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An Injectable Collagen Scaffold Delivering Exogenous microRNA as a Therapy to Modulate Extracellular Matrix Remodelling

A thesis submitted to the National University of Ireland for the

Degree of Doctor of Philosophy

By

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Network of Excellence for Functional Biomaterials

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<td>4S-StarPEG</td>
<td>4-arm Poly (Ethylene Glycol) Terminated Succinimidyl Glutarate</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>AMD</td>
<td>Age-related Macular Degeneration</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Expressing Cell</td>
</tr>
<tr>
<td>AR</td>
<td>Adrenergic Receptor</td>
</tr>
<tr>
<td>ATRP</td>
<td>Atom Transfer Radical Polymerization</td>
</tr>
<tr>
<td>BW</td>
<td>Body Weight</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C chemokine Receptor Type 5</td>
</tr>
<tr>
<td>CDP</td>
<td>Cyclodextrin-based Polymer</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic Guanosine Monophosphate</td>
</tr>
<tr>
<td>CHO-E</td>
<td>Chinese Hamster Ovary Expressing E-Selectin</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective Tissue Growth Factor</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>deATRP</td>
<td>Deactivation Enhanced Atom Transfer Radical Polymerization</td>
</tr>
<tr>
<td>DGCR8</td>
<td>DeGeorge Syndrome Critical Region 8</td>
</tr>
<tr>
<td>DMAEMA</td>
<td>Dimethylamino Ethyl Methacrylate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco Modified Eagles’s Minimum Essential Medium</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylflormaide</td>
</tr>
<tr>
<td>DNA</td>
<td>De-oxy Ribonucleic Acid</td>
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<tr>
<td>DOTAP</td>
<td>1,2-bis-Oleoylxy)-3-(Trimethylammonium Propane)</td>
</tr>
<tr>
<td>dPAMAM</td>
<td>Partially Degraded Poly (Amino Amine)</td>
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<tr>
<td>dsRNA</td>
<td>Double Stranded RNA</td>
</tr>
<tr>
<td>DTNB</td>
<td>5, 5'-Dithiobis (2-Nitrobenzoic Acid)</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EBM</td>
<td>Endothelial Basal Media</td>
</tr>
<tr>
<td>EBriB</td>
<td>Ethyl 2-Bromoisobutyrate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDC</td>
<td>Ethyl (Dimethylaminopropyl)</td>
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<td>EDTA</td>
<td>Ethylenediaminetetra Acetic Acid</td>
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<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
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<td>EMA</td>
<td>European Medical Association</td>
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<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
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<tr>
<td>EPR</td>
<td>Enhanced Permeation and Retention</td>
</tr>
<tr>
<td>Fab</td>
<td>Antibody Fragment</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>Fc</td>
<td>Fragment, Crystallisable</td>
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<td>FCS</td>
<td>Fetal Calf Serum</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>GAPDH</td>
<td>Glyceraldehydes 3- Phosphate Dehydrogenase</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>GP</td>
<td>Glycoprotein</td>
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<tr>
<td>GPC</td>
<td>Gel Permeation Chromatography</td>
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<tr>
<td>GTA</td>
<td>Glutaraldehyde</td>
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<tr>
<td>HBSS</td>
<td>Hanks Balanced Salt Solution</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HDMEC</td>
<td>Human Dermal Microvascular Endothelial Cells</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin &amp; Eosin</td>
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<td>HEK</td>
<td>Hamster Embryonic Kidney</td>
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<td>hMGFP</td>
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<td>Human Mesenchymal Stem Cell</td>
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<td>HPLC</td>
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<td>HuR</td>
<td>Human Stabilizing Protein</td>
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<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cell</td>
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<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
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<td>Interleukin</td>
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<td>Inducible Nitric Oxide Synthase</td>
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<td>L-Ascorbic Acid</td>
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<td>Lysogeny Broth</td>
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<td>LNA</td>
<td>Locked Nucleic Acid</td>
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<td>Left Ventricle</td>
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<td>mAb</td>
<td>Monoclonal Antibody</td>
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<td>Mhy6:</td>
<td>Alpha Heavy Myosin Chain</td>
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<td>Myocardial Infarction</td>
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<td>Mammalian Target of Rapamycin</td>
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<td>Multi-Vinyl Monomers</td>
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</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Transcription Factor kappa B</td>
</tr>
<tr>
<td>NHE1</td>
<td>Cardiac Na⁺/H⁺ Exchanger</td>
</tr>
<tr>
<td>NMN-A-R2B</td>
<td>N-Methyl-D-Aspartate Receptor Type 2B</td>
</tr>
<tr>
<td>NOD</td>
<td>Non Obese Diabetic</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleocapsid</td>
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<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>P2X7</td>
<td>Purinergic P2X7 Receptor</td>
</tr>
<tr>
<td>P4H</td>
<td>Prolyl-4 Hydroxylase</td>
</tr>
</tbody>
</table>
PACT  Protein Kinase RNA Activator
PAGE  Poly (Acrylamide) Gel Electrophoresis
PBS   Phosphate Buffered Solution
PCL   Poly (ε-Caprolactone)
PCM   Primary Cardiomyocyte
PCR   Polymerase Chain Reaction
PDCD4 Programmed Cell Death-4
PDE5a  Phosphodiesterase 5a
pDMAEMA Poly (Dimethylamino Ethyl Methacrylate)
pDNA  Plasmid DNA
PECAM Platelet Endothelial Cell Adhesion Molecule
PEG   Poly (Ethylene Glycol)
PEGDA Poly (Ethylene Glycol) Diacrylate
PEGM  Poly (Ethylene Glycol) Methyl Ether
PEGMEA Poly (Ethylene Glycol) Methyl Ether Acrylate
PEI   Poly (Ethilenimine)
PGE2  Prostaglandin E2
PLGA  Poly (Lactic-co-Glycolic Acid)
PLK-1  Polo-like Kinase 1
PLL   Poly (L-Lactide)
PMDETA Pentamethyldiethylenetriamine
Pol II Polymerase II
Pre-miRAs Precursor miRs
PVA   Poly (Vinyl Alcohol)
PVDF  Poly Vinlylidene Fluoride
q-PCR Quantitative PCR
RI    Refractive Index
RISC  RNA Induced Silencing Complex
RNA   Ribonucleic Acid
RNAi  RNA Interference
ROS   Reactive Oxygen Species
RSV   Respiratory Syncytial Virus
RT    Real-time
RT    Reverse Transcription
scFv  Single Chain Variable Fragment
SCID  Severe Combined Immunodeficiency
SDS   Sodium Dodecyl Sulphate
SG    Succinimydil Glutarate
SH    Thiol
SH2   Src Homology Domain 2
shRNA Short Hairpin RNA
siRNA  Short Interfering RNA
STAT3 Signal Transducer and Activator of Transcription 3
TAT   Tyrosine Aminotransferase
TBE   Tris-Borate
Tfr   Transferrin Receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>TIPA</td>
<td>Radio Immunoprecipitation Assay</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptors</td>
</tr>
<tr>
<td>TNBSA</td>
<td>2,2,6-Trinitrobenzenesulfonic Acid</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tissue Necrosis Factor alpha</td>
</tr>
<tr>
<td>TR</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TRBP</td>
<td>TAR RNA Binding Protein</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per Volume</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
</tr>
<tr>
<td>Vif</td>
<td>Virion Infectivity Factor</td>
</tr>
<tr>
<td>VS</td>
<td>Vinyl-Sufone</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per Volume</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight to Weight Ratio</td>
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</table>
Abstract
Cardiovascular disease is the leading cause of death in the developed world and is responsible for approximately 36% of Irish mortality. Myocardial infarction (MI), which is literally the death of cardiac tissue due to lack of oxygenation, accounts for the majority of deaths associated with cardiovascular disease. This death of cardiac tissue leads to a loss of cardiac function as the damaged area becomes a non-contractile scar. Amelioration of this process is a main aim of regenerative cardiac strategies such as anti-fibrotic therapies. Thus, anti-fibrotic interfering RNA (RNAi) therapy with exogenous microRNA (miR)-29B was proposed as a method to modulate extracellular matrix (ECM) remodelling following MI. It was hypothesized that miR-29B scaffold delivery will efficiently modulate the ECM remodelling response and reduce maladaptive remodelling, such as aggressive deposition of collagen type I, after injury. The primary objective of this doctoral project was to develop a scaffold-based, controlled release gene therapy system. A co-polymer of a linear poly (dimethylamino) ethyl methacrylate (pDMAEMA) block and a hyperbranched poly (ethylene glycol) methyl ether acrylate (PEGMEA) based unit with poly (ethylene glycol) diacrylate (PEGDA) as the branching agent (pDb/PDA) was synthesised, with the purpose of complexing miRs, using deactivation enhanced atom transfer radical (De-ATRP) synthesis. Non-viral complexes of miR-29B and pDb/PDA were optimized for both monolayer and three-dimensional delivery from a crosslinked collagen-based scaffold in vitro. The release of these complexes from the scaffolds was assessed and their ability to silence collagen type I and collagen type III expression was evaluated. When cardiac fibroblasts were cultured with complex loaded scaffolds, relatively low levels of collagen type I and collagen type III mRNA expression were observed for up to two weeks of culture. When scaffolds loaded with miR-29B or miR-29B complexes were applied to a rat excisional wound model, reduced wound contraction, improved collagen type III/I ratios and a significantly higher MMP-8: TIMP-1 ratio were detected. From these investigations it was concluded miR-29B functionalization of the scaffold significantly increased the ratio of collagen type III/I and MMP-8/TIMP-1 ratios. Furthermore, these effects were not improved through the use of pDb/PDA, and were significantly influenced by the dose of miR-29B in the collagen scaffold (0.5 μg versus 5 μg). This is the first study to describe a biomaterial scaffold combined exogenous miRs capable of improving ECM remodelling following injury. There is significant potential for further development of this platform which could ultimately represent a step towards the realization of true cardiac regeneration.
Chapter One

Literature Review

The majority of this chapter has been previously published in


1.1 Introduction

Tissue engineering is the use of a combination of biomaterial scaffolds, cells, and engineering, with suitable biochemical and physio-chemical factors to improve or replace biological functions \(^1\). Scaffolds employed in tissue engineering act as platforms that can recapitulate the *in vivo* milieu, allowing cells to influence their own microenvironments and/or retain cells and biochemical factors. These scaffolds can provide structural support, porosity to enable cellular infiltration, and possible release of embedded biomolecules and cues for the surrounding tissues to regenerate. Scaffolds can be further ameliorated by the incorporation of cells to replace those that have been lost or to offer paracrine support to those that are compromised at the site of delivery \(^2\). Biomaterial-based scaffolds have played a central role in regenerative medicine and tissue engineering and several key requirements for scaffolds have been identified. Scaffolds fabricated from a range of natural and synthetic materials are desired to be biodegradable to obviate the need for a removal procedure. In tandem, predictable biodegradation of the scaffold can facilitate controlled release of biomolecules embedded within the scaffold. The degradation time of the scaffold should ideally mirror the time necessary for tissue regeneration. Degradation of the biomaterial can create a path for tissue ingrowth and this can be further facilitated by a highly porous scaffold which can also allow for the initial transport of oxygen and nutrients, as well as for the removal of metabolic waste and degradation products.

The current paradigm of tissue engineering incorporates the use of biomolecules, which can be growth factors, pharmaceutical agents, or mediators of nucleic acid therapy. Therefore it is intuitive that scaffolds act as reservoirs in the delivery of nucleic acid such as interfering RNA (RNAi) (see Figure 1.2). RNAi delivery from a scaffold enables localized treatment, as the scaffold, acting as a reservoir of RNAi, facilitates delivery of this therapeutic molecule in comparison to delivery via a systemic approach \(^3,^4\). Targeting a cell population or anatomical location by injection or systemic delivery is complex and poses many challenges; direct delivery of a therapy from a scaffold, however, can surmount these barriers. Cells surrounding a scaffold are considered for targeting and become exposed to the therapeutic agent limiting unwanted exposure in other areas. Additionally, scaffold-based delivery has the potential to maintain effective levels of payload and nucleotide bioactivity for extended periods which broadens the opportunity for cellular uptake and increases the likelihood of nucleic acid transfer. Delivery from the majority of biomaterial systems most likely occurs by means of a combination of payload interaction with the matrix and subsequent release, with the payload and material designed to regulate these interactions.
Another advantage of a scaffold is its role in the protection of its payload from attack by immune responses, and limitation of degradation by serum nucleases or proteases. The use of scaffolds in the delivery of nucleic acid therapy has been extensively reviewed previously \(^4\)\(^-\)\(^5\); and the basic criteria, concepts and interactions between scaffolds and their therapeutic loads do not change in the context of RNAi delivery.

1.2 RNA Interference

RNAi is a fundamental pathway in eukaryotic cells by which sequence-specific double stranded RNA targets, binds to, and inhibits the translation of a complementary mRNA. Many similar RNAi mechanisms exist which pursue distinct pathways inside eukaryotic cells. In general, however, most pathways terminate at a common end; in that they inhibit the translation of messenger RNA (mRNA) (see Figure 1.1). Synthetic approaches employing antisense oligonucleotides have evolved to double stranded RNAs (dsRNAs) following seminal discoveries by Fire et al. in 2001 \(^6\), illustrating a ten-fold improvement in silencing target genes when compared to a single stranded RNA alone. These exogenous molecules manipulate an existing regulatory pathway: that of microRNA (miR).

The miR pathway begins in the nucleus with transcription of long primary miR (pri-miRs) by RNA polymerase II (Pol II) \(^7\) and subsequent processing into ~70 nucleotide (nt) stem-loop structures termed ‘precursor miRs’ (pre—miRs) by the RNase III enzyme Drosha which functions with the dsRNA binding protein of DiGeorge syndrome critical region 8 (DGCR8) \(^8\). Exportin-5, another dsRNA- binding protein, chaperones these pre-miRs to the cytoplasm in a Ran-GTP-dependent manner. Once in the cytoplasm; Dicer, an endoribonuclease in the RNAse III family, and its associated dsRNA-binding protein partners, HIV-1 and the TAR RNA binding protein (TRBP) \(^9\), cleave pre-miR to a mature miR duplex ~22 nt long and load this mature miR into a RNA induced silencing complex (RISC) \(^10\),\(^11\). miR interaction with RISC involves cleavage of the miR passenger strand by Ago2 although imperfect sequence complementarities between the mature miR strand and its complementary passenger strand may inhibit Ago2 from cleaving the passenger strand \(^12\). Alternatively, this interface employs a circumventing mechanism using helicase activity to unwind and remove the passenger strand. Regardless of the mechanism, the removal of passenger strand enables the binding of the mature miR to its target mRNA 3’ UTR. Gene silencing is then catalysed by RISC through translational repression and consequent mRNA degradation.

The seed sequence of a mature miR encompasses the first 2 - 7 or 2 - 2 nucleotides from its 5’ end and needs to have full complementarity with its target to enable RNAi, whereas imperfect matches in the 3’ end of the miR strand are tolerable \(^13\). As a result, a single miR can regulate multiple cellular
targets and, conversely, each gene can be controlled by many miRs, suggesting an elaborate and multifaceted mechanism of gene regulation. Numerous hypotheses toward the effects of imperfect binding of a miR to its target mRNA and its effect on gene silencing have been put forward, including interruption of translation at the initiation or elongation step, co-translational degradation, transportation to cytoplasmic processing bodies (P-bodies), and/or de-capping or deadenylation\textsuperscript{14-16}. On the other hand, when complete sequence complementarities occur; silencing is achieved via cleavage of the mRNA through RISC activity\textsuperscript{17}. In both cases, miR targeting and binding results in post-transcriptional silencing of the target gene.

