced vessel density due to differing distances for growth factors to travel (considerable variability within each group was observed for capsule size, capsule sizes such as in Fig 13A and 13D were observed in each group), or it could simply represent an inadequate growth factor release rate. As an important aim of these types of cell dependent degradable hydrogels is to generate new vessels in the region previously occupied by the hydrogel, a new strategy was required where the hydrogels would degrade more rapidly but retain the aspect of cell dependent invasion. For these reasons, a novel formulation of the PEG hydrogels that might allow for sufficient ingrowth into the PU scaffold and an increased release of growth factor was developed. Thus, a hybrid scaffold was formed with different combinations of 8-arm PEG Acrylate (PEG-AC) and PEG-VS molecules cross-linked by the MMP degradable peptides. The PEG-AC molecule forms hydrolytically degradable crosslinks with thiol cross-linkers whilst the PEG-VS forms very stable crosslinks (Dobner et al., 2009) (van Rensburg et al., 2016). This novel approach therefore allows for both bulk hydrolytic breakdown and cell directed enzymatic degradation to occur simultaneously.

20. Control of Tissue Invasion into Hybrid Hydrolytically and Enzymatically Degradable PEG Hydrogel

To analyse which ratios of PEG-AC and PEG-VS would be ideal for both desired degradation rate for tissue invasion, an experiment was set up to analyse the *in vivo* degradation rates of hybrid hydrogels. It was desirable to determine what ratio of AC and VS molecules would allow for complete ingrowth into the porous PU discs over a 28-day period. This is a period used by our group that allows for the detection of stable vessel formation, and also roughly corresponds with 4-5 weeks time period in which remodelling of cardiac tissue is complete in a rat heart, the ultimate target for these hydrogels. In this experiment, hydrogels polymerised from ratios of PEG-VS (%): PEG-AC (%) of 100:0; 75:25; 50:50; 25:75 and 0:100. Heparin loaded at 1.5% m/m of total polymer was used exclusively in this and further experiments because this heparin loading had shown the highest growth factors binding and release potential. These various formulations were polymerized within the porous PU scaffolds and implanted subcutaneously for 14 and 28 days. H&E staining allowed for clear visualisation of newly formed tissue and the presence of non-degraded hydrogel (Figure 15).