Appreciation of endogenous RNAi pathways has enabled improvement in synthetic strategies to silence gene expression. One approach is to, albeit stably or transiently using plasmid DNA (pDNA) encoding the expression of short hairpin RNA (shRNA)\textsuperscript{18-20} or delivery to the cytosol of shRNA/siRNA and dsRNA. The utilization of either approach needs to account for the stability, the therapeutic target and the efficacy of delivery. Efficacy of shRNA-expressing pDNA requires entry into the cell, endosomal escape and subsequent entry to the nucleus via a nuclear pore, as in the case of non-dividing cells\textsuperscript{21,22} or nuclear uptake during cell division\textsuperscript{23}. An alternative approach is to deliver dsRNAs exogenously which become cleaved in the cytoplasm by Drosha to siRNA and subsequently loaded into the Argonaut/RISC complex. Therefore, dsRNAs are advantageous in delivery compared with shRNA-expressing pDNA as they obviate the necessity for nuclear entry and can therefore enable a more efficient process of RNAi. Silencing gene expression using long dsRNAs has been demonstrated in an array of organisms including plants and drosophila. However, in mammalian cells, it has been observed that dsRNAs longer than \(\sim30\) nt elicit an antiviral associated interferon response which ultimately results in non-specific suppression of gene expression through activation of RNase L and general degradation of RNA molecules\textsuperscript{24}. This response can be evaded however, by the design of the dsRNA. Elbashir \textit{et al.} observed that smaller dsRNAs, less than 30 nt are capable of bypassing the mammalian immune response while inducing RNA interference to achieve specific gene silencing\textsuperscript{25}. This realisation progressed to the hypothesis that synthetic dsRNAs of very short length (siRNAs) with perfect homology to the mRNA of a target gene can be delivered as an alternative mediator of gene silencing. However, early innate immuno-stimulatory activity with these synthetic molecules remains a concern. siRNAs containing certain nucleotide sequence motifs (GU) can stimulate toll-like receptors (TLRs) in the endosomal pathway. Obviation of this is desirable because if it is not carefully avoided, siRNAs capable of triggering immuno-stimulatory activity\textsuperscript{26}. The antigenic potential of siRNAs can occur via TLR-3, and has been reported as a possible safety concern\textsuperscript{27}. This has brought scrutiny to some studies where anti-angiogenic specific siRNAs have been reported as being successful as other pre-clinical studies.
have concluded that non-specific siRNAs also induce an anti-angiogenic effect due to interaction with TLRs, and engaging the TLR3/interleukin pathway.\(^\text{28}\)

The exogenous introduction of short siRNAs with appropriate length and 2-nt 3’ overhang can be loaded directly onto RISC for RNAi function, obviating interaction with Dicer, TRBP or protein kinase RNA activator (PACT); however, the loading process is ten times less efficient than that of shRNA or longer dsRNAs. Increasing the length of the siRNA duplex to 29-30 nt with a 2-nt 3’ overhang only at the antisense end of the duplex appears to improve efficacy.\(^\text{29}\) Dicer processing before RISC loading can increase the potency of post-transcriptional gene silencing and can be achieved by using longer chemically synthesized siRNAs (27 nt) and shRNAs (28 nt)\(^\text{29,30}\). It is likely that Dicer and TRBP-PACT present an effective basis for RISC formation and shuttle their cleaved products directly to RISC\(^\text{12,31}\). Employing this loading stem in exogenous RNAi therapies can elicit a more potent gene-silencing effect through the use of these Dicer substrates. Twenty seven nt dsRNAs are intended to be asymmetrical, with one 2-nt 3’ overhang and one blunt end. A single stranded siRNA results from Dicer processing due to Dicer recognition of the 2-nt 3’ overhang for processing. However, the blunt end, which includes DNA bases, might trigger low levels of interferon induction; but the potency of this longer dsRNA means that lower concentrations of 27 mers are required to silence gene expression which may avoid or minimise such an interferon response.\(^\text{32}\) Furthermore, it is possible that increasing the length of a siRNA duplex with an unprocessed end dictates directionality as a result of imposed thermodynamic instability determining the guide strand shape and thereby enhancing its association with Dicer/TRBP/PACT complex for more efficient loading onto RISC. shRNA on the other hand, assimilates into the endogenous miR pathway and in doing so is significantly more efficient.\(^\text{33,34}\)

### 1.3 Non-viral Delivery

Current clinical applications of RNAi are focused on using previously mentioned 21–nt siRNA duplexes with 2-nt 3’ overhangs for Dicer recognition which are suitable for large-scale synthesis that enables uniform production of these chemically synthesized molecules. The clinical relevance of siRNA is supported by its ability to transfect relatively non-proliferating cells in which the nuclear entry of shRNA-expressing pDNA is limited. For example, successful target gene knockdown in cells such as bone marrow-derived dendritic cells\(^\text{35}\) and primary T lymphocytes\(^\text{36}\), for which transfection with pDNA is difficult due to low permeability in their nuclear envelope has been reported with siRNA. For a quantitative comparison of the duration of effect of siRNA and shRNA-expressing pDNA, McAnuff \textit{et al.} compared the potency of siRNA and shRNA-expressing pDNA mediated gene-silencing \textit{in vivo} by co-administration of siRNA or shRNA-expressing pDNA with the pDNA.
Figure 1.1: Schematic illustrating non-viral RNAi delivery mechanisms. Red arrows show the mechanism of siRNA and miR mimics entering the cell, uptake and activation by RISC, binding to target mRNA and inhibiting translation. Blue arrows indicate the delivery of pDNA encoding shRNA, which must enter the nucleus to transcribe shRNA from the nucleus. shRNA becomes cleaved by DICER, uploaded by RISC and terminates at the same endpoint as the red arrow pathway. Finally, black arrows indicate the delivery of antagomiRs, designed to bind to endogenous miRs and inhibit them from binding to their target mRNAs.

encoding a target reporter gene. The extent of the reduction in the target gene expression was comparable to that between siRNA and shRNA-expressing pDNA at a 10 mg dose; however on a molar basis, the shRNA was 250 fold more effective than the siRNA, at day one and day three after administration. A concern in this study was that the expression of the reporter gene was transient, and therefore it was difficult to conclude whether these compounds were effective and, furthermore, were comparable with each other for longer than three days. pDNA transient transfection of cells in vitro exhibits gene silencing at day three and day five in rapidly dividing cell lines and using naked siRNAs achieves transient gene knockdown for at day three and day seven due to dilution of the siRNAs below a therapeutic level with repeated cell division. In quiescent or non-dividing cells, the silencing effect can remain at three weeks after which the siRNAs are hypothesized to have been naturally degraded. In vivo, a similar trend was observed using rapidly dividing subcutaneous tumours vs. non-dividing hepatocytes.

A significant hindrance to the progression of siRNA therapies is delivery of these biomolecules to the desired cell type, tissue or organ, because their negative charge and size prevents passive endocytosis across the cellular membrane. Furthermore, their progress has been obstructed due to their rapid clearance and lack of target tissue specificity. It is evident from the plethora of research reported on the delivery of gene encoding pDNA by non-viral methods; overlapping and additional considerations are required when introducing synthetic RNAs or RNA encoding plasmid DNA to eukaryotic cells. Plasmid DNA is often several kilo base pairs long, and possesses molecular characteristics that allow it to be condensed into particles, from submicron up to just a few microns in size, by complexation with a cationic agent. This condensation is driven for the most part by electrostatic attraction and ensures that pDNA is protected against enzymatic degradation as it becomes entirely encapsulated by the cationic agent. Furthermore, the condensing of large pDNA constructs enables a favourable size for cellular uptake via the endosomal pathway. However, RNA and DNA are different types of nucleic structures and their physico-chemical properties are determinants of their ability to complex.

The persistence length of siRNA and pDNA is an important consideration in the delivery of these molecules. This is the length over which chains of nucleic acid base pairs act as individual rigid elements. dsDNA has a persistence length of about 50 nm, whereas dsRNA is ~70 nm which lends RNA to be a stiffer molecule. Translating this to a nucleotide (nt) scale, the persistence length of RNA is about 260 nt. This suggests that a 21 nt siRNA does not condense further and, that when improperly fractioned with cationic agents, will form inefficient large complexes or insufficient encapsulation of the siRNA. The maximum size for clatherin mediated cellular uptake is 150 nm, and particles larger than this do not diffuse passively across the cell membrane. In fact, with respect
to siRNA complexes, it has been shown that particles of siRNA complexed with poly(ethylenimine) (PEI) greater than 150 nm in size are unable to cause gene silencing \textit{in vitro}.

RNAi can be therapeutically viable with the use of a non-viral vector by prolonging the serum half-life and intracellular buffering of siRNA by improving pharmacokinetics and nuclease resistance. For example, it has been reported that chemically modified siRNAs encapsulated within a lipid core have extended serum half-life (6.5h) compared to those without protection (0.8h). Non-viral vectors available for siRNA/pDNA delivery, such as cationic polymers, lipid based approaches, nanoparticle approaches or biomolecular chaperoning as these areas have been covered in exhaustive reviews; and key developments in pre-clinical and clinical studies will be elaborated in the next section.

1.4 RNAi as a Therapeutic

The use of RNAi has undergone a rapid transition to pre-clinical studies due to advances made previously in non-viral nucleic acid delivery and the current understanding of cellular pathways during states of pathogenesis, and due to the availability of a wide range of \textit{in vivo} models. Although clinical trials are in progress, the number of these trials is relatively few and almost all of these trials employ naked or slightly modified siRNAs to deliver RNAi.

The most advanced clinical trials are in the treatment of age-related macular degeneration (AMD) which is a leading cause of blindness. These trials involve the injection of naked siRNA into the macula of the eye, targeted to genes for vascular endothelial growth factor (VEGF-A) and the VEGF receptor (VEGFR), and have shown some therapeutic potential in their inhibition of excessive vascularization of the eye that leads to AMD. The first clinical trial involving siRNA was carried out by Acuity Pharmaceuticals Inc. for the treatment of AMD, for which phase I results have been completed. The completed phase II trials reported tolerated doses with a lack of adverse systemic effects. Testing had advanced into phase III trials, when Acuity Pharmaceuticals Inc. was taken over by OPKO Health Inc.; however, the company decided to terminate its phase III clinical study of bevasiranib (a naked siRNA) for the treatment of AMD. No systemic safety issues were identified and local ocular risks were generally considered ‘unremarkable’ by the company. However, a careful review of the data by the Independent Data Monitoring Committee (IDMC) concluded that the trial, as structured, was unlikely to meet a significant beneficial primary end point.

Allergen Inc. is conducting a phase II clinical trial on a siRNA therapy for AMD, with completed phase I results indicating minimal side effects and improved vision in some of the patients. In the phase I clinical trial targeting VEGFR-1 in patients with choroidal neovascularisation resulting from
AMD; mild to moderate adverse effects and an adjusted mean foveal thickness within two weeks after the study treatment was reported. However, the trial co-ordinators, Allergen Inc., halted development of AGN211745, a chemically modified siRNA, after the drug failed to meet a key efficacy endpoint in a phase II study. No safety issues were associated with AGN211745 targeting VEGF-1; but as the drug did ‘not meet its efficacy hurdle’, its development was halted. Silence Therapeutics Plc. also had a siRNA product to treat AMD in development, but have now refocused their phase II clinical study toward the treatment of diabetic macular edema, which is another complication caused by leaky vasculature within the eye.

The anti-angiogenic effect reported in AMD clinical trials has been called into question by a study conducted by Kleinman et al. Here, the authors reported that the efficiency of siRNA targeting VEGF in the eye, in patients with choroidal neovascularisation resulting from AMD, is not due to specific gene silencing, but is instead caused by non-specific stimulation of the TLR-3 pathway which can reduce angiogenesis. This raises concern over the nature of the anti-angiogenic effects reported in other AMD clinical trials; however, it should not compromise therapeutic effects described when other mechanisms (unrelated to angiogenesis) are targeted. Clinical evidence for the effectiveness of RNAi as a therapeutic approach for AMD is questionable; however there are some recent publications showing promising results in addressing other therapeutic targets which are summarized in Table 1.

For instance, in a recent phase II randomized, double-blind placebo-controlled study of ALN-TSV01, a siRNA directed against mRNA of the respiratory syncytial virus (RSV) nucleocapsid (NP) protein was administered daily via a nasal spray to subjects two days before and three days after inoculation with RSV. It was reported that independent of other factors, including pre-existing immunity to the virus or secondary inflammation, the RSV-NP siRNA resulted in a 38% decrease in the number of culture-defined RSV infected patients.

Progress of a phase I clinical trial in systemically delivering targeted siRNA nanoparticles toward metastatic melanoma have been documented by Davis et al. This platform consisted of a cyclodextrin-based polymer (CDP) particle, loaded with a siRNA for silencing expression of RRM2, decorated with PEG to improve stability for systemic delivery, with a human transferrin protein (Tf) targeting ligand displayed on the exterior to engage TF receptors on the surface of cancer cells. Tumour biopsies showed localized presence of nanoparticles delivered systemically, and furthermore, a reduction was found in both the specific mRNA and RRM2 protein levels when compared with predosing tissue. This is the first study that illustrated the localization of systemically delivered nanoparticles and, furthermore, is the first clinical trial to use a biomaterial approach in the delivery of
Table 1.1: Selected examples of pre-clinical studies with non-viral delivery of siRNAs

<table>
<thead>
<tr>
<th>Mode of delivery</th>
<th>Application/disease</th>
<th>Target</th>
<th>Observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid/Liposomal</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Liposomal (i-FECT™)</td>
<td>Japanese encephalitis virus, West Nile virus</td>
<td>Viral envelope gene</td>
<td>Protection against both viruses when administered</td>
<td>54</td>
</tr>
<tr>
<td>Lipofectamine™</td>
<td>Ocular neovascularisation</td>
<td>VEGF-A</td>
<td>Severity of neoangiogenesis was reduced</td>
<td>55</td>
</tr>
<tr>
<td>Lipofectamine 2000™ in Agarose matrix</td>
<td>Wound healing</td>
<td>Mapk-1 and lamin A/C</td>
<td>Target proteins, and their gene expression were silenced at days 14 and 21(Mapk-1 and lamin A/C)</td>
<td>56</td>
</tr>
<tr>
<td>LipoTrust™</td>
<td>Liver cirrhosis</td>
<td>Gp46</td>
<td>Liver fibrosis was reversed in experimental models</td>
<td>57</td>
</tr>
<tr>
<td>PEGylated cationic lipid particles</td>
<td>Ovarian cancer</td>
<td>Claudin-3</td>
<td>Reduced tumour size and tumor burden when siRNA silencing claudin-3 was administered</td>
<td>58</td>
</tr>
<tr>
<td>Lipofectamine 2000™</td>
<td>Hepatitis C</td>
<td>GB virus-B</td>
<td>Dose dependent repression was achieved</td>
<td>59</td>
</tr>
<tr>
<td>Oligofectamine™</td>
<td>Post-ischemic cerebral inflammation</td>
<td>STAT3</td>
<td>Silencing of target mRNA and reduction in volume infarct</td>
<td>60</td>
</tr>
<tr>
<td>Cationic Polymers</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PEI</td>
<td>Formalin induced pain</td>
<td>NMDA-R2B</td>
<td>Target mRNA levels were reduced and behavioural pain was minimized</td>
<td>61</td>
</tr>
<tr>
<td>PEGylated PEI micelles</td>
<td>Prostate cancer</td>
<td>VEGF_{165}</td>
<td>Enhanced uptake in tumour tissue and inhibition of VEGF expression</td>
<td>62</td>
</tr>
<tr>
<td>β₁,3-D-glucan/PEI particles</td>
<td>Systemic inflammation</td>
<td>TNF-α</td>
<td>Systemic reduction of TNF-α mRNA levels was reduced and serum levels of TNF-α</td>
<td>63</td>
</tr>
<tr>
<td>PEI</td>
<td>Post-operative abdomino-plevic adhesions</td>
<td>HIF-1α</td>
<td>Complexed siRNA (with PEI) had a significant reduction of abdominal adhesion</td>
<td>64</td>
</tr>
<tr>
<td>siRNA Conjugates</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Anti-CD7 antibody</td>
<td>HIV</td>
<td>Vif, TAT, CCR5</td>
<td>Suppression of endogenous virus and maintenance of CD4 cell counts</td>
<td>65</td>
</tr>
<tr>
<td>Aptamer</td>
<td>Pancreatic cancer</td>
<td>PLK-1</td>
<td>Reduction in tumour size was observed in a mouse model</td>
<td>66</td>
</tr>
</tbody>
</table>

Abbreviations: VEGF: vascular endothelial growth factor; Mapk-1: Mitogen-activated protein kinase 1; GP: glycoprotein; STAT3: Signal transducer and activator of transcription 3; NMDA-R2B: N-methyl-D-aspartate receptor type 2B; TNF-α: tissue necrosis factor α; CCR5: C-C chemokine receptor type 5; PLK-1: Polo-like kinase 1; Vif: virion infectivity factor; TAT: tyrosine aminotransferase.
RNAi. Currently, the use of non-viral vector systems is being employed to improve the efficacy of siRNA delivery and to improve targeting to tissues of interest. Table 1.1 summarizes selected examples in pre-clinical trials to date. Overall, while clinical trials have been conducted, none of these trials have demonstrated the full efficacy of this therapy. As previously discussed, most trials are in their formative stages, while others have been terminated or discontinued. It becomes imperative; therefore, that optimisation of delivery, together with careful selection of target gene, is addressed before translation to the clinic.