14 Days
VS : AC (%)
A
100 : 0
B
75 : 25
C
50 : 50
D
25 : 75
E
0 : 100
F

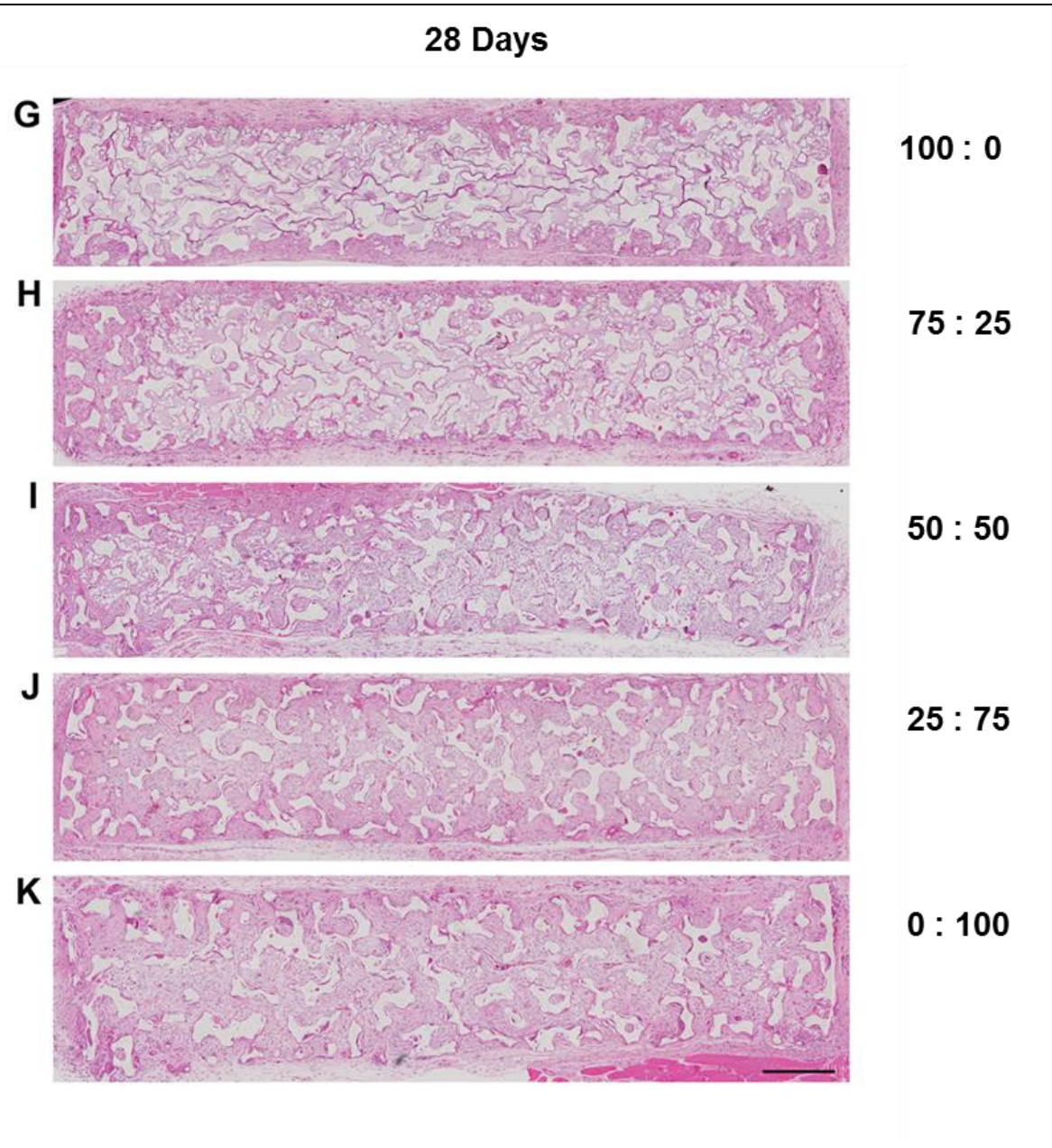


Figure 15: H&E staining of PU discs containing heparinised PEG hybrid hydrogels explanted at 14 (A-F) and 28 days (G-K). PEG-VS %: PEG-AC %: 100:0 (A;G); 75:25 (B;H) 50:50 (C;I) 25:75 (D;J) 0:100 (E;K). size bar 500 µm (E; K). (F) Higher magnification of blocked region in (B). Arrowhead indicating intact hydrogel; arrow indicating tissue. Size bar 100 µm.

As is clear from the micrographs, the percentage acrylate had a marked influence on the invasion of tissue into hydrogels that were formulated with the same percentage of total polymer. Thus, hydrogels containing 50% to 100% of PEG-VS hydrogel showed very little to no tissue ingrowth at 14 days, and hydrogels with lower PEG-VS

incorporation (25% and 0%) showed essentially complete tissue invasion. After 28 days implantation, the 50% VS group now showed complete tissue ingrowth with occasional traces of hydrogel remaining. PEG-VS 25% and PEG-VS 0% hydrogels were, of course, fully ingrown at 28 days as was already seen at 14 days. The 75 and 100 % VS groups showed a small increase in invasion, the invasion for the 100% VS group was also expected from the first *in vivo* angiogenesis study described above (See Section 19).

Quantitative image analysis of the cross-sections confirmed the initial observations. After 14 days, hydrogels containing 100%, 75% and 50% PEG-VS had tissue ingrowth of 2%, 12% and 11% respectively, showing minimal cellular invasion, while hydrogels containing 25% and 0% PEG-VS showed tissue ingrowth of 97% and 100% respectively. After 28 days, hydrogels containing 100% and 75% PEG-VS had 29% and 30% tissue invasion, whereas hydrogels containing 50%, 25% and 0% PEG-VS showed 87%, 100% and 100% tissue ingrowth (Figure 19). These findings show that controlled invasion of tissue into the hydrogel can be obtained by titrating hydrolytic groups with groups that can only be broken apart by enzymatic activity. This, to our knowledge, novel approach complements previous work by our group where PEG-VS hydrogels were cross-linked with differing combinations of a readily degradable MMP cleavage sequence, with one with low degradation potential (Goetsch et al., 2015). The use of differentially degradable peptides allowed for a finer control over invasion than observed for the hydrolytic/enzymatic approach described above. However, it is possible that the use of different ratios to those used might allow for further control. Thus, hydrogels that are expected to be mechanically similar could allow for different ingrowth rates. This is useful as ingrowth control is usually obtained by varying the stiffness of the gel, which, by necessity, varies other parameters (Goetsch et al., 2015). Though similar stiffness is expected based on previous work in the laboratory, this has yet to be confirmed by rheological analysis.

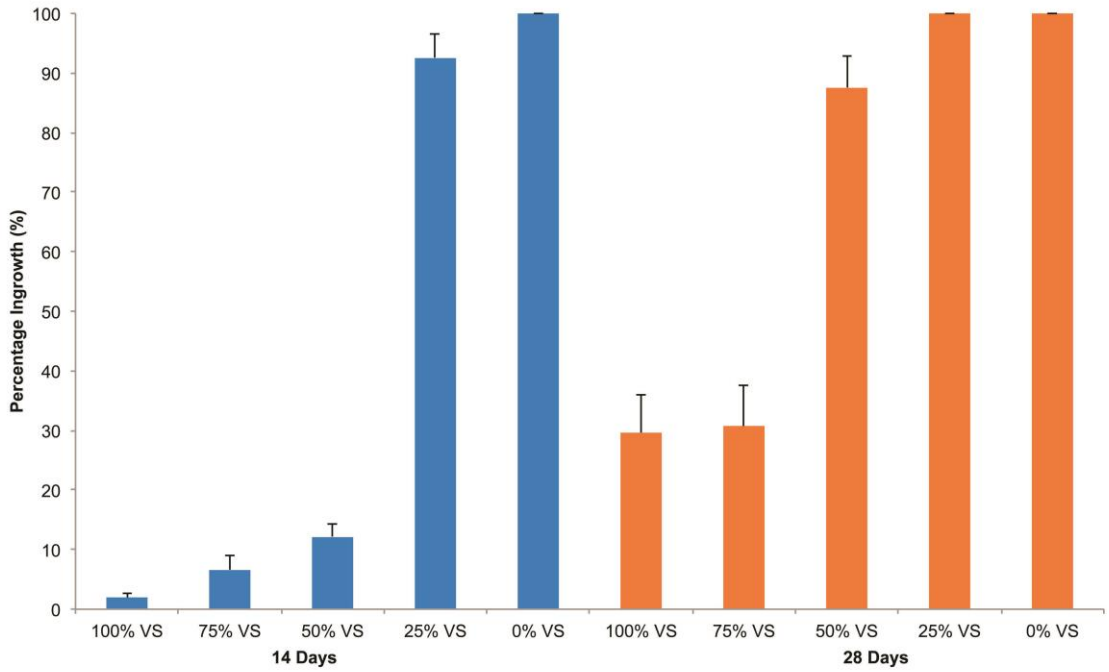


Figure 16: Hybrid Hydrogel *In vivo* Degradation. Image analysis of tissue ingrowth into the porous PU discs as a percentage of ingrowth area available. (n=4)

The results assessed above led the selection of the 50% hybrid hydrogel construct as the most desirable in controlling for degradation. This is because it appeared to persist for almost 28 days, with traces of hydrogel still visible suggesting that growth factors should be delivered over the entire period This hydrogel was therefore assessed for its growth factors binding ability and sustainable release over 28 days.