1.5 RNAi Delivery through Scaffolds

Many biomaterials used for controlled drug delivery can also be used to fabricate scaffolds. Release from the scaffold occurs by combinatorial processes of polymer degradation and vector diffusion. A key requirement associated with the encapsulation of RNAi and non-viral vectors (if any) is that the methods employed to fabricate the scaffold is compatible with the integrity of the biomolecules and their vectors. Methods can involve high temperatures, organic solvents and the generation of free radicals or shear stresses that may damage the payload. Even if the nucleic acid (pDNA or RNA) is stably encapsulated, the nucleic acid can still be damaged by the degradation products of a vector. The release kinetics of encapsulated RNAi from tissue engineering scaffolds is also reliant upon a number of factors such as the concentration of the scaffold, its degree of cross-linking, and the scaffold material which can make its degradation responsive to pH, temperature, cellular enzymatic products and/or hydrolysis. This degradation rate can influence the time-course release of the embedded RNAi as the scaffold loses volume and its products become metabolised by surrounding tissue. Furthermore, charge interaction between the scaffold material and the RNAi which can be further encapsulated using a delivery vector can affect the release

Although the advent of RNAi based-therapy is relatively recent, there have; nevertheless, been numerous efforts to deliver this therapy via scaffolds and hierarchical structures. Several polymeric materials are utilized in this field; and for convenience, both natural and synthetic polymer based scaffold will be discussed, which are also summarized in Table 1.2. Furthermore, these materials can be manipulated to obtain a wide range of biomaterials with different structural properties and function-based morphologies; and therefore this discussion will explore the various types of scaffold attainable.

1.5.1 Hydrogels

Hydrogels are formed by crosslinking or self-assembly of a variety of natural or synthetic hydrophilic
Figure 1.2: Schematic of scaffold-mediated delivery of RNAi. A: scaffold implanted at a target site, B: scaffold loaded with non-viral RNAi vectors and C: degradation of scaffold enabling cell ingrowth and release of therapeutic RNAi.
polymers to produce structures that contain over 90% water. Many natural materials, such as collagen or fibrin, have been widely used as matrix components for the formulation of hydrogels. These hydrogels have been used in tissue engineering applications due to their innate cellular interactions and cell-mediated degradation. Synthetic materials, such as poly (ethylene glycol) (PEG), lack intrinsic cellular signals yet allow for greater manipulation of the hydrogel properties relative to natural materials. Hydrogels can be fabricated ex vivo for subsequent implantation, or can be formed in situ allowing for a less-invasive approach via catheter delivery. In addition, they can act as a delivery vehicle for implanted cells and many studies have encapsulated cells within hydrogels prior to gelation with minimal effects on cell viability. Degradation of hydrogels can be tailored to occur through a variety of mechanisms including digestion by cell secreted enzymes and hydrolysis, and can be manipulated by the size of the interconnecting pore network within the hydrogel, ion exchange, and/or the strength of crosslinking interactions. Therapeutic release from a hydrogel is dependent upon the physical structure of the hydrogel, its degradation, and interactions between the matrix of the hydrogel, nucleic acids and/or non-viral vector. In general, hydrogels produce high encapsulation efficiencies with release occurring through either diffusion alone (non-biodegradable hydrogels), or through a combination of hydrogel degradation and payload diffusion.

The use of hydrogels as a reservoir of RNAi has been illustrated in many studies due to their potential as an injectable therapeutic. Their use in this respect has been demonstrated by Krebs et al. who employed collagen hydrogels, photo-crosslinked alginate hydrogels, and calcium crosslinked alginate hydrogels as reservoirs of siRNA to silence GFP expression. The silencing efficacy of siRNA released into supernatant and applied to cells stably expressing GFP and the silencing effect on these cells stably expressing GFP embedded within the hydrogels was investigated. A point to note in this particular study is that serum deprivation conditions were employed which does limit the translation of this technology to a pre-clinical setting if the objective is to deliver RNAi in vivo. Regardless, when the hydrogels were loaded with naked siRNA, they offered maintenance of its bioactivity even after six days. The authors reported that silencing of the reporter gene was even more pronounced at six days than at three days, with greater than 80% silencing. An additional investigation of this work was complexing the siRNA with PEI and chitosan to offer better protection in vivo and also to delay its release from the hydrogel due to charge interaction of the positively charged PEI/chitosan with negatively charged alginate. However, the remaining bioactivity of this siRNA was not determined. Cells embedded within the hydrogels also had prolonged silencing of GFP expression at six days, particularly when embedded in a collagen hydrogel. This study demonstrated the efficacy of loading siRNA into hydrogel reservoirs to enable a prolonged silencing and also elucidated the electrostatic effect of using a vector and siRNA and embedding within a hydrogel. The influence of the
electrostatic charge between PEI and chitosan charged siRNA complexes mirrored that of hydrogel mediated delivery of pDNA complexes \(^7_2\), showing that through this parameter there is further control over the release rate of a therapeutic payload.

Combinatorial approaches in delivering both pDNA to transduce the expression of a gene of interest while silencing that of another using RNAi is an approach that is gaining popularity as knowledge of cellular events accumulates. A combinatorial scaffold approach has been reported by Singh \textit{et al.} albeit where the pDNA was also delivered as a reporter of siRNA gene silencing \(^7_3\). In their study, hydrogels were constructed of Dextran vinyl-sulfone (Dextran-VS) crosslinked with tetra-functional PEG thiol (PEG4SH), and PEG diacrylate, also crosslinked with PEG4SH. These hydrogel scaffolds were impregnated with a chemoattractant biomolecule; MIP3α, and within the hydrogel; poly (lactic-co-glycolic acid) (PLGA) microparticles encapsulating siRNA against interleukin10 (IL-10) and surface-coated with pDNA-PEI complexes, were embedded. Although this study also focused on the chemoattractant potential of the scaffold, examination of the gene silencing ability of this platform using RT-PCR to analyse gene expression of IL-10 found that primary antigen expressing cells (APCs) had up to 80% silencing with 10% (w/v) crosslinked dextran hydrogels five days post transfection which was comparable to microparticles alone. Furthermore, a trend was observed in that silencing of IL-10 was reduced when the concentration of the dextran hydrogel was increased (20 and 30%) indicating that the release and permeability kinetics of the hydrogel affects the uptake of siRNA.

\subsection*{1.5.2 Sponges}

The use of a sponge-based construct to deliver siRNA has been reported. Crosslinked collagen sponges as reservoirs of siRNA complexes to silence SNAIL1 expression have been studied \(^7_4\) and these platforms have previously been used as reservoirs of pDNA \(^7_5\). A crosslinked collagen sponge served as a reservoir of complexed siRNA directed toward the silencing of SNAIL1 with a cumulative release of 65% at day seven. Although no significant effect was observed at day one and two when seeded with 3T3 murine fibroblasts, at seven days a significant reduction in SNAIL1 expression was observed in cells incubated with release supernatants from anti-SNAIL1 siRNA loaded scaffolds when compared to negative control siRNA loaded scaffolds. Again, similar to effects observed with hydrogels, sponge-based scaffolds were shown to prolong the silencing effect of siRNA whilst acting as a support for cellular attachment and growth.

Although most of the efforts discussed have reported down-regulating reporter genes or a certain gene of interest, recent reports in the field have illustrated the use of siRNA loaded matrices in directing stem cell differentiation. Andersen \textit{et al.} reported that poly (e-caprolactone) (PCL) scaffolds
functionalized with a siRNA nano-particulate can deliver a range of target siRNAs to enhance differentiation of hMSCs in vitro and in vivo and create a spatio-temporal pattern to generate areas of defined differentiation. Previously, the authors had coated surfaces with lyophilized siRNA complexed with TransIT-TKO™, a commercial, lipid based delivery vector. In vitro, delivering a lyophilized polymer/lipid-based nano-particulate with EGFP silencing siRNA, embedded within a structured PCL scaffold, to cells stably expressing EGFP, achieved up to 60% silencing when compared to non-functionalized scaffolds. Furthermore, delivery of either BCL2L2 or TRIB2 silencing siRNAs from the structured PCL scaffolds, reduced expression of these genes by 41% and 51% respectively, after 72 hours, which was quantified using q-PCR. The silencing of BCL2L2 and TRIB2 was postulated to direct osteogenic and adipogenic differentiation of hMSCs on scaffolds, which had been reported in previous studies using monolayer cultures. The researchers found, that after seven days in adipogenic differentiation media, hMSCs on scaffolds with TRIB2 siRNA had significantly increased expression of adipogenic markers when compared with controls. Similarly, when hMSCs were subjected to osteogenic differentiation, scaffolds coated with BCL2L2 siRNA particles had the greatest expression of osteogenic markers at day seven. The authors then translated this platform of a PCL scaffold loaded with target siRNAs (TRIB2 and BCL2L2) and non-targeting siRNA nanoparticles, seeded with hMSCs, into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. The in vivo results demonstrated that TRIB2 and BCL2L2 targeted siRNA initiated adipogenic and osteogenic differentiated respectively but could not drive terminal differentiation as a sole differentiation cue.

Similar results were observed in an in vitro experiment in which the authors reported that the TRIB2 siRNA- coated scaffold stained positive for markers of adipogenic differentiation and BCL2L2 siRNA scaffolds strongly exhibited markers for osteogenic differentiation compared to controls. Gradient controlled differentiation was also controlled using this scaffold both in vitro and in vivo. In vitro hMSCs were seeded on a scaffold containing TRIB2 siRNA on one side and BCL2L2 siRNA on the other half. Measurement of mRNA levels elucidated that the expression of one adipogenic marker (AP2) was four-fold upregulated in the TRIB2 siRNA-coated part, and ALP was 45% upregulated in the BCL2L2 siRNA coated part. In vivo, staining showed that the cells could still be induced to commit to alternative differentiation (osteogenic and adipogenic) in specific locations within the same implant by placing the different siRNAs in distinct locations.

1.5.3 Fibers

Choice of scaffold morphology and architecture for a specific clinical target is dictated by the end application, desired release, and mimicry of the native tissue in order to guide neighbouring cell
infiltration and differentiation. Hence researchers in the field have developed various constructs with varying architecture and form.

One such study employed PCL nano-fibers as reservoirs of both naked and complexed siRNA against glyceraldehydes 3 – phosphate dehydrogenase (GADPH) some of which were PEGylated. Up to 80% silencing of GADPH was observed when release supernatants were added to HEK cells up to 30 days post incubation. When cells were directly seeded on scaffolds impregnated with naked siRNA and siRNA complexed with a commercial transfection reagent (TransIT-TKO™), GAPDH silencing was achieved with naked siRNA but to an average of ~20% efficiency; whereas the impregnation of complexed siRNA impregnated scaffolds attained a silencing efficiency of ~75% and comparable with a bolus delivery of complexed siRNA after 96 hours. Although this study realises the concept that siRNA delivery via a reservoir matrix can be improved using a complexing agent, the full extent to its use as a therapeutic has not been demonstrated due to the lack of time points and duration of the study during which the cells were seeded on the nano-fiber scaffolds.

1.5.4 Microspheres

Nucleic acids (such as RNAi) can be introduced into microspheres during the fabrication process in which a water-in-oil-in-water multiple emulsion/solvent evaporation or extraction technique is employed. Preliminary studies have shown that the molecule size and structure (bases etc.), the dosage of loading and the size of microspheres, greatly affects the encapsulation efficiency and release properties. Higher loading is generally associated with a larger size, but is independent of oligonucleotide length.

The in vitro release profile of antisense oligonucleotides from microspheres has been shown to be affected by the size of the spheres, drug loading, oligonucleotide length and molecular weight of the polymer. Small microspheres (1-2 μm) fabricated from PLGA have been reported to release most entrapped oligonucleotide within four days, compared with 40 days for larger spheres (10- 20 μm). In the same report a triphasic release was observed with larger PLGA spheres: a burst release in the first 48 hours, attributed to the rapid release of the oligonucleotides present on or close to the microsphere surface; a sustained release phase (about 25 days) in which oligonucleotide diffusion through pores present in the microsphere’s shell material occurred; and a final more rapid release phase (up to a complete release at about 60 days) due to degradation of the polymer that accelerated oligonucleotide release. In the case of smaller spheres, increased surface area to volume ratio results in a greater concentration of oligonucleotides at or near to the surface with subsequent rapid release. Another factor to consider is the mass of oligonucleotides loaded into microspheres as there have been reports that an increase in nucleotide loading results in a higher burst effect and a more rapid release.
rate in PEG/PLGA blend microspheres. The release profile is only slightly dependent on nucleic acid length, with a release rate that increases as the nucleic acid molecular weight decreases. Finally, a high burst effect and a rapid in vitro release rate have been attributed to microsphere porosity. Pores on the microsphere surface occur when an osmotically active agent is added to the internal aqueous phase. The use of an osmotic agent, such as NaCl, into the external aqueous phase allows one to reduce microsphere surface porosity, thus reducing the initial burst effect and decelerating in vitro release rate.

One study has reported the encapsulation of double stranded oligonucleotides within PLGA microspheres. In this study, microspheres encapsulating a decoy dsRNA against the transcription factor-κB (NF-κB) showed a negligible burst effect and had exhibited a gradual release profile, with complete release after about 40 days. Khan et al. have encapsulated double-stranded siRNA into PLGA microspheres and found that in vitro the release of siRNA was much slower and had a very limited burst effect compared with that obtained with a single-stranded RNA. The difference between the release profiles was ascribed to the different chemical nature of the two nucleic acids, while only a modest effect was attributed to variation in molecular weight. Increase in oligonucleotide encapsulation within PLGA or poly (L-lactide) (PLL) microspheres, as well as optimization of their in vitro release properties, has been achieved by using cationic additives which were able to complex the nucleic acid within the matrix. Rather than releasing siRNA all at once, the microspheres protect against serum nucleases and release a slow continual supply of bioactive siRNA to cells, sustaining RNAi.

### 1.5.5 Surface Coatings

Surface functionalization is another approach in reservoir delivery for RNAi and offers much versatility in translation to current implants and scaffolds being used clinically. Zhang et al. have delivered shRNA encoding pDNA via a multilayer reservoir of pDNA/calcium phosphate scaffolds. Briefly, polyelectrolyte multilayered films were prepared on glass cover slips, and alternating layers of poly (L-lysine) and nanoparticles were built up by alternated immersions. The use of one and six alternate layers was investigated with shRNA for the silencing of osteopontin expression and another for the silencing of osteocalcin expression in vitro. Employing both RT-PCR and immunocytochemistry the authors reported that qualitatively, the expression of osteopontin and osteocalcin had greater silencing with six alternate layers in the platform than it had with one layer. Furthermore, the overall use of a multilayer reservoir, silenced osteopontin and osteocalcin compared to negative controls and free dissolved shRNA pDNA.
Many studies have employed biomaterial coatings harnessing RNAi to improve their performance or to elicit a therapeutic response. Takahashi *et al.* have reported a PEG methyl ether (PEGM) hydrogel coating for loading siRNA targeted toward a mammalian target of rapamycin (mTOR) \(^90\). The authors observed promising results *in vitro* wherein mTOR expression was silenced up to seven days. The authors also investigated the effect of varying the concentration of the hydrogels and found that prolonged and sustained release could be achieved with a higher concentration of hydrogel. However, when investigated as a surface coating for subcutaneous implants in a mouse model, a significant reduction on the formation of a fibrous capsule around the implant was not achieved. This is due to the choice of siRNA, although TGF-\(\beta_1\) silencing siRNA was also investigated, or, alternatively, may be due to a response to the hydrogel material. A limitation in this study is that a control implant without any hydrogel coating was not included hence the results cannot be interpreted.

Anderson *et al.* have also employed lyophilized *TransIT-TKOl*-siRNA complexes as coatings for silencing of reporter EGFP and TNF-\(\alpha\) \(^77\). This coating, which additionally retains bioactivity ~two months under room temperature storage, was effective at silencing EGFP expression (~85%) but, more importantly, was effective at silencing TNF-\(\alpha\) expression in a macrophage cell line. This approach could be harnessed to effectively mediate a macrophage response to biomedical devices and other tissue engineering approaches.

One of the most common types of device currently in clinical use are stents and their emergence as reservoirs of anti-mitotic drug delivery is well established. Recent investigations have not only harnessed their capability as reservoirs of nucleic acid delivery agents \(^91\) but their use as a reservoir of RNAi has also been reported. In one such study \(^92\), bare metal stents were coated with a cationized pullulan hydrogel loaded with siRNA targeted at silencing matrix-metalloproteinase-2 (MMP-2) expression in vascular cells. The cationized matrix retained siRNA for an extended period. This retention was due to the charge interaction of the matrix and siRNA. When implanted into a rabbit model of neointimal hyperplasia, although MMP-2 expression was not significantly silenced when compared to a scrambled control, proMMP-2 expression was silenced up to 28 ± 13%.

1.5.6 *Topical Delivery*

From a tissue engineering perspective, most scaffolds aim to occupy an anatomical location to add structural support and/or deliver a therapeutic agent. However, some areas of tissue engineering aim to employ biomaterials as active dressings for the delivery of therapeutic agents both topically and transdermally. Although not traditionally tissue engineering applications the therapeutic delivery of siRNA across the skin epithelium has been reported by Takanashi *et al.* \(^93\) where siRNA was delivered using a commercially available lipid/alcohol-based formulation (‘GeneCream™’). After dermal
<table>
<thead>
<tr>
<th>RNAi</th>
<th>Scaffold type</th>
<th>Target</th>
<th>Study type</th>
<th>Outcome</th>
<th>Reference</th>
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<tbody>
<tr>
<td>siRNA</td>
<td>Calcium crosslinked alginate hydrogel, photo-crosslinked alginate hydrogel, collagen hydrogel</td>
<td>GFP</td>
<td>In vitro</td>
<td>Up to 80% silencing was achieved at six days post transfection</td>
<td>71</td>
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<td>shRNA</td>
<td>Multilayer poly-(l-lysine)/PLL films with calcium phosphate-shRNA nanoparticles</td>
<td>Osteopontin, Osteocalcin</td>
<td>In vitro</td>
<td>Quantitative analysis found significant silencing of targets, an increase in layer number resulted in an increase in silencing</td>
<td>89</td>
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<td>siRNA</td>
<td>Dextran vinyl-sulfone (Dextran-VS) crosslinked with tetra-functional PEG thiol (PEG4SH), and PEG diacrylate also crosslinked with PEG4SH hydrogels</td>
<td>IL-10</td>
<td>In vitro</td>
<td>Up to 80% silencing with 10% (w/v) crosslinked dextran hydrogels five days post transfection</td>
<td>73</td>
</tr>
<tr>
<td>siRNA</td>
<td>Crosslinked collagen sponge with dPAMAM complexes</td>
<td>SNAIL1</td>
<td>In vitro</td>
<td>Significant silencing of target protein at seven days</td>
<td>74</td>
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<tr>
<td>siRNA</td>
<td>PCL nanofibers functionalized with PEG/ loaded with siRNA complexed and uncomplexed</td>
<td>GAPDH</td>
<td>In vitro</td>
<td>Silencing achieved with up to 80% with scaffold/complexed siRNA matrix</td>
<td>82</td>
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<tr>
<td>siRNA</td>
<td>Polymeric surfaces functionalized with lyophilized chitosan/siRNA particles or TransIT-TKO™/siRNA particles</td>
<td>EGFP, TNF-α</td>
<td>In vitro</td>
<td>Silencing of EGFP was achieved using both chitosan and TransIT-TKO™ as vector systems (~70% and 85% respectively) and silencing of TNF-α was also effective using this platform</td>
<td>77</td>
</tr>
<tr>
<td>siRNA</td>
<td>PCL porous sponge scaffold with TransIT-TKO™/siRNA particles</td>
<td>EGFP, TRIB2, BCL2L2</td>
<td>In vitro</td>
<td>Scaffold induced gene silencing for all targets</td>
<td>76</td>
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<td></td>
<td></td>
<td></td>
<td>and in vivo</td>
<td>siRNA guided differentiation of cells seeded on scaffolds was achieved in vitro and in vivo</td>
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<tr>
<td>siRNA</td>
<td>Commercially available ‘Genecream™’</td>
<td>Osteopontin</td>
<td>In vivo</td>
<td>Dermal penetration achieved using scaffold, suppressed target mRNA levels nine fold in the skin and subcutaneous tissue and serum levels of protein levels were reduced (25 – 50%). Furthermore symptoms of arthritis were alleviated</td>
<td>93</td>
</tr>
<tr>
<td>siRNA</td>
<td>Bare metal stent with cationized pullulan hydrogel coating embedded with siRNA</td>
<td>MMP-2</td>
<td>In vivo</td>
<td>ProMMP-2 expression was silenced up to 28 ± 13%.</td>
<td>92</td>
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<tr>
<td>siRNA</td>
<td>Agarose gel doped with siRNA complexed with Lipofectamine™</td>
<td>Mapk-1 and lamin A/C</td>
<td>In vivo</td>
<td>Histological, western blot and RT-PCR analyses reported significant silencing of target proteins when compared with controls</td>
<td>56</td>
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<td>RNAi</td>
<td>Scaffold type</td>
<td>Target</td>
<td>Study type</td>
<td>Outcome</td>
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<td>siRNA</td>
<td>Low molecular weight poly (ethyleneimine)-poly (organophosphazene) as a</td>
<td>Cyclin B1</td>
<td>In vitro</td>
<td>In vitro, at 21 days, Cyclin B silenced to a level of ~20% which was determined by western blot</td>
<td>94</td>
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<td></td>
<td>complexing agent. Polyplexes form a hydrogel structure at 37 °C</td>
<td></td>
<td>and</td>
<td>At four weeks, following a single injection of the hydrogel, an anti-tumour effect was achieved delivering Cyclin B1</td>
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<td>siRNA</td>
<td>Electrospun fibers (400 nm diameter) of a copolymer of caprolactone and</td>
<td>GAPDH</td>
<td>In vitro</td>
<td>Sustained release of siRNA up to 28 days. Co-polymerization with ethylene phosphate significantly improved the release of siRNA. In direct culture, naked siRNA was effective at GAPDH silencing, and TransIT-TKO™ improved this silencing further</td>
<td>95</td>
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<td></td>
<td>ethylene phosphate with naked siRNA or siRNA complexed with TransIT-TKO™</td>
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<td>siRNA</td>
<td>pDMAEMA based siRNA micelles embedded within a polyurethane foam disc</td>
<td>GAPDH</td>
<td>In vitro</td>
<td>80 % cumulative release from the scaffolds was observed at 21 days. The authors checked silencing only up to four days at which GAPDH expression was down to ~65%</td>
<td>96</td>
</tr>
<tr>
<td>siRNA</td>
<td>PEGylated lipoplexes embedded in freeze dried alginate scaffolds</td>
<td>GFP</td>
<td>In vitro</td>
<td>In vitro, there was 50% entrapment of the siRNA and GFP expression was reduced to 65 %</td>
<td>97</td>
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<td></td>
<td>during the freeze-drying process</td>
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<td>In vivo, the system was effective at enabling 85 % reduction of Lamin A/C expression which was verified by western blot</td>
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<tr>
<td>siRNA</td>
<td>Low molecular weight poly (ethyleneimine) methacrylate macromolecules</td>
<td>GFP</td>
<td>In vitro</td>
<td>The use of a complexing agent had a significant effect on the release profile of the siRNA from the gels. Increasing the concentration of the hydrogels prolonged the release profile of the siRNA to almost double the time (18 days). The silencing efficiency of the platform was significantly influenced by the dose of siRNA employed. GFP positive cells embedded within the platform had significantly higher GFP silencing with siRNA complexed versus naked siRNA within the dextran gels</td>
<td>98</td>
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<td>complexed with siRNA, embedded within dextran 2-hydroxyethyl methacrylate</td>
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<td>photo-crosslinked gels</td>
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<td>siRNA</td>
<td>Electrospun fibers of a copolymer of caprolactone and ethylene phosphate</td>
<td>Collagen 1a1</td>
<td>In vitro</td>
<td>In all platforms, sustained release of siRNA was achieved up to 28 days. In vitro, the scaffold prolonged the delivery of siRNA up to three times more than bolus delivery. There was a significant decrease in fibrous capsule thickness at week two and four when compared with plain electrospun fibers</td>
<td>99</td>
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<td>RNAi</td>
<td>Scaffold type</td>
<td>Target</td>
<td>Study type</td>
<td>Outcome</td>
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<tr>
<td>siRNA</td>
<td>Chitosan nanoparticles encapsulating siRNA embedded within electrospun PLGA fibers (diameter ~ 900 nm)</td>
<td>EGFP</td>
<td><em>In vitro</em></td>
<td>The release profile of siRNA from the platform was significantly dependent of the pH of the release buffer. 50% EGFP silencing occurred after 48 hours</td>
<td>100</td>
</tr>
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</table>

**Abbreviations:** GFP: green fluorescent protein; Dextran-VS: Dextran vinyl-sulfone; Dextran-VS; PEG: poly (ethylene glycol); IL-10: interleukin 10; dPAMAM: partially degraded poly (amino amine); PCL: poly (ε-caprolactone); GAPDH: glyceraldehydes 3 – phosphate dehydrogenase; EGFP: enhanced green fluorescent protein; TNF-α: tissue necrosis factor α; MMP-2: matrix-metalloproteinase.
application of GeneCream™ containing a siRNA sequence for detection via in situ hybridization, the delivered siRNA was detected in the epidermis at one hour, the dermis at four hours, and in the fat tissue of the subcutis ten hours after application. The same platform was then used to deliver siRNA silencing osteopontin resulting in suppression of symptoms of arthritic mice joints 20 days after an initial administration of siRNA cream. Delivery of the siRNA osteopontin cream also suppressed synovial hyperplasia, leukocyte infiltration and joint cartilage erosion compared to GeneCream™ containing a scrambled siRNA control. Furthermore, the osteopontin siRNA cream suppressed osteopontin mRNA levels nine fold in the skin and subcutaneous tissue and serum levels of osteopontin protein levels were reduced (25 – 50%). Another topical delivery of siRNA has been reported to modulate local gene expression of cutaneous disorders while avoiding systemic complications. In an excisional mouse wound model, an agarose gel formulation loaded with siRNA complexed with Lipofectamin™ was employed as a topical therapeutic. The experimental groups in this study investigated the silencing effect on two ubiquitously expressed proteins: Mapk-1 and lamin A/C. Histological, western blot and RT-PCR analyses reported greater silencing of target proteins than that found in controls. Furthermore, greater localized topical silencing was demonstrated when compared with that of other treatment groups.

Other novel attempts are being made at overcoming transdermal delivery of nucleic acids. Gonzalez-Gonzalez et al. have employed micro needles fabricated from polyvinyl alcohol (PVA) loaded with therapeutic and reporter nucleic acids (siRNA or pDNA) which, as needle tips pierce the outer skin surface, become embedded in the dermis and hydrate below the skin surface to form a viscous gel. Delivery throughout the dermis and epidermis was achieved using these PVA depots. Furthermore, delivery of siRNA to silence humanized Montrastrea green fluorescent protein (hMGPF) in a transgenic mouse skin model found that the use of this platform resulted in a noticeable reduction in hMGFP mRNA levels (25 – 50%) after 13 days when compared to nonspecific siRNA controls.

1.6 RNAi for Treatment of Myocardial Infarction

Ischemic heart disease leading to acute myocardial infarction (MI) is a leading cause of death in the developed world. Compensatory mechanisms such as formation of a structural scar, hypertrophy of non-ischemic cardiac tissue and development of collateral blood supply aim to maintain the functional role of the heart after MI. Likewise, clinical approaches such as reperfusion, angioplasty, stent placement, coronary artery bypass surgery, heart transplantation and established pharmaceutical interventions attempt to minimize this damage: but heart failure due to insufficient and adverse remodelling remains a common complication following MI. Genetic engineering technologies are making advances in the field by continually identifying oligonucleotides with higher potencies,
particularly RNAi; more effective cellular targets, typically in signalling pathways; and delivery approaches with the next generation of drug platforms. These advances have enabled detailed genomic investigations of endogenous miR expression profiles in impaired myocardium yielding new aberrantly expressed gene targets for silencing using RNAi technology. In addition, an increased understanding of surface-expressed markers offers the opportunity to develop more specific, cell-directed delivery technologies for genetic payloads.

1.6.1 siRNA

Circulating inflammatory cells, including neutrophils and monocytes/macrophages, arrive to the infarct site in the sub-acute phase following MI, initiated by complement components from ischemic tissue, and release of cytokines that are triggered by mechanical deformation, ischemic stimuli, reactive oxygen species (ROS) and amplification of cytokine pathways. This inflammatory response is initiated significantly within an hour of MI, peaks at two weeks post-MI, and this response tapers off as inflammatory cells disappear from the infarct site; modulating fibroblast phenotype transition and activation of proteinases that remodel the myocardium during the remodeling response within four weeks following MI. These fibroblasts play a significant role in this remodeling phase, laying down a fibrous collagenous scar to compensate for the loss of myocardium due to MI.

The clinical focus of MI nucleic acid therapy research has been on upregulation or overexpression of therapeutic genes, whereas siRNA delivery studies have reached the clinic only in the areas of cancer and AMD, among other targets. Currently RNAi research in the pathogenesis of MI is at the investigatory stage in which tissue is being profiled for overexpressed and underexpressed miRs. Considering the infancy of this research field, the large majority of the upcoming studies related to RNAi addressing the different phases following MI discussed are in pre-clinical animal models.

Manipulating inflammation is an approach that has gained particular interest post-MI. Using lentiviral particles, Krishnamurthy et al. knocked down human stabilizing protein (HuR – known to stabilize mRNA of proinflammatory cytokines) by intramyocardial delivery of HuR-specific shRNA into the infarct borderzone at five different locations immediately following MI. This was followed by daily intravenous injections of the same shRNA for one week, and resulted in a significant reduction in fibrotic area and improved left ventricular function at day 28. HuR can be suppressed by anti-inflammatory interleukin 10 (IL-10); therefore, a parallel strategy with IL-10 knockout mice was studied with similar results which the authors ascribed to the HuR knockdown mimicking the anti-inflammatory effects of IL-10.
Another anti-inflammatory approach by Van Tassell et al. \textsuperscript{108} aimed at inhibiting the myeloid differentiation factor 88 (MyD88), an innate adaptor protein that binds to Toll/IL-1 receptor complexes and activates the IL-1 associated kinase complex. Pharmacologic inhibition of MyD88 was achieved by intraperitoneal administration of ST2825 (which mimics the TIR domain of the MyD88 protein, thereby inhibiting homodimerization), IMG2005 (a synthetic oligopeptide with similar structure to ST2825) and a siRNA designed to silence MyD88 prior to induction of MI in mice and seven days following surgery. The authors reported protection of left ventricular dilation but no measureable reduction in infarct size with any of the silencing approaches (ST2825, IMG2005 and siRNA). This result can be attributed to the premature endpoint (seven days) as remodeling is still at an early stage.

A final RNAi intervention of inflammation has also been reported by Mezzaroma et al. This report describes the formation of a multiprotein complex, termed an inflammasome, necessary for caspase-1 activation and IL-1\_p release (players in the initial inflammatory response) \textsuperscript{106}. The inflammasome is formed by recruitment of an apoptosis speck-like protein containing a caspase-recruitment domain, by cryopyrin (an intracellular Nod-like receptor activated in response to tissue injury), which the study sought to silence using siRNA. Additionally, the authors investigated the silencing of the purinergic P2X7 receptor which is activated by extracellular ATP and leads to K\textsuperscript{+} efflux and cryopyrin activation. P2X7 and cryopyrin inhibition using siRNA prevented the formation of the inflammasome and limited the infarct size and cardiac enlargement after acute MI. However, the effect of this treatment on the ejection fraction of the infarcted myocardium following MI has not been reported to date.

β-blockers are routinely used in the prevention of MI in patients with coronary heart disease and in treating patients following MI \textsuperscript{109}. Although extremely beneficial, they also pose negative side effects such as aggravation of asthma and peripheral vascular diseases due to antagonism of the β\textsubscript{2} adrenergic receptor (β\textsubscript{2}AR). Therefore, a highly specific β\textsubscript{1} antagonist is desirable for clinical testing, something which is achievable using siRNA technology. Arnold et al. \textsuperscript{110} have reported successful delivery and functional knockdown of the β\textsubscript{1}AR using a siRNA/liposomal formulation (1,2-bis-oleoyloxy)-3-(trimethylammonium) propane, (DOTAP), both \textit{in vitro} and \textit{in vivo} and in treating spontaneously hypertensive rats and rats subjected to MI. Briefly, one month after hypertensive rats received a single siRNA dose, a significant decrease (10\%) in LV/BW ratio (which signified a decrease in cardiac hypertrophy) was observed compared with that of saline and scrambled siRNA control animals, suggesting a capacity of β\textsubscript{1} siRNA to inhibit hypertension-related LV-hypertrophy. In the MI model, siRNA injected intravenously three days prior to induction of MI considerably improved LV function. Furthermore, the number of apoptotic cells per field reduced significantly with siRNA treatment.
These findings alone suggest a potential of RNAi as a next generation pharmaceutical in the case of specific β-blocking.