21. Characterization Heparinized Hybrid Hydrogel on VEGF and bFGF binding as an injectable scaffold

The heparinised 50% hybrid hydrogel pre-polymerised with VEGF and bFGF, was assayed for its ability to bind and release said GFs over 28 days.

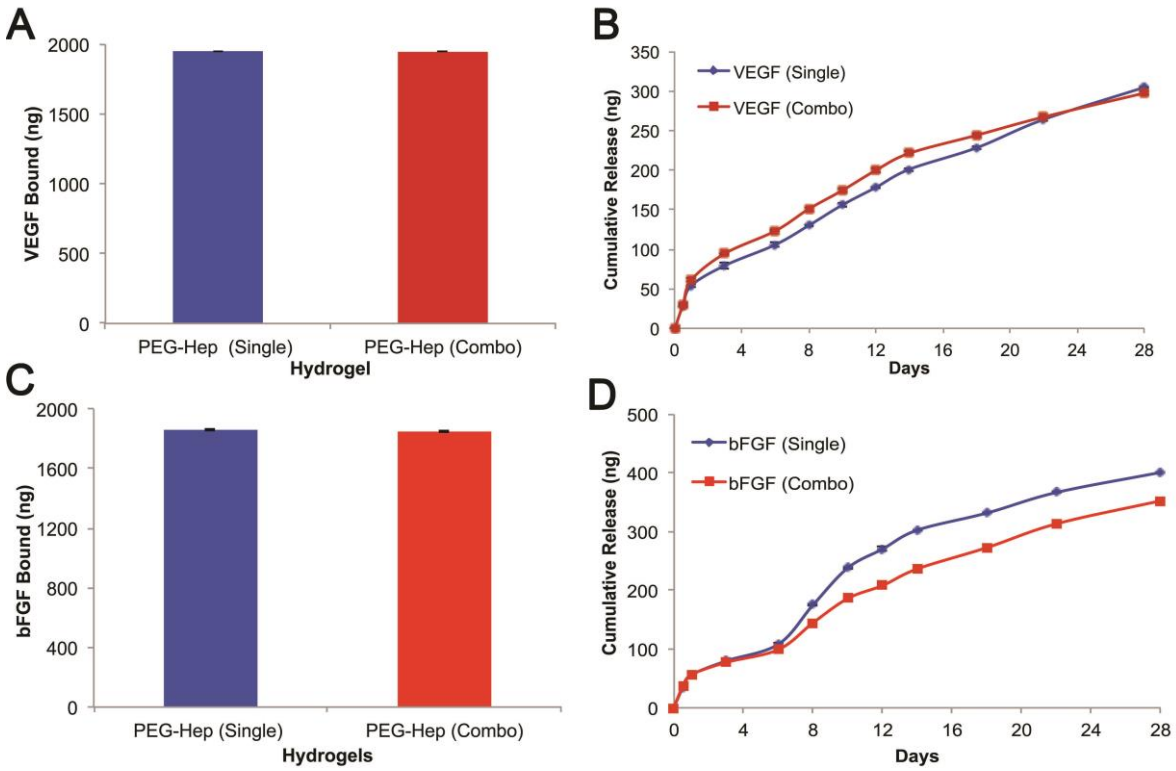


Figure 17: Characterization of Interaction of VEGF and bFGF mixed in 50% PEG-VS/PEG-AC Hybrid Hydrogels. (A) PEG-Hep Hybrid Hydrogel interaction with VEGF alone and in combination with bFGF (B) Cumulative release of VEGF from the respective hydrogels over a 28 day period, (C) PEG-Hep Hybrid Hydrogel interaction with bFGF alone and in combination with VEGF (D) Cumulative release of bFGF from the respective hydrogels over a 28 day period. (n=4)

VEGF binding potential to PEG-Hep 1.5% hybrid hydrogel was measured *in vitro*. In Figure 17A, 1950ng ± 3.43ng (97.5% bound) and 1945ng ± 10.1ng (97% bound) of VEGF bound to hybrid hydrogel as a single growth factor, and in combination with bFGF respectively.

Figure 17B shows the sustained cumulative release of VEGF from the respective hybrid hydrogels over a period of 28 days. VEGF, singly bound to the hybrid hydrogel, showed an initial burst release of 53.99ng in the first day, followed by a further 9.28ng released per day over the remaining 27 days, with 304.69ng released in total. The VEGF bound in combination with another growth factor showed a burst release of 61.05ng on day 1, followed by an average release of 8.75ng released per day over 27 days. 297.42ng of VEGF was released in total. As seen in figure 17B, there was a consistency observed in the release rate over the 28 days, and VEGF released alone or in combination with bFGF showed a similar trend in cumulative release.

In Figure 17C, 1860ng ± 14 (93% bound) and 1849ng ± 3.61ng (92% bound) of bFGF bound to hybrid hydrogel as a single growth factor, and in combination with VEGF respectively. Figure 17D showed the sustained cumulative release of bFGF from the respective hybrid hydrogels over 28 days. bFGF bound to the hybrid hydrogel alone showed an initial burst release of 55.87ng on the first day, followed by a further 12.78ng released per day over the remaining 27 days, with 400.93ng released in total. The bFGF bound in combination with VEGF showed a burst release of 56.47ng on day 1, followed by an average release of 10.96ng released per day over 27 days. 352.57ng of VEGF was released in total.

Double the amount of hydrogel (20ul) was assessed in this experiment, with a corresponding increase in growth factor (2ug) loaded in comparison to the 10 µl hydrogels loaded with 1 µg growth factors used for the previously described release assays. This was, to show more clearly the true amounts expected to bind and release in an *in vivo* model, as this is the quantity of hydrogel and growth factor that was, and would be used, in the angiogenesis assays. According to figure 17, it is possible that hybrid hydrogel constructs allowed for an increase in GF binding compared to pure PEG-VS hydrogels (VEGF binding: hybrid 97%; PEG-VS 82%. bFGF binding: hybrid 92%; PEG-VS 75%). A potential reason for this could be the incorporation of AC moieties into the hydrogel. But as both AC and VS bind to sulfhydryls via a Michael addition with similar kinetics, it is not expected that PEG-AC would have a marked influence on growth factor binding to what has already been observed with PEG-VS. However, in future experiments, this potential influence should be assayed (van Rensburg et al., 2016).

Hybrid hydrogels showed similar VEGF release rates compared to PEG-Hep 1.5% (100% VS) hydrogels in both the paracrine model (Figure 8 and 9) and mixed GF experiments (Figure 10). The bFGF release rate however showed a 3-fold increase in hybrid hydrogels compared to that observed in the pure VS PEG-Hep 1.5% hydrogels. It is somewhat slightly surprising that bFGF showed such a substantial increase in release rate whilst VEGF did not. As noted above, bFGF is known to have a higher binding affinity for heparin and is therefore expected to release more slowly. A slower release was indeed observed for the 100% VS hydrogels (Figure 10). To potentially gain a clearer understanding of this possible discrepancy, as mentioned above, further experiments with non-heparinised hybrid hydrogels should be performed.

As the 50% hybrid hydrogel released both growth factors at similar or even elevated levels than the pure PEG-VS hydrogel previously analysed in the subcutaneous angiogenesis model, and also showed suitable degradation, this formulation was assessed for its pro-angiogenic potential.