Li et al. have employed an adenoviral shRNA as a more direct pharmacological inhibitor of phosphodiesterase 5a (PDE5a) than tadalafil. PDE5a degrades cyclic guanosine monophosphate (cGMP) by hydrolysis and therefore its inhibition enhances the advantageous effects of cGMP such as regulation of apoptosis, vasodilation and regulation of ion channel conductance. It has been found that mice treated with shRNA silencing PDE5a showed significant preservation of ejection fraction and fractional shortening, and reduction in infarction size and fibrosis. The authors obviated any concerns with regard the effect of the therapeutic in off target organs (as it would have similar effects as tadalafil), by delivering the shRNA intramyocardially.

Another anti-apoptotic approach looked at inhibiting the Src homology domain 2 (SH2) - containing tyrosine phopatase-1 (SHP-1) which has a key role in apoptosis and decreases the phosphorylation of Akt (which exerts a powerful cardioprotective effect after ischemia). The authors delivered a siRNA vector targeting SHP-1 directly into the left ventricular wall immediately following MI. Silencing was confirmed in vivo and, more significantly, a reduction in infarct size and a significantly higher fractional shortening one and two days after MI with the group treated with siRNA targeting SHP-1 was evident. The authors concluded that as SHP-1 is required for Fas-mediated cytotoxic signaling; SHP-1 silencing had an anti-apoptotic effect.

Hypoxia-inducible factor-1 (HIF-1) - a transcription factor consisting of two basic-helix-loop-helix-PAS transcription factors, HIF-1α and HIF-1β - mediates tissue responses to ischemia and has garnered interest in RNAi approaches. It promotes the transcription of in excess of 100 genes, notably vascular endothelial growth factor (VEGF-A), which is beneficial for angiogenesis, and inducible nitric oxide synthase (iNOS), whose role is being debated as it is likely to have a beneficial effect (attributed to a decrease in neutrophil and platelet adhesion or vasodilation) or a detrimental effect (related to the production of free radicals and inactivation of mitochondrial enzymes). Considering its pivotal role in these processes, HIF-1α has become a focus of numerous RNAi approaches to improve recovery following MI; it has been shown to induce therapeutic angiogenesis, limit infarct size and improve myocardial function after acute coronary occlusion in a transgenic mouse model. Therefore, increasing HIF-1 expression can be an approach in the treatment of MI and also in the case of RNAi silencing factors that in turn would normally be inhibiting HIF-1α. One such approach investigated whether cardiac preconditioning via prolyl-4 hydroxylase-2 silencing (to activate HIF-1α) would improve cardiac function following MI. Administering a siRNA silencing prolyl-4 hydroxylase-2 intraperitoneally 24 hours prior, to 30 minutes ischemia and 120 minutes reperfusion,
the authors reported reduced infarct sizes and a decrease in polymorphonuclear leukocyte infiltration (associated with diminished chemokine and adhesion molecule expression). Unfortunately, further analysis revealed the drawback that the murine knockdown target was pro-collagen prolyl-4 hydroxylase-2 rather than HIF prolyl-4 hydroxylase-2, and the authors were not able to conclusively explain a link between the knockdown of pro-collagen prolyl-4 hydroxylase-2 and HIF-1α upregulation and the reduced infarct sizes. A more robust strategy was attempted by others using a shRNA plasmid to silence HIF prolyl-4 hydroxylase-2 whose selection was confirmed by GenBank database 115 which had not been conducted in the previous similar study 114. Activity of the shRNA plasmid, via a reporter luciferase gene was monitored and plasmid-mediated transgene expression was observed for up to five weeks. Fractional shortening and increased angiogenesis in the infarcted heart compared with a scrambled sequence was improved at four weeks and was associated with higher levels of HIF1-α, verified by western blot analysis. The study concluded that RNAi of HIF prolyl-4 hydroxylase-2 increases HIF1-α expression, leading to improved recovery following MI.

1.6.2 miRs Mimicking Nature – miR Manipulation in the Treatment of Myocardial Infarction

Recently, the focus of RNAi approaches has encompassed the use of synthetic sequences to mimic or silence endogenous miRs that are abruptly deregulated following MI. Van Rooij et al. first described regulation of miR expression in heart disease by the use of microarray profiling in murine cardiac hypertrophy/failure 116. In the same study, selected miR patterns in human cardiomyopathy were described. miRs are attractive therapeutic targets because of the significant control exercised by individual miRs or miR clusters over pathways that regulate cardiac hypertrophy, cardiomyocyte apoptosis, myocardial vascularization, and cardiac fibrosis.

The first report of delivering miRs towards a therapeutic end is the delivery of miR-1, miR-12 and miR-24, which were revealed by the authors to have been upregulated during ischemic preconditioning in a mouse model 117. The authors isolated this cohort of miRs from preconditioned myocardium and injected them intramyocardially into mice 48 hours prior to ischemia/reperfusion injury. This miR preconditioning led to an upregulation of eNOS, HSP70 and the HSP70 transcription factor HSF-1. Furthermore, the treatment group had a significantly reduced infarct size when compared to the negative controls. The beneficial miRs in this study conflict with papers that have since been published. For instance, miR-24 is shown to be slightly upregulated in response to ischemic preconditioning and the authors postulate that miR-24 could be a therapeutic miR although this is not conclusively discussed in the results. In a more recent study, miR-24 has been shown upregulated in endothelial cells following cardiac ischemia. Forced overexpression of miR-24 by delivering pre-miR-24 mimics in vitro induced apoptosis 118. Furthermore, the administration of antagoniRs targeting miR-24, zero and two days following MI resulted in an improved capillary
density and smaller significant infarct size 14 days after MI and improved fractional shortening compared to the scrambled control. Although the previous study does demonstrate a reduced infarct size with an ischemic preconditioning cocktail of miRs, one must fully elucidate the role of each individual miR to achieve a therapeutic goal.

There exists a family of miRs, coined myomiRs, which are the three highly expressed muscle-specific miRs: miR-208a, -208b, and -499, shown to control myosin heavy chain isoform expression\(^\text{119}\). Specifically, miR-208 is a miR encoded within an intron of the Myh6 (α-myosin heavy chain), which is downregulated in response to cardiac injury and is essential for the expression of Myh7 (β-myosin heavy chain) and Myh7b (a closely related isoform)\(^\text{120}\). AntagomiRs, in a locked nucleic acid (LNA) format to target miR-208a, delivered subcutaneously during hypertension-induced heart failure, hindered myosin switching and cardiac remodeling while improving cardiac function, overall health and survival\(^\text{121}\). Although the study monitored the survival of a hypertension-induced heart failure model, application of this treatment in the context of MI appears promising.

miR-126 has been demonstrated to be downregulated within the infarcted myocardium but upregulated at the periphery of the infarct (borderzone). A crucial function of miR-126 in the development of the fetal vascular system was documented in a study showing that miR-126 knockout in mice impaired vascularization after experimental MI\(^\text{122}\), suggesting that miR-126 directs both initial vascular development and angiogenesis. miR-126 expression occurs exclusively in endothelial cells, whereas miR-210 is expressed in cardiomyocytes, where it is induced by HIF1-α, and is associated with increased survival/decreased apoptosis. Delivery of a minicircle DNA vector encoding the miR-210 precursor decreased ventricular remodeling, improved ventricular ejection performance, diminished cardiomyocyte apoptosis and enhanced myocardial neovascularization following MI in mice\(^\text{123}\). Conversely, Bonauer et al. described an increase in expression of miR-92a after experimental MI and observed that it decreased neovascularization. Consequently, administration of a miR-92a antagomiR showed increased border zone vascularization, diminished infarct size and enhanced post-infarction ventricular function following MI in a mouse model\(^\text{124}\).

Typically, myocardial fibrosis comes after significant cardiomyocyte death due to MI, and several miRs (fibromiRs) modulate cardiac fibrosis. Expression of miR-29 is higher in cardiac fibroblasts than in cardiomyocytes, but the expression of miR-29B in cardiac fibroblasts is downregulated in infarct border zone of infarcted hearts\(^\text{125}\). By contrast, miR-21 expression is increased in the border zone of infarcted myocardium and in the fibroblasts of failing human hearts, where miR-21 enhances fibroblast survival by oppressing programmed cell death-4 (PDCD4) and sprouty homolog-1, and promotes myocardial fibrosis by activating the extracellular signal-regulated and mitogen-activated
Table 1.3: Summary of studies reporting the use of siRNA following cardiac ischemia/remodeling

<table>
<thead>
<tr>
<th>Target</th>
<th>Presumed role</th>
<th>Observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuR</td>
<td>Stabilizes mRNA of proinflammatory cytokines</td>
<td>shRNA injected at the infarct borderzone immediately following MI and intravenously for one week following resulted in significant reduction in fibrosis and improved cardiac function at day 28. HuR silencing mimicked anti-inflammatory effects of IL-10</td>
<td>107</td>
</tr>
<tr>
<td>MyD88</td>
<td>Binds to Toll/IL-1 receptor and activates IL-1 associated kinase complex</td>
<td>Protection of left ventricular dilation but no measureable reduction in infarct size after seven days</td>
<td>108</td>
</tr>
<tr>
<td>Cryopyrin and P2X7</td>
<td>Cryopyrin regulates the formation of a multiprotein complex called an inflammasome necessary for caspase-1 activation and IL-1α release. P2X7 leads to K⁺ efflux and cryopyrin activation</td>
<td>Both P2x7 and cryopyrin inhibition independently prevented the formation of the inflammasome, reduced caspase activity and limited the infarct size and cardiac enlargement after acute MI</td>
<td>106</td>
</tr>
<tr>
<td>β₁-AR</td>
<td>Responsive to adrenaline, controls cardiac conduction velocity, automaticity. Pharmacological blockers of this receptor are routinely used in cardiovascular disease</td>
<td>siRNA injected intravenously three days prior to MI exhibited considerable improved LV functions. The number of apoptotic cells per field of view reduced significantly</td>
<td>110</td>
</tr>
<tr>
<td>PDE5a</td>
<td>Degrades cGMP and therefore its inhibition enhances cGMP effects such as regulation of apoptosis, vasodilation and regulation of ion channel conductance</td>
<td>shRNA treated mice had significant preservation of ejection fraction and fraction shortening, and reduction in infarction size and fibrosis</td>
<td>111</td>
</tr>
<tr>
<td>P4H</td>
<td>P4HL-2 knockdown inhibits the maturation of HIF-1α, therefore inhibiting it would increase HIF-1α</td>
<td>Reduced infarct sizes and decrease in polymorphonuclear leukocyte infiltration. However, further analysis revealed that the target was incorrectly determined and was in fact procollagen prolyl-4 hydroxulase-2 rather than HIF prolyl-4 hydroxylase-3</td>
<td>114</td>
</tr>
<tr>
<td>P4HL-2</td>
<td>shRNA plasmid sequence confirmed by GenBank</td>
<td>Activity of shRNA plasmid monitored up to five weeks. In vivo, fractional shortening and increased angiogenesis in the infarcted heart was improved at four weeks; associated with higher levels of HIF-1α</td>
<td>115</td>
</tr>
<tr>
<td>NHE1</td>
<td>Removes H⁺ from the cytosol in exchange for Na⁺, thereby regulating intracellular pH and Na⁺ concentration</td>
<td>This study was not in a diseased model, however, a single injection of naked siRNA into the left ventricle in a mouse model spread through the left ventricle (through diffusion in the myocardium from cell to cell through gap junctions). Effective silencing was achieved as verified by ion exchange measurement and western blots</td>
<td>127</td>
</tr>
</tbody>
</table>

**Abbreviations:** siRNA: short interfering RNA; HuR: human stabilizing protein; mRNA: messenger RNA; shRNA: short hairpin RNA; MI: myocardial infarction; IL-10: interleukin 10; MyD88: myeloid differentiation factor 88; IL-1: interleukin 1; P2X7: purinergic P2X7 receptor; β₁-AR: β₁-adrenergic receptor; LV: left ventricular; PDE5a: phosphodiesterase 5a; cGMP: cyclic guanosine monophosphate; P4H: prolyl-4 hydroxylase; HIF-1α: hypoxia-inducible factor-1α; NHE1: Cardiac Na⁺/H⁺ exchanger
Figure 1.3: Dysregulation of endogenous microRNAs following myocardial infarction. Colours correspond to the remote myocardium (red), borderzone of infarct (blue), ischemic tissue (green) and globally (black), and where appropriate according to each cell type. miRs on the lower side of the scales are downregulated, whereas those on the upper side of the scales are upregulated.

15* denotes the miR-15 family which includes miRs 15a, 15b, 16-1, 16-2, 195 and 497*.

Table 1.4: Summary of effort at mimicking miRs following cardiac ischemia/remodeling

<table>
<thead>
<tr>
<th>miR</th>
<th>Presumed role</th>
<th>Observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>210</td>
<td>Upregulated in hypoxic conditions (postulated for cell survival in hypoxic conditions)</td>
<td>Delivery of exogenous miR-210 via a mini-circle vector improved angiogenesis and reduced cardiomyocyte apoptosis in a rat model of MI at eight weeks. Ventricular shortening was also reduced in the treatment group</td>
<td>123</td>
</tr>
<tr>
<td>208/208a</td>
<td>Linked to the transition of Myh6 to Myh7 during cardiac stress</td>
<td>Inhibition of miR-208a using LNA reduced remodeling and Myh7 expression following cardiac stress and led to improved cardiac function</td>
<td>121</td>
</tr>
<tr>
<td>126</td>
<td>Currently deemed endothelial specific, it has been linked to maintaining vascular integrity and postnatal angiogenesis</td>
<td>miR-126 mutant mice exhibited increased mortality due to ventricular rupture and defective cardiac neovascularization after MI</td>
<td>122</td>
</tr>
<tr>
<td>92a</td>
<td>A component of the 17-92 cluster of miRs, the overexpression of 92a blocks angiogenesis and vessel formation in vitro and in vivo. Upregulated after induction of acute MI in a mouse model</td>
<td>Administration of miR-92a antagonomiRs reduced infarct size, reduced apoptosis and augmented the number of perfused lectin positive cells in the borderzone of infarcted mouse myocardium</td>
<td>124</td>
</tr>
<tr>
<td>29</td>
<td>A family of miRs postulated to be intrinsically connected with ECM production</td>
<td>miR 29, specifically miR 29B, was significantly reduced in borderzone and remote myocardium in response to MI at three and 14 days in a mouse model. Similar trends existed in human cardiac tissue. Administration of antimiR-29b oligonucleotides resulted in a significant increase in ECM protein expression in vivo. In vitro, delivery of miR-29b mimics led to reduced expression of ECM proteins</td>
<td>125</td>
</tr>
<tr>
<td>21</td>
<td>Decreased at six hours and 24 hours within infarct tissue and increased expression in borderzone tissue and remote myocardium. Ischemic preconditioning caused an increase in the expression of miR-21 in all areas</td>
<td>Adenoviral delivery of miR-21 reduced infarct size after 24 hours following administration after MI. The cytoprotective effect of miR-21 was verified using loss- and gain-of-function tests in vitro using hypoxic conditions in cardiomyocytes</td>
<td>128</td>
</tr>
<tr>
<td>133</td>
<td>Reported anti-hypertrophic effect. Exclusively expressed in cardiomyocytes and not in fibroblasts. However, regulates cardiac fibrosis through a presumed paracrine effect</td>
<td>Its role following MI has yet to be elucidated. In an in vivo model of transverse aortic constriction, however, cardiomyocyte-specific overexpression of miR-133a resulted in decreased levels of apoptosis and fibrosis, together leading to improved diastolic performance. In addition, reduced expression of miR-133 was noted in hypertrophic models with a marked increase in hypertrophy when inhibited using antagoniRs</td>
<td>129-130</td>
</tr>
<tr>
<td>30</td>
<td>Consistently downregulated in rodent and human hypertrophy and heart failure. Highly expressed in cardiac fibroblasts and has been shown to bind to the 3' UTR of the profibrotic connective tissue growth factor (CTGF) to inhibit its expression</td>
<td>In vivo studies are warranted to confirm whether cardiac fibrosis can be modulated by loss- or gain-of-function of miR-30</td>
<td>131</td>
</tr>
<tr>
<td>IPC-miRs</td>
<td>miRs was extracted from myocardium subjected to ischemic preconditioning. miR-1, -21, and -24 were found to be upregulated</td>
<td>Preconditioning with IPC-miRs prior to ischemia reperfusion resulted in reduction of infarct size and increased expression of eNOS, HSF-1 and HSP70</td>
<td>117</td>
</tr>
</tbody>
</table>
### Literature Review

<table>
<thead>
<tr>
<th>miR Cluster</th>
<th>Presumed role</th>
<th>Observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>23-27~24</td>
<td>Implicated in the regulation of angiogenesis during vascular disorders and ischemic heart disease. However, there are conflicting observations in the literature with regard to the specific action, most notable with miR-24</td>
<td>Two studies present that blocking of miR-24 in a mouse model of MI limits infarct size by preventing endothelial apoptosis and enhancing vascularity, leading to preserved cardiac function and survival. However, one reports that overexpression of miR-24 by local delivery of miR-24 mimics resulted in inhibition of cardiomyocyte apoptosis, reduced infarct size and improved LV function in a mouse model of MI</td>
<td>118, 132-133</td>
</tr>
<tr>
<td>494</td>
<td>Previously found to be downregulated in human failing hearts and animal models of hypertrophy and ischemia</td>
<td>A transgenic mouse model overexpressing miR-494 had reduced apoptosis and infarct size following MI. Furthermore, inhibition of miR-494 using antagomiRs increased cardiac injury following MI</td>
<td>134</td>
</tr>
</tbody>
</table>

**Abbreviations:** miR: microRNA; MI: myocardial infarction; LNA: locked nucleic acid; ECM: extracellular matrix; Myh6: α-heavy myosin chains; Myh7: β-heavy myosin chains; CTGF: connective tissue growth factor; IPC: ischemic preconditioning; eNOS: endothelial nitric oxide synthase; HSF-1: heat shock transcription factor-1; HSP70: heat shock protein 70; LV: left ventricular.
protein kinases. miR-21 expression is downregulated by hypoxia in cardiomyocytes (but not in fibroblasts), possibly as part of the Akt survival pathway. The same study employing a cholesterol-modified miR-21 antagomiR led to reduced myocardial fibrosis and pathological remodeling in the mouse aortic banding pressure overload model, although this effect was not proven to be exclusively mediated by the inhibition of miR-21.

1.7 Delivery Strategies

Although relatively few studies using RNAi delivery to improve recovery following MI exist to date, the delivery of RNAi molecules has achieved some significant improvements in cardiac function in pre-clinical models. In many of the studies discussed above, the intravenous delivery route necessitates high doses which raise concerns as to potential gene silencing in non-target organs. This calls for development of more localized delivery systems to reduce dosing regimens and eliminate unwarranted off-target effects, especially in healthy organs. This can involve local intracoronary administration following reperfusion interventions; engraftment onto stents, intramyocardial injection or functionalization of carriers with targeting moieties and ligands, delivered systemically, and designed to reach target tissues as described in the next section. The intramyocardial delivery route is feasible as researchers have shown that a single injection of naked siRNA into the left ventricle of a mouse model spread through the left ventricle (possibly through diffusion in the myocardium from cell to cell through gap junctions) aimed to specifically silence the cardiac Na⁺/H⁺ exchanger and was effective when verified by ion exchange measurements and western blots. Although an MI model was not employed, considerations and knowledge towards intramyocardial delivery are highlighted.

RNAi molecules in their naked unprotected form are subject to rapid degradation in the circulation, whereas the encapsulation of these therapeutics within protective carriers can enhance their efficacy. Non-viral nucleic acid carriers can be loosely grouped into three main categories: liposomes, cationic polymers and nanoparticles.

Cationic liposome-DNA complexes (lipoplexes) have been widely investigated in pre-clinical and clinical trials due to their modest in vitro transfection efficiency. Liposomes in their unmodified form; however, are non-specific in terms of cell targeting and, depending on the dosage, may have cytotoxic effects. Cationic polymers are macromolecules with a highly branched three-dimensional architecture, consisting of branches with terminal reactive end groups. These reactive end groups are usually amine end-groups that facilitate complexation to negatively charged DNA. The third non-viral carrier; nanoparticles, are engineered vehicles of 1 nm – 200 nm that have the capacity to
transport pharmaceuticals and genetic material. They can be produced in the form of rods, tubes and spheres/shells and can be fabricated from synthetic or natural polymers and metals, depending on considerations such as large scale production requirements and their intended application. These structures can be used in a variety of clinical applications, including that of improved in vivo localization using magnetic manipulation.

The functionality of these synthetic carriers such as these is increasingly improved through processes such as PEGylation, charge manipulation and ligand conjugation. An important aspect of this functionality is the addition of receptor engaging ligands to offer improved targeting and binding to specific cell types. This gives rise to the need to identify cell-specific surface molecules, and this aspect is discussed in the next section.

1.8 Engineering Ligands for Improved Uptake

Attaching cell-targeting ligands to nucleic acid carriers as discussed above can offer selectivity to a particular cell type/accumulation at target tissue, and control the route of internalization into the target cell. Endocytosis is the main route of cellular internalization of non-viral vectors and multiple mechanisms have been described to date. Internalized molecules typically traffic in intracellular vesicles and eventually fuse with lysosomes to be degraded, presenting a further challenge in reaching intracellular target sites.

Cell-targeting ligands include natural ligands based on transferrin, peptide formulations (RGD) to engage integrins and antibodies, and functional fragments thereof. In MI, many markers of inflammation and endocytic receptors receptive to specific ligands can allow for improved and controlled uptake of non-viral vectors carrying pDNA for transcription of desired shRNA and miR molecules, or direct siRNA payloads. Synthetic vectors have shown potential for improved delivery using ligand-based nucleic acid therapy (Table 1.5). It is worth noting here that the misnomer ‘targeted’ is avoided, especially in the context of intravenous administration as this would suggest delivery specifically to, and unique to, one particular target; in fact, less than 5% of delivered drug particles engage with their intended cell or tissue and instead accumulate in the liver, lungs, kidney and spleen. Passive targeting is achieved in tumour tissue via the enhanced permeation and retention (EPR) effect, but in the context of MI, this effect is less pronounced, and therefore engagement with cell-specific markers and receptors is desirable for specific delivery.

1.8.1 Antibodies

Hybridoma technology and the development of chimeric antibodies (mouse antigen-binding domains fused to human constant regions) have enabled large-scale production of antibodies, specific for a
diverse range of targets. The use of monoclonal antibodies (mAbs) is advantageous over most fragments due to the higher binding avidity that results from their bivalency, and the presence of the Fc domain appropriate to tether the mAb to a delivery vehicle in an appropriate orientation. Conversely, the presence of exposed Fc domains that can bind Fc receptors in non-target tissues, particularly on macrophages, leads to high uptake in the liver and spleen has the potential to increase the immunogenicity of such an antibody functionalized delivery vehicle. Extensive engineering of Fab and single-chain Fv (scFv) fragments has led to the development of bivalent \(^{145}\) and even bispecific \(^{146}\) variants for improved target binding while obviating immunogenic responses. The lack of availability of an Fc domain for orientation of attachment to carriers can be overcome by chemical and/or genetic modification of the fragments to create active groups distant from the target-binding pocket \(^{147}\).

Advantages of such fragments are their smaller size (which allows denser packing on vectors and greater penetration of tissues), their ease of production and modification in recombinant – particularly bacterial – expression systems \(^{148}\) and, increasingly, the development of large combinatorial libraries of fragments that can be screened relatively easily and rapidly to isolate binders against any molecule of interest \(^{149}\). In addition, antibody fragments can be engineered to improve their linking by covalent or ionic interactions to reservoirs of genetic material.

Béduneau et al. have engineered liposomes conjugated with monoclonal antibodies and Fab fragments with specificity for the transferrin receptor (Tfr) \(^{150}\). Both liposome populations demonstrated enhanced accumulation at Tfr sites 24 h post-injection in a healthy rat model, most notably in the brain, and liposomes functionalized with whole antibodies proved to be more efficient. Lau et al. reported improved extravasation of siRNA-albumin conjugates into organs with fenestrated sinusoidal endothelia (including the myocardium) to silence an insulin-like growth factor type I receptor (IGF-IR) \(^{151}\). These findings provide an important insight into targeting cardiac tissue using siRNA, although it would be interesting to see the results of similar experiments carried out with a cell-specific targeting ligand (as opposed to a more general protein such as albumin) or in a pathological model. In principle, while past research efforts have focussed primarily on monoclonal antibodies, in theory any monoclonal antibody, Fab fragment or scFv can be linked to a synthetic vector to engage with specific cells.

1.8.2 Cell-targeting Peptides

Phage display technology generates and screens large libraries of peptides or proteins for binders to a specific, immobilized ligand and is used to identify peptides rather than antibodies or fragments that can bind specifically to many molecular targets. Liu and co-workers have identified peptides specific for atherosclerotic lesions induced in apoE knockout mice \(^{152}\), with GRP78 identified in \textit{ex vivo}
studies as the endothelial cell surface target of one such peptide. Meanwhile, Tepe et al.\textsuperscript{153} have described a radiolabeled endothelin derivative which selectively accumulates on atherosclerotic plaques after intravenous administration. PEI has been conjugated to the cyclic RGD-4C to effect delivery to endothelial cells expressing αυβ3 while the use of RGD-containing peptides and a short polylysine sequence for increased electrostatic binding of DNA has been demonstrated to enhance DNA delivery by up to five-fold\textsuperscript{154}. Other researchers have also documented the use of cationic nanoparticles conjugated to the αυβ3 integrin-ligand to selectively deliver a mutant Raf protein to inhibit endothelial cell survival by blocking angiogenesis\textsuperscript{155}.

In terms of the myocardium, cardiomyocyte prostaglandin receptors using prostaglandin E2 (PGE2) as a ligand\textsuperscript{156} have been used as potential targets. A primary cardiomyocyte (PCM)-specific peptide generated by phage display\textsuperscript{157} and investigated \textit{in vitro}. Both delivery strategies (PGE2 or PCM specific peptide as ligands) resulted in significant silencing of \textit{Fas} when utilized with cationic complexes to deliver siRNA against the \textit{Fas} gene (a transmembrane protein which belongs to the TNF-α family that when ligand/receptor engagement occurs apoptosis is induced). \textit{Fas} silencing using these systems resulted in a significant reduction in cardiomyocyte apoptosis compared to controls \textit{in vitro}. Although this system was also an \textit{in vitro} demonstration of the effect, it indicates a therapeutic promise of the approach that warrants further investigation.

The lack of successful clinical strategies employing ligand-based drug delivery is a concern for researchers aiming to develop cancer-based therapeutics and for those in the field of MI. However, there are many options available to the cardiovascular researcher that can enhance delivery to the infarcted myocardium. The coronary vasculature is an anatomically defined network enabling local catheter-based delivery which obviates pre-clearance of therapeutics by other organs and provides direct interaction with the infarcted myocardium. Systemic delivery with the aim of a successful targeted tissue is still beyond realization until optimization of the more fundamental aspects of carriers such as physico-chemical properties of the bulk material, size and charge is performed to fully understand the interaction \textit{in vivo} after systemic delivery.

1.9 Fibrosis

Fibrosis is the final, common pathological outcome of tissue insult and/or inflammation and a central target in the development of the platform developed in this thesis. Although collagen deposition is an indispensable and, typically, a reversible element of wound healing, normal tissue repair can evolve into a progressively irreversible fibrotic response if the tissue injury is severe, repetitive, or if the wound-healing response itself becomes dysregulated. Fibrosis is defined as the excessive accumulation of fibrous connective tissue (components of the ECM such as collagen and fibronectin)
Figure 1.4: Schematic mechanisms of hypoxia-induced collagen deposition. Hypoxia stimulates the production of collagens via oxidative stress or transforming growth factor beta (TGF-β) signalling pathway (in blue). Oxidative stress can also activate TGF-β, which might induce the expression of pro-fibrogenic genes, including those encoding collagens. Matrix metalloproteinases (MMPs) and the endogenous tissue inhibitors of metalloproteinases (TIMPs) can be regulated at transcriptional levels through epigenetic mechanisms (i.e. DNA methylation and histone modification) in response to hypoxia. In addition, hypoxia activates several transcriptional factors (e.g. nuclear factor-kappaB (NF-κB), activating protein 1 (AP-1), signal transducers and activators of transcription-1 (STAT) and TGF-β) that subsequently bind to some of the key transcriptional binding sites, regulating MMP gene expression (in orange). The MMPs digest collagens and reduce collagen deposition; as an autoregulation, collagens bind to their discoidin domain receptor (DDR) to upregulate MMPs levels. In addition to the inhibitory effect on MMPs, TIMPs also have a key role in cell proliferation and cell death. Adapted from Tong et al. 184.
in and around inflamed or damaged tissue, which can lead to permanent scarring, organ malfunction and, ultimately death, as seen in heart failure \(^{158}\). In cardiac tissue, fibroblasts are recognised as key regulators of ECM components and also are a major source of MMPs and TIMPs (Figure 1.4). In the inflammatory environment following MI, cytokines, grown factors, and other environmental stimuli modulate fibroblast function by changing the production patterns of ECM components, MMPs and TIMPs, by stimulating their migration and proliferation, and by mediating the interaction between cardiac fibroblasts and other cell types.

Cardiac remodelling is the most common underlying cause of end-stage heart failure morbidity and mortality worldwide after MI \(^{159-161}\). Significant fibrosis, occurring in both the infarcted and non-infarcted myocardium, represents a characteristic pathological alteration of post infarct remodelling and is recognised to be a major determinant of the progressive deterioration of ventricular function after MI. This fibrosis is modulated by a cascade of biochemical intracellular signalling processes that are triggered by necrotic myocardium in the context of MI in combination with increased preload and afterload. It also reduces the electrical coupling between cardiomyocytes as fibroblasts produce smaller or larger collagenous septa, which electrically insulate cardiac cells or muscle bundles. Due to this, the normal myocardial architecture becomes disrupted and is transformed into a pathological substrate characterized by the presence of multiple insulating barriers, which force the depolarization wave to spread non-uniformly \(^{162,163}\).

Without performing an exhaustive review of the many factors that play a key role in the pathological post infarct remodelling process, key events and signalling molecules emerge that play key roles. For instance, TGF–β is a key signalling molecule that induces cardiac fibrosis by activating the proliferation and collagen production of cardiac fibroblasts \(^{164}\). Intracellular mitogen-activated protein kinase (MPAK) signalling cascades have also been proven to play an important role in the pathogenesis of cardiac fibrosis \(^{165}\).

The importance of fibrosis as a determinant of myocardial performance and disease outcome is increasingly appreciated. Nevertheless, efforts to develop novel therapies that specifically target the cardiac fibroblast are at a relatively early stage in common with approaches to fibrotic disease in other organ systems. Current pharmaceutical attempts at modulating fibrosis include inhibitors of rennin-angiotensin-aldosterone \(^{166,169}\), endothelin receptor antagonists \(^{170}\), statins \(^{171,172}\), cytokine therapy \(^{173-175}\), and inhibitors of MMPs \(^{176,177}\), all of which are extensively reviewed elsewhere \(^{178}\).

miRs are emerging as key players in the pathogenesis of fibrosis. For instance the miR-29 cluster in humans includes hsa-miR-29A, hsa-miR-29B-1, hsa-miR-29B-2 and hsa-miR-29C. miR-29B-1 and mir-29B-2 have identical mature sequences, which are together called miR-29B. Mature miR-29s share identical sequences at nucleotide positions 2-7, the seed region that plays a key role in determining which protein-coding-genes a microRNA would target \(^{179}\). Therefore, predicted target
Figure 1.5: A model for the role of miR-29 in cardiac fibrosis. In response to cardiac stress, TGF-β1 is activated and triggers the down-regulation of miR-29 in cardiac fibroblasts and consequent up-regulation of the expression of collagens and other ECM proteins involved in fibrosis. Adapted from van Rooij et al. 125.
### Table 1.5: Summary of non-viral nucleic acid delivery employing ligands to improve efficacy