22. Hybrid Hydrogel Angiogenesis Potential

The potential of the growth factors bound to the 50% hybrid hydrogel to induce angiogenesis was again assayed in the rat subcutaneous angiogenesis model. PU discs were filled with growth factors containing 50% hybrid hydrogels and implanted for 28 days. In this study, an empty PU disc group was included as an additional control. The full cross-sections (Figure 18) show that, as predicted, there was substantial ingrowth into the hydrogel filled discs with vessel invasion throughout for all groups. This was optimal for quantification because it allowed for the measurement of vessel density in a well-defined area, as previously used in our laboratory.

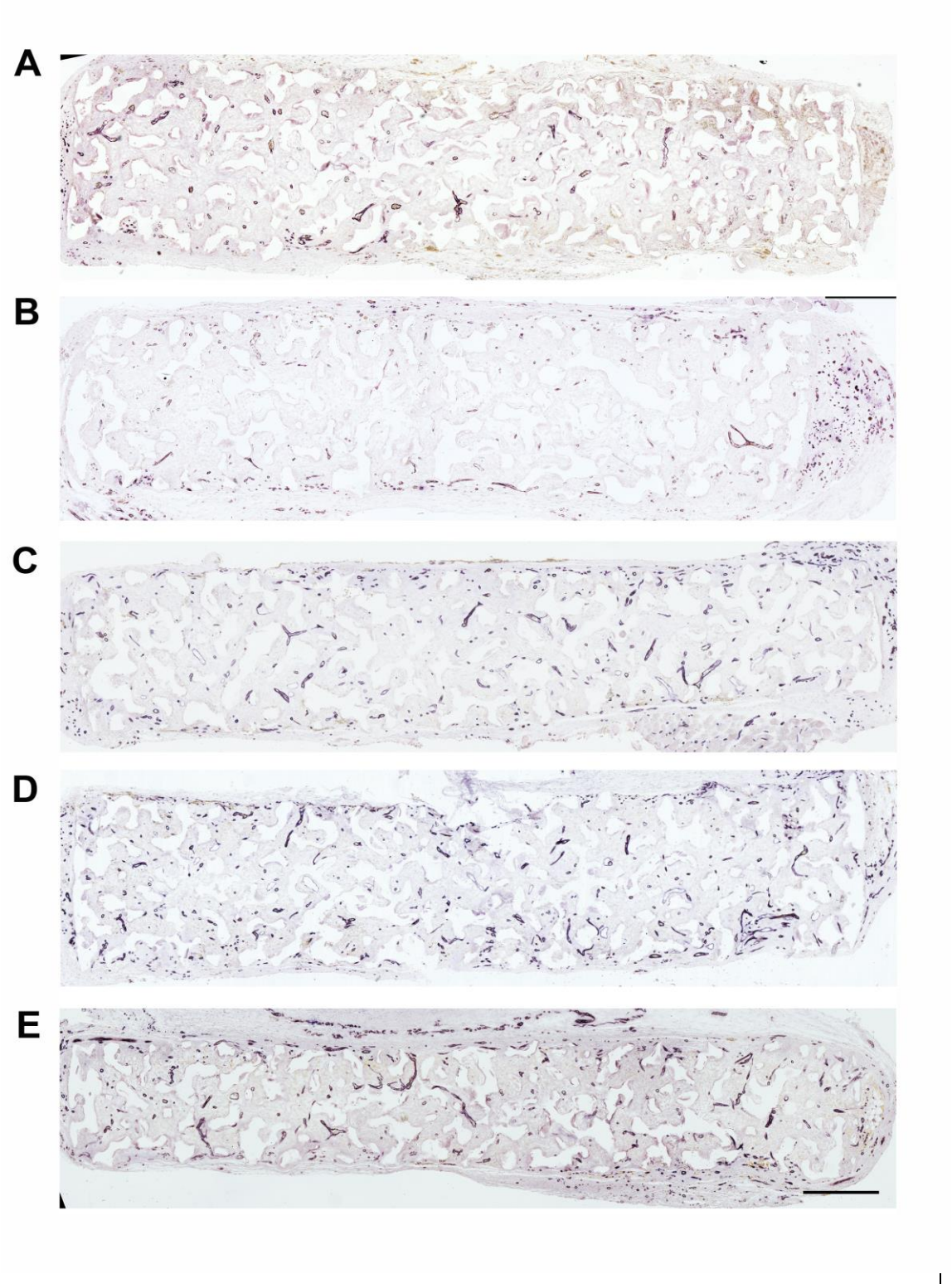


Figure 18: CD31 immunohistology of PU dics containing heparinised hybrid PEG hydrogel explanted after 28 days (complete cross sections). (A) Empty control disc (B) PEG-Hep hybrid (C) PEG-Hep hybrid VEGF (D) PEG-Hep hybrid bFGF (E) PEG-Hep hybrid bFGF/VEGF. Size bar 500 µm

Blinded image analysis of area occupied by vessels in the various groups showed that the incorporation of VEGF, bFGF or their combination, significantly increased the neovascularisation within the hydrogels (Figure 19). Empty PU discs and PEG-Hep hybrid control showed 3.2% and 3.02% in average vessel percentage area, in comparison to the hydrogel area. Growth factor containing hydrogels showed significant increases to 4.3%, 4.8% and 4.7% percentage