<table>
<thead>
<tr>
<th>Platform</th>
<th>Antibody</th>
<th>Target</th>
<th>Method</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposomes</td>
<td>Anti-E-Selectin (mAb)</td>
<td>E-selectin on TNF activated vascular cells, CHO-E cells and vascular tissue explants.</td>
<td>Coupling antibodies to liposomes complexed to DNA via mild heat treatment. TNF administered to vascular cells to upregulate E-selectin and P-selectin.</td>
<td>Addition of anti-transferrin receptor mAbs increased internalization of liposome-DNA complex into cells. mAbs against both transferrin receptor and E-selectin augmented transfection of cells expressing appropriate antigens.</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>Anti TfR (mAb)</td>
<td>E-selectin on TNF activated vascular cells, CHO-E cells and vascular tissue explants.</td>
<td>Coupling antibodies to liposomes complexed to DNA via mild heat treatment. TNF administered to vascular cells to upregulate E-selectin and P-selectin.</td>
<td>Addition of anti-transferrin receptor mAbs increased internalization of liposome-DNA complex into cells. mAbs against both transferrin receptor and E-selectin augmented transfection of cells expressing appropriate antigens.</td>
<td>185</td>
</tr>
<tr>
<td>Anti-endoglin (CD105)</td>
<td>CD105 endoglin on HUVECs and HDMECs.</td>
<td>Covalent binding of antibody scFv to liposome.</td>
<td>Immunoliposomes displayed rapid and strong binding to endoglin expressing cells (HUVEC, HDMEC) whereas no binding was observed with control lines. Immunoliposomes had increased binding and internalization when compared to controls.</td>
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<td>186</td>
</tr>
<tr>
<td>Anti-VCAM-1 (mAb)</td>
<td>Overexpressed VCAM-1 on human endothelial cells.</td>
<td>Anti-VCAM-1 antibody conjugated via carbodiimide/EDC crosslinking onto liposome.</td>
<td>Immunolipoplexes bound selectively and specifically to TNF-activated endothelial cells. Binding of immunolipoplexes induced an increase in Ca^{2+} and reorganization of actin filaments. A small percentage of non-modified liposomes migrated across endothelial cells.</td>
<td>Immunolipoplexes bound selectively and specifically to TNF-activated endothelial cells. Binding of immunolipoplexes induced an increase in Ca^{2+} and reorganization of actin filaments. A small percentage of non-modified liposomes migrated across endothelial cells.</td>
<td>187</td>
</tr>
<tr>
<td>Branched Polymers</td>
<td>Anti-E/P Selectin (mAb)</td>
<td>E-selectin and P-selectin on TNF-activated vascular cells, CHO-E and vascular tissue explants.</td>
<td>Antibodies coupled to PAMAM complexed with plasmid via biotinylation.</td>
<td>Polyplexes demonstrated increased transfection efficiency in CHO-E cells, activated HSVEC and saphenous vein segments ex vivo when coupled with anti E/P selectin.</td>
<td>188</td>
</tr>
<tr>
<td>PAMAM</td>
<td>αβ3/αβ5 integrin-binding RGD peptide</td>
<td>Angiogenic endothelial cells overexpressing αβ3 and αβ5.</td>
<td>αβ3/αβ5 integrin-binding RGD peptide incorporated into PEI through a PEG spacer.</td>
<td>In vitro transfection experiments showed an approximate fivefold increase in transfection efficiency compared to controls.</td>
<td>189</td>
</tr>
</tbody>
</table>

**Abbreviations:** mAb: monoclonal antibody; TNF: tissue necrosis factor; CHO-E: Chinese hamster ovary expressing E-selectin; scFv: single chain variable fragment; HUVEC: human umbilical vein endothelial cells; HDMEC: human dermal microvascular endothelial cells; VCAM: vascular cell adhesion molecule; EDC: ethyl (dimethylaminopropyl) carbodiimide; PAMAM: poly (amido amine); HSVEC: human saphenous vein endothelial cells; PEI: poly (ethylenimine); PEG: poly (ethylene glycol).
genes for the miR-29 family members largely overlap. It has been confirmed that miR-29B-1 and miR-29A are transcribed together as a polycistronic pri-miR and likewise miR-29B-2 and miR-29C are transcribed together. It has collectively been shown that the miR-29 family members target at least 16 genes related to extracellular matrix (ECM) which encode for several key proteins involved in the physiological or pathological formation of ECM including: a large number of collagen isoforms, laminin γ, fibrilin 1, elastin, matrix metalloproteinase 2, and integrin β1. This is unique to the miR-29 family as no other miR is predicted to target more than 11 of the 20 collagen genes (Figure 1.5).

1.10 Project Rationale

The preceding sections have illustrated the use of RNAi as an intervention in the treatment of the infarcted myocardium and the delivery of RNA from biomaterial scaffolds in various applications. From a biomaterials approach, non-viral vectors are discussed, with focus on the various methods of delivering and functionalizing these vehicles. Furthermore, the use of biomaterial platforms on the macro-scale as scaffold reservoirs of delivery is presented. The project described herein combines many elements of this introduction. A type I atelocollagen scaffold is proposed as a delivery platform for RNAi that is injectable and crosslinks in situ. The RNAi investigated in this study is in the form of an exogenous miR mimic of the 29B sequence (miR-29B) which is downregulated following ECM remodelling and has a pivotal role in ECM components synthesis. Finally, a poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA) based transfection agent (pD-b-P/DA) which can conjugate antibody fragments receptive to the CD90 antigen is synthesized as a protective vehicle of the exogenous miR. As anti-fibrotic therapy can be effective in promoting beneficial remodelling of the infarcted myocardium, promotion of recovery of cardiac function is possible and was investigated via systemic delivery in the appendix chapter.

1.10.1 Rationale for the use of a miR Mimic

Various non-viral delivery methods of miRs exist such as pDNA encoding shRNA, pre-miRs and mature double stranded miR mimics. It was decided to employ mature miR mimics in order to obviate the necessity for nuclear entry and transcription of pre-miRs. Furthermore, as miR-29B is transcribed along with other members of the miR-29 family, therefore the direct delivery of a mature miR sequence allows the determination of the effect of miR-29B alone.
1.10.2 Rationale for the use of miR-29B

Fibrosis caused by a dysregulation in the wound healing response is most likely due to traumatic injury, such that is present following MI. This fibrosis is mediated by the excessive deposition of collagen type I fibrils and re-organization of the ECM. The focus in this thesis is on reducing fibrosis by delivering miR-29B which is directly related (and also downregulated) during increased ECM remodelling, by delivering exogenous miR mimics of miR-29B.

1.10.3 Rationale for the use of pDMAEMA

A non-viral delivery approach composed of miR mimics and a pDMAEMA based transfection agent was developed. While viral systems can be among the most effective agents available for nucleic acid transfer, they have associated health risks, high production costs, and a variety of other drawbacks. Thus, a non-viral delivery system was selected. pDMAEMA based transfection agents have shown much efficacy as non-viral transfection agents posing minimal toxicity while also maintaining efficient delivery of nucleic acid. pDMAEMA has been shown to efficiently transfect a variety of cells; however there is a drawback of toxicity due to the cationic nature of this agent. The incorporation of poly (ethylene glycol) into such a structure can offer a steric shield to non-viral complexes from interaction with blood components and also contributes to reduced toxicity levels. Deactivation enhanced atom transfer radical polymerization enables the controlled synthesis and propagation of multi-vinyl monomers allowing for the creation of highly ordered structures with vinyl functionality. Chapter Two of this thesis provides further details in how this was envisaged for this thesis; briefly, vinyl functionality can allow for the incorporation and decoration of targeting moieties (in this case antibody fragments) which increases transfection efficacy by mediating receptor mediated endocytosis.

1.10.4 Rationale for the Selection of an Antibody Fragment Directed towards CD90

Considering that fibrosis is the therapeutic target of the platform developed in this thesis, it was sought to enhance the delivery of miR-29B to cardiac fibroblasts which are predominant cell type in the myocardium and play an active role in extracellular matrix remodelling. Cardiac fibroblasts, as with all fibroblasts, express CD90, a cell surface antigen which is not present in cardiomyocytes or the endothelium of the coronary vasculature. As previously discussed, at the end of the literature review; the functionalisation of gene delivery vehicles with cell receptive moieties (such as monoclonal antibodies, antibody fragments, peptides and scFvs) can improve their efficiency and uptake into cells preferentially. CD90 was selected due to its association with cardiac fibroblasts and
also as it was available through a hybridoma cell line which facilitates a continuing source of parent antibody through which antibody fragments can be derived in large amounts.

1.10.5 Rationale for the use of a Crosslinked Atelocollagen Type I Scaffold

Type I collagen was selected as the optimal scaffold because it is a well-characterised natural biomaterial that is commonly used in the field. Furthermore, many collagen-based biomaterial products are approved for medical use in humans by the US Food and Drug Administration (FDA) and European Medical Association (EMA). Atelocollagen is a less immunogenic form that is prepared by removing the telopeptides from the end of the collagen fibrils. Type I collagen is also the principal constituent of native extracellular matrix (ECM) which promotes growth, attachment, and proliferation of a variety of cell types. Finally, there are multiple reports of collagen being used as an effective nucleic acid delivery system, further recommending its use in this application.

1.10.6 Rationale for the use of Scaffold-Based RNAi Delivery

Individually, each of the elements (scaffold, miR mimics, non-viral delivery agent) of the proposed combinatorial RNAi delivery system have shown significant potential for use in tissue engineering applications. However, each component has limitations when delivered in isolation. It has been hypothesized that implanted collagen may play a minor role in modulating the production of tissue MMPs as part of the foreign body response. This role is exceptionally minor however, and collagen scaffolds have relatively minor effects on ischemic tissue and are ideal as a delivery system as they induces minimal foreign body response, are biodegradable, and can be prepared, stored and sterilized with relative ease. Hence, the use of a collagen scaffold reservoir system will act as a tuneable reservoir of miR-29B complexes and will also add functional support to the treated areas.

The combination of biomaterial scaffold and pDMAEMA based delivery agent should furthermore improve the efficacy of the RNAi, thereby allowing the miRs to act more efficiently. This thesis reports the development of each individual component and the combination thereof to improve delivery of miRs to effect a therapeutic treatment in the inhibition of fibrosis.

1.11 Objectives and Hypotheses

The overall hypothesis of this thesis is that an effective and efficient delivery of miR-29B complexes can be achieved to silence the production of collagen type I through an injectable non-toxic type I atelocollagen hydrogel scaffold with/without the use of a pDMAEMA based complexing agent. The specific objectives of the in vitro studies were to develop and characterize a pDMAEMA based delivery agent for complexation of miR mimics and conjugation of antibody fragments. The in vitro
studies were designed to develop and characterize an atelocollagen type I scaffold crosslinked using poly (ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG) and were evaluated for their ability and effectiveness as a delivery reservoir of RNAi in various forms towards a goal of silencing miR-29B. The in vivo studies investigated the effects of miR-29B on ECM remodelling in a rat syngeneic dermal excisional wound model.

Objectives: Phase I - Delivering Exogenous miRNA (Chapter Two)

- Synthesize a block co-polymer composed of a linear poly (dimethylamino) ethyl methacrylate (pDMAEMA) block and a hyperbranched poly (ethylene glycol) methyl ether acrylate (PEGMEA) based unit with poly (ethylene glycol) diacrylate (PEGDA) as the MVM branching agent (pD-/PDA) using De-ATRP synthesis. This resultant pD-/PDA will be characterised during polymer synthesis using gel permeation chromatography and its structure evaluated using proton nuclear magnetic resonance (\(^1\)H NMR).
- Demonstrate effective complexation of miR mimics with pD-/PDA by characterising its electrophoretic mobility, prove and demonstrate Michael-type addition of thiols to pD-/PDA using Ellman’s Assay, and antibody fragments to pD-/PDA by SDS-PAGE.
- Optimize and deliver miRs with pD-/PDA to effect silencing in a dual reporter system of Renilla Luciferase and Firefly Luciferase using cardiac fibroblasts as an experimental cell type.

Hypotheses: Phase I - Delivering Exogenous miRNA (Chapter Two)

- A co-polymer composed of a linear poly (dimethylamino) ethyl methacrylate (pDMAEMA) block and a hyperbranched poly (ethylene glycol) methyl ether acrylate (PEGMEA) based unit with poly (ethylene glycol) diacrylate (PEGDA) as the MVM branching agent (pD-/PDA) can complex and delivery miR mimics with reduced cytotoxicity and also offer the possibility of facile conjugation of additional functional groups by Michael type addition.

Objectives: Phase II- An Injectable Hydrogel Delivery System (Chapter Three)

- Develop an atelocollagen type I hydrogel and characterise the effect of crosslinking that can be achieved using 4S-StarPEG by determining the amine content of the scaffolds using a trinitrobenzenesulfonic acid (TNBSA) assay, the effect on the degradation profile of the hydrogels using collagenase degradation and the effect on mechanical properties using rheology.
Investigate the effect of crosslinker density on the release profile of interfering RNA from hydrogels and the effect of complexing the RNA with a complexing agent on the release of the RNA from the hydrogels, spectrophotometrically, using Cy™3 labelled siRNA.

Investigate the ability of this hydrogel acting as a reservoir in vitro of miR-29B, characterising its release profile and the subsequent effect of specifically determining the effect on production of collagen type I and type III expression in vitro.

**Hypotheses: Phase II - An Injectable Hydrogel Delivery System (Chapter Three)**

- An atelocollagen type I hydrogel crosslinked with 4S-StarPEG will act as a reservoir of miR-29B in naked form, miR-29B complexed with a poly (2-(dimethylamino) ethyl methacrylate) (pDMAEMA) based polymer (pD-b-P/DA- developed in Phase I) and miR-29B complexed with PEI.

- This scaffold/miR platform will effectively silence ECM genes; namely collagen type I and collagen type III.

**Objectives: Phase III – System Evaluation in a Rat Excisional Wound Model (Chapter Four)**

To investigate the effect of the components in this platform (miR-29B, 4S-StarPEG-collagen scaffold, pD-b-P/DA) on a number of key parameters involved in wound healing, using a number of techniques. Specifically:

- Evaluate, using histological analysis, wound contraction and granulation tissue formation following full thickness excisional skin wounding in a rat model.
- Quantify collagen type I and III deposition by polarised light microscopy and qualitative immunohistochemistry for collagen type III within the wound bed.
- Employ an antibody labelled membrane protein array to elucidate the up/down regulation of factors associated with apoptosis, inflammation and ECM remodelling.
- Use enzyme linked immunosorbert assays (ELISA) to quantify the expression of TGF-β1, TIMP-1, MMP-8 and determine the ratio of MMP-8: TIMP-1.
- Determine the effect of the components of this platform on genes associated with wound healing using a rat wound healing PCR Array.
Hypotheses: Phase III- System Evaluation in a Rat Excisional Wound Model (Chapter Four)

- Incorporation of miR-29B complexes (with or without the use of pDγ-P/DA) into a crosslinked collagen hydrogel will have significant benefit on wound closure, collagen type I and collagen type III deposition and key ECM remodelling mediators (TGF-β1, TIMP-1, and MMP-8) in a rat excisional wound model.
- These parameters will all be responsive to the dose of miR-29B incorporated within the scaffold and therefore a dose response will be observed through the parameters investigated.
Project Overview:

Delivery System for Injectable Reservoir of miR-29B Complexes

Phase I
- Delivering Exogenous miRNA
  - pDβ-P/DA Synthesis and Characterization
  - pDβ-P/DA-miR Optimisation
  - Antibody Fragment Functionalisation

Phase II
- An Injectable Hydrogel Delivery System
  - Hydrogel Development
  - Temporal Release Profiles with pDβ-P/DA
  - Temporal Silencing Profiles of Primary Cardiac Fibroblasts

Phase III
- System Evaluation in a Rat Excisional Wound
  - Evaluation of Remodelling Response to miR-29B Dose
  - Evaluation of Wound Contraction
  - Effect of System Components on ECM Remodelling
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Delivering Exogenous miRNA

The majority of this chapter has been previously published in:

2.1 Introduction

RNA interference (RNAi) is recognized as another gene therapy in manipulating the proteomic behaviour of cells and tissues at the post-transcriptional level. There is significant interest in the manipulation of endogenous small interfering microRNAs (miRs) and their subsequent increase and/or decrease in levels in states of development, regeneration, remodelling and pathogenesis \(^1\)\(^-\)\(^4\). These small, non-coding, naturally occurring RNAs negatively regulate the stability and translation of target protein-coding mRNAs at the 3` untranslated region. They typically affect a cluster of genes rather than one specific gene, and this allows them to play critical roles in a variety of biological processes.

Intuitively, delivery of such a therapeutic is a criterion that needs to be addressed as miRs are highly prone to degradation by ubiquitous RNases, have a short half-life in serum, must be internalized into eukaryotic cells and require obviation of lysosomal compartmentalisation. Additionally, they must reach their intended target effectively and persist for an extended time as in the case of systemic delivery \(^5\). The delivery of exogenous miRs, similar to plasmid based gene therapy, is achievable through both a viral and non-viral approach. Viral delivery systems exist have been tested in clinical studies to date, to effect long-term expression of a therapeutic gene with excellent efficiency, but sometimes transient regulation of aberrant genes during acute illnesses may be desired. Viral delivery also has inherent safety concerns such as immunogenicity and possible mutation; and despite advances in the field to overcome these concerns \(^6\), these factors still remain contentious. A non-viral vector approach, although less efficient than viral vectors, offers transient, and sometimes stable expression, is better suited to the mass production and the scalability necessary for clinical translation \(^7\). Non-viral vectors can prolong the serum half-life and intracellular buffering of the nucleic acid cargo, thereby improving pharmacokinetics and nuclease resistance. Previously, exogenous miRs have been complexed (encapsulated via electrostatic interactions between a cationic polymer and the negatively charged nucleic acid) and delivered using copolypeptides \(^8\). More recently, studies have reported on the therapeutic effect of delivering non-viral miRs in the form of duplex mature miR sequences using liposomal based carriers \(^9\) and plasmid DNA (pDNA) transcribing candidate miRs delivered systemically using liposomes \(^10\). As with the delivery of exogenous DNA to cells, efforts are being made to deliver interfering RNA by a number of non-viral methods which include liposomes \(^11\), nanoparticles \(^12\) and cationic polymers \(^13\). Specifically, the use of cationic polymer structures offers a facile approach that can enable tuneable, efficient and potentially large-scale production to be achieved.
To complex miR with a cationic polymer, a charge interaction transpires with that of the negatively charged phosphate groups of nucleic acid, usually facilitated by tertiary or secondary amines of the complexing agent. Efforts at reducing the toxicity of these polymers include PEGylation to enable systemic delivery and adequate interaction with blood components (thereby increasing circulation times) and the decoration of these structures with peptide and/or protein sequences. However, both these approaches present limitations in both processing and in the efficacy of the carrier. PEGylation of carriers does improve serum interaction and reduces toxicity with cells. Their functionalisation with peptides or monoclonal antibodies can introduce issues of immunogenicity and requires secondary linking which can be cumbersome and often introduces hazardous compounds.