area for VEGF, bFGF and their combination respectively relative to both the empty disc and PEG-Hep controls ($p < 0.05$)(Davies et al., 2011). It is clear that the incorporation of GF to the heparinised hybrid hydrogel induces substantial increases in vessel ingrowth of 44, 59 and 55% as observed for VEGF, bFGF and their combination relative to PEG-Hep. This demonstrates that the PEG hybrid hydrogels are able to control the release of these two important growth factors in a manner that can achieve a robust angiogenic response. The extent of the response is similar to other studies assaying angiogenesis stimulation by release of these two factors from scaffolds and other hydrogels (Pike et al., 2006) (Davies et al., 2011) (Elia et al., 2010). It had been expected that the combination of VEGF and bFGF would show a synergistic effect (Pike et al., 2006) but this was not observed. However, other researchers have also failed to discern a synergism in combinations of bFGF and VEGF (Zieris et al., 2010) (Cao et al., 2003).

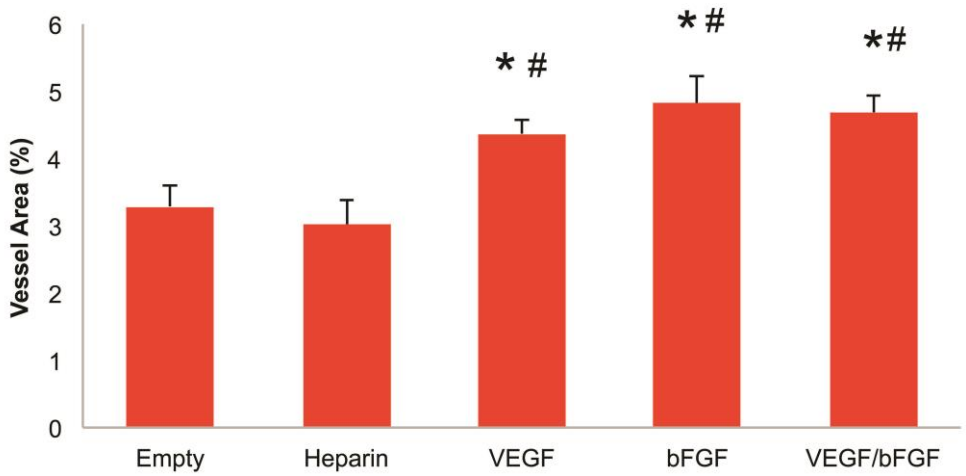


Figure 19: Hybrid *in vivo* Angiogenesis Assay. CD31 treated PU discs filled with GF containing hybrid hydrogel after 4-week period. Discs analysed for vessel ingrowth were analysed qualitatively and quantitatively. Percentage area of ingrowth was quantified and represented graphically. (n=6,). * = $p<0.05$ in comparison to PEG-Hep Control and # = $p < 0.05$ in comparison to Empty PU.

The above findings indicate that the heparinised hybrid hydrogels allow hydrogel degradation to be controlled, and that the resultant controlled release of VEGF and bFGF is sufficient to generate a substantial increase in vessel ingrowth. This suggests that this type of hydrogel would be a useful candidate for injection into damaged cardiac tissue after infarction, and may allow for regain of function and limitation of pathological remodelling.

23. Conclusion

The above work details a characterisation of the potential of heparinised PEG based hydrogels for either potentiation of the stem cell paracrine effect or as injectable GF release vehicles.

In the first study, the utility of these hydrogels was explored in a manner that simulated the situation, if these hydrogels were used as an injectable vehicle for stem cell delivery to an infarcted heart. Fully polymerised heparinised hydrogels were found to be able entrap and release significant levels of VEGF and bFGF. This indicates their promise as stem cell delivery vehicles with the ability to potentiate the paracrine effect. Though non-heparinised PEG hydrogels entrapped substantially less growth factors and had very low release rates, it was still somewhat unexpected that they were able to entrap growth factors after polymerisation. It is assumed this entrapment must derive from interaction of growth factors with unreacted VS moieties, this finding requires further investigation.

In the main body of the research, the capability of the heparinised hydrogels as injectable growth factors delivery vehicles was investigated. Here again, significant increases in entrapment and release rates were observed relative to non-heparinised PEG hydrogels. As the first therapeutic target would be increased neovascularisation, their efficacy was assessed in a rat subcutaneous angiogenesis model. Surprisingly, considering the level of VEGF and bFGF release observed *in vitro*, no enhancement of angiogenesis was observed. It was speculated that this might have resulted from the minimal tissue invasion observed into the hydrogels utilised that require cell based enzymatic degradation.

Thus, a novel approach towards possibly increasing invasion rates and also release rates was explored. Heparinised PEG hydrogels were formed that had variable proportions of PEG molecules that can break down hydrolytically, namely PEG-AC. These hydrogels were indeed found to be able to control tissue invasion in a subcutaneous model. Hydrogels that contained higher proportions of the hydrolytic component were fully invaded within 14 days whilst an intermediate version had a degradation period of 28 days. As expected, those with higher ratios of the enzymatically degradable component persisted to at least 28 days. Certainly, further investigations are warranted into the fine-tuning of this system with other ratios of the two monomers.

The intermediate hybrid hydrogel with the desirable degradation profile was then assayed for VEGF and bFGF entrapment and release. Though the hydrogel was equivalent to the pure enzymatic hydrogels in the initial studies in entrapment as expected, similarly it did not have a higher release rate. This was unexpected, as the loss of the hydrolytic component should have reduced steric hindrance to growth factors escape. This finding requires further assessment of the influence of the PEG-AC moieties and a thorough biomechanical analysis of the hydrogels over the release period.

Nevertheless, as the intermediate hybrid hydrogel had the required tissue invasion rate, a subcutaneous angiogenesis assay was performed. Here a pronounced and significant increase was observed for both VEGF, bFGF and their combinations. Thus, the novel hybrid heparinised PEG hydrogels show promise as another means of controlling growth factors release from an injectable hydrogel.

Because the threshold at which protein will no longer bind is not known. There would be some value to knowing the threshold and a scientific approach focused on testing effects of various concentrations of protein on hydrogel binding would be analysed. This was not explored due to time constraints and the very substantial expense required to run such an experiment. Conducting a binding curve assay would clarify this further.

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