The use of Michael-type addition reaction offers a facile ‘click chemistry’ approach in which an alkaline reaction solution behaves as a base donor and can enable covalent linkage, most commonly between vinyl groups and thiol (SH) moieties. Previous approaches have employed this technique in decorating surfaces with SH functionalized peptides with effective outcomes. This reaction enables a facile and orientated conjugation of SH terminated antibody fragments (Fab’s), derived from a monoclonal parent. It additionally would eradicate any complement-activated response by the Fc region of the antibody and orientate the antigen-binding region effectively on the surface of the structure. Attaching cell-targeting ligands (such as antibody fragments) to gene carriers can offer selectivity to a particular cell type/accumulation at target tissue, and control the route of internalisation into the target cell.

Previous efforts have reported a deactivation enhanced atom transfer polymerization (De-ATRP) approach, which can suppress the gelation and produce hyperbranched polymers from the homopolymerization of multivinyl monomers (MVMs). Furthermore, the employment of De-ATRP in can achieve hyperbranched transfection agent that have competitive efficiency when compared with commercial transfection agents. Based on these observations, it is hypothesized that a co-polymer composed of a linear poly (dimethylamino) ethyl methacrylate (pDMAEMA) block and a hyperbranched poly (ethylene glycol) methyl ether acrylate (PEGMEA) based unit with poly (ethylene glycol) diacrylate (PEGDA) as the MVM branching agent (pD-b-PDA) can reduce cytotoxicity and also offer the possibility of facile conjugation of additional functional groups by Michael-type addition.

Therefore, the objectives of the work presented in this chapter are:

i. Synthesize a block co-polymer composed of a linear poly (dimethylamino) ethyl methacrylate (pDMAEMA) block and a hyperbranched poly (ethylene glycol) methyl ether acrylate
Delivering Exogenous miRNA

(PEGMEA) based unit with poly (ethylene glycol) diacrylate (PEGDA) as the MVM branching agent (pD\textsubscript{b}/PDA) using De-ATRP synthesis. This resultant pD\textsubscript{b}/PDA will be characterized during polymer synthesis using gel permeation chromatography and its structure evaluated using proton nucleic magnetic resonance (\textsuperscript{1}H NMR).

ii. Demonstrate effective complexation of miR mimics with pD\textsubscript{b}/PDA by characterising its electrophoretic mobility, prove and demonstrate Michael-type addition of thiols to pD\textsubscript{b}/PDA using Ellman’s Assay, and antibody fragments to pD\textsubscript{b}/PDA and by SDS PAGE.

iii. Optimise and deliver miRs with pD\textsubscript{b}/PDA to effect silencing in a dual reporter system of Renilla Luciferase and Firefly Luciferase using cardiac fibroblasts as an experimental cell type.

2.2 Materials and Methods

2.2.1 Materials

All laboratory consumables were obtained from Sigma Aldrich (Dublin, Ireland), unless otherwise stated. Monomers 2-(dimethylamino) ethyl methacrylate (DMAEMA M\textsubscript{W} = 157), poly (ethylene glycol) diacrylate (PEGDA M\textsubscript{W} = 258), poly (ethylene glycol) methyl ether acrylate (PEGMEA M\textsubscript{W} = 454) were purchased from Sigma Aldrich Corporation. Ethyl 2-bromoisobutyrate (EBriB, 98%, Sigma Aldrich), Pentamethyldiethylenetriamine (PMDETA, 99%, Sigma Aldrich), copper (II) chloride (CuCl\textsubscript{2}, 97%, Sigma Aldrich), L-ascorbic acid (L-AA, 99%, Sigma Aldrich), d-Chloroform (99.8%, Sigma Aldrich), 2-Butanone (HPLC grade, LabScan), Dimethylflormaide (DMF, HPLC grade, Thermo Fisher Inc., Dublin, Ireland) were used as received. SYBR® Safe Gel stain (Invitrogen, Dun Laoghaire, Ireland) and alamarBlue® (Invitrogen) were used as received. pmirGlo™ Vector, restriction enzymes and DualGlo® luciferase assay kit were purchased from Promega Corporation (Southampton, United Kingdom). Oligonucleotides were purchased from Eurofins MWG GmbH (Ebersberg, Germany).

2.2.2 ATRP of pDMAEMA

EBriB (0.2732 g, 1 equiv) was used as the radical initiator and the reaction was catalysed PMDETA (0.061 g, 0.5 equiv)/ CuCl\textsubscript{2} (0.0347 g, 0.25 equiv). DMAEMA was added at a corresponding molar equivalent of 127 (27.96 g) with an equal volume of butanone acting as the reaction solvent, and mixed in a double-necked round bottomed flask. To this the EBriB and PMDTA were added and, the mixture was purged with argon for 20 minutes to remove any oxygen in the mixture. CuCl\textsubscript{2} was added to the inert mixture and the reaction initiated by immersion in an oil bath at 50 °C stirring at 700 rpm. The reaction was halted when monomer
conversion of ~50% was achieved by purging oxygen into the system. The resultant polymer was precipitated drop-wise in a five-fold excess volume of hexane. After it had stood for 20 minutes, the excess hexane was decanted and residual hexane removed by vacuum drying. (Appendix E.1).

2.2.3 De-ATRP of pD$_v$-P/DA

pD$_v$-P/DA was synthesized via a modified deactivation enhanced ATRP reaction as previously described $^{18}$. Briefly, this modification allows the formation of a hyperbranched structure in a simple ‘one-pot’ reaction, giving the advantages of a branched structure while avoiding the complications of dendrimer synthesis and purification. Unlike conventional ATRP that uses a halogen –Cu$^I$/Ligand catalyst, deactivation enhanced ARTP (De-ATRP) uses a halogen – Cu$^I$/halogen-Cu$^{II}$ mixture that enhances the deactivation of polymerization. The approach in this study utilizes CuCl$_2$ as the sole catalytic reagent which is reduced to the Cu$^I$ state by the use of a reducing agent: L-ascorbic acid (L-AA). Thus, the ATRP deactivation/activation equilibrium can be easily adjusted by the L-AA ratio, which facilitates controlled chain grown. (Appendix E.2)

Linear pDMAEMA, synthesized and as described in the previous paragraph, was employed as a macro-initiator in a De-ATRP reaction. pDMAEMA was dissolved in a four-fold excess of butanone as the reaction solvent and added to a double-necked round bottomed flask. To this, poly (ethylene glycol) methyl ether acrylate (PEGMEA) and poly (ethylene glycol) diacrylate (PEGDA) were added at ratios of 90 and 10 respectively corresponding to the original EBriB quantity added in the previous ATRP reaction (57.204 g and 3.612 g respectively). The mixture was purged with argon for 20 minutes to remove any oxygen in the mixture and dynamic equilibrium was set by adding L-AA (100 μl of 48.6 mg/ml L-AA/ deionized water solution, 0.025 equiv) under positive pressure of argon to reduce Cu$^{II}$ (deactivated state) to Cu$^I$ (propagation state). The reaction (see Figure 2.1) was carried out in a two-neck round bottomed flask under argon following addition of L-AA, and immersed in an oil bath at 50 °C stirring at 700 rpm. Samples were withdrawn at the start and after every hour for gel permeation chromatography (GPC,Varian™ 920-LC, Agilent Technologies, USA), with an additional final sample being taken at the end for proton nuclear magnetic resonance analysis ($^1$H NMR , 300 MHz NMR Spectrometer, Bruker, UK). Copper was removed from the GPC samples by running them through a silica gel column, followed by dilution in Dimethyl Formaldehyde (DMF) for analysis. The reaction was halted by purging oxygen into the system. The polymer was precipitated dropwise into five-fold excess of cooled 1:1 hexane: di-ethyl ether. Re-dissolved polymer (in acetone) was then dialyzed with distilled water for several days using a 6000-8000 M$_w$ cut-off dialysis membrane (Spectra/Por®, Spectrum Laboratories®, Breda, The Netherlands) before being
Figure 2.1: Schematic diagram of deactivation enhanced – ATRP showing the activated and deactivated routes, controlling the formation of a branched structure, which will enhance the uptake of these complexes. Antibody fragment binds to the free vinyls available in a Michael type addition whereas cationic attraction between pDMAEMA and miR enables complexation.
freeze dried for subsequent studies. The final polymer product was stored at -20 °C and protected from light until use.

2.2.4 Molecular Weight Determination by Gel Permeation Chromatography
Small samples were withdrawn from the reaction at specific intervals using a glass syringe with luer needle under positive pressure of argon. These were then diluted in DMF and filtered through alumina for chromatography followed by a 0.2 μm filter before analysis. The molecular weight and molecular weight distribution of each sample was determined using a Varian™ 920 LC instrument with a refractive index S2 detector (RI). Chromatograms were run at 50 °C using DMF as eluent with a flow rate of 1 ml/min. The machine was calibrated with linear polystyrene standards. A detailed description of GPC sample preparation is available in Appendix E.3.

2.2.5 $^1$H Nuclear Magnetic Resonance
$^1$H NMR was performed using a 300 MHz Bruker NMR with MestRec™ processing software. The polymer was dissolved in Deuterated chloroform (CDCl₃, Sigma Aldrich) for $^1$H NMR analysis and all chemical shifts are reported in ppm relative to dilute tetramethylsilane (TMS). The $^1$H NMR spectrum confirmed the presence of each monomer in the polymer structure and the presence of free vinyl groups. The NMR spectrum was used to determine the degree of branching within the polymer structure via Equations 2.1.

2.2.6 Agarose Gel Electrophoresis
Complex formation of miR/ pD-b-P/DA was observed by polyacrylamide gel retardation. This procedure enables the user to see the effect of complexation on electrophoretic mobility of oligonucleotides. Various weight ratios of pD-b-P/DA, from 0 to 20, were added to 200 ng of miR-29B (Syn-rno-miR-29B; miScriptmiRNA Mimic™ (MSY0000801), Qiagen, Hilden, Germany) in phosphate buffered solution (PBS), vortexed briefly and incubated for 30 minutes at room temperature. After incubation, each sample was mixed proportionately with a loading dye and electrophoresed on a 13% polyacrylamide gel (w/v) for one hour at 100 V. 89 mM Tris-borate (TBE), 2 mM ethylenediaminetetraacetic acid (EDTA) buffer was used as the electrophoresis buffer. Following SYBR® Safe (1:10000) staining for ten minutes, the gel was illuminated with a UV illuminator to show the location of migrated miR complexes. This experiment was repeated under the same conditions except that the direction of charge was reversed in order to verify complex formation. A detailed description of this protocol is available in Appendix F.2.
2.2.7 Thiol Binding of Polymer

The predicted presence of vinyl groups on this structure enables the covalent binding of thiols via Michael type addition. In a model system, cysteine hydrochloride monohydrate was employed as a model of cysteine, having a free thiol group present in its structure. The binding efficacy of pD-β-P/DA was evaluated by monitoring the consumption of cysteine over time and measured indirectly using Ellman’s assay (Appendix N.1). This assay is a colorimetric reaction between Ellman’s reagent (5, 5’-dithiobis (2-nitrobenzoic acid), DTNB) and free thiol (SH) groups. 0.4 mg of DTNB dissolved in 1 mL of the corresponding Ellman’s buffer (0.2 M NaH₂PO₄ with 1 mM EDTA at pH 8.0, purged with argon for 30 minutes) was used as stock solution. For baseline measurement, Ellman’s stock diluted 1:10 in Ellman’s buffer was taken as blank. Standards were created using cysteine hydrochloride monohydrate (Sigma Aldrich) dissolved in Ellman’s buffer (0 – 1.5 mM final concentration). Known concentrations of cysteine were incubated with optimal ratios of pD-β-P/DA, and corresponding weights of poly (ethylenimine) (PEI), using Ellman’s buffer as the reaction buffer. Samples were serially diluted in Ellman’s buffer and 10% (v/v) of the stock solution and incubated at 37 °C. At each time point (0, 0.5, 1, 2 and 3 hours), 50 μl of Ellman’s reagent was added to the reactant. Solutions were measured at 412 nm and the concentration of thiol groups was extrapolated from the standard curve.

2.2.8 Fab’ Generation

Hybridoma cultures expressing antibodies against CD90 (Health Protection Agency, Salisbury, UK) were expanded in RPMI containing 20% serum. The CD90 antigen was chosen as it is expressed on the cell surface of fibroblasts which would enable more cell surface attachment of non-viral vectors with a CD90 antibody fragment attached. These cultures were weaned in graduations of 10, 5, 2.5 and gradually to 0.5 % serum concentrations. Cells were maintained at 0.5% fetal bovine serum (FBS) concentration for six weeks at a concentration of 5 x 10⁵ cells/ml to produce antibodies. At the end of incubation, remaining cells and debris were removed from the media through centrifugation and the protein fraction of the media separated on Centricon® (MWCO = 100 kDa, Millipore Corp., Billenica, MA, USA) filter devices. Antibodies within this fraction were isolated using an antibody purification kit according to the manufacturer’s specifications (Nab™ Protein A/G Spin Kit, Thermo Scientific, Dublin, Ireland). Generation of F(ab’)₂ was performed using the Pierce F(ab’)₂ Preparation Kit™ (Thermo Scientific) according to the protocol provided. Fab’ generation was performed using Reduce-Immobilized Reductant Columns™ (Thermo Scientific) according to the protocol provided by the manufacturer. Molar quantification of antibody fragments was based on free thiol determination based on Ellman’s assay. Detailed descriptions of these protocols are available in Appendices J.1, J.2, and J.3.
2.2.9 **Fab’ Conjugation**

Known molar concentrations (deduced from Ellman’s assay) of Fab’s were reacted with pDγ-P/DA in a reaction buffer of 0.2 mM NaH₂PO₄ with 1 mM EDTA at pH 8.0 (purged prior to reaction with argon for 30 minutes). Briefly, reactions were performed in which molar ratios of Fab’ thiol concentration (from Ellman’s assay) and moles of free vinyls (calculated from ¹H NMR data) were reacted at ratios of 1:1 and 1:2. Fab’s alone were used as a control. Samples were reacted for one hour under gentle rocking (Figure 2.2).

2.2.10 **Characterization of miR-Complexes Decorated with Antibody Fragments**

Sodium dodecyl sulfate poly (acrylamide) gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions was conducted to confirm conjugation of Fab’s to pDγ-P/DA. Briefly, 1 μg of each sample was removed (confirmed by absorbance at 280 nm) denatured via heat treatment and, in the case of reducing conditions, reduced using β-mercaptoethanol. Samples were loaded onto 10 % acrylamide gels (Bio-Rad Laboratories Inc., Hertfordshire, UK) and electrophoresis was performed at a constant voltage (150 V) for approximately one hour for both reducing and non-reducing conditions. The proteins were revealed by silver staining (SilverQuest™ Silver Staining Kit, Invitrogen) and subsequently micrographed. Refer to Appendix K for detailed protocol.

Agarose gel electrophoresis was performed to see the effect of antibody fragment conjugation on complex formation. Briefly, 1 μg of miR was complexed at varying w/w ratios with pDγ-P/DA conjugated with antibody fragments at a w/w ratio of 1:1 (moles antibody fragments free thiols: moles vinyl groups available in PEGDA portion of pDγ-P/DA). An agarose gel (1% agarose in TBE buffer, with SYBR®Safe DNA stain) was made to analyze the weight/weight ratio for polyplex formation. 5 ml of each polymer/miR solution were added along with 5 μl loading dye to each well and subjected simultaneously to 80 mV for up 30 minutes.

2.2.11 **Reporter Plasmid Construction, Transformation, Propagation and Isolation**

For construction of a vector containing miRNA target sequence fused downstream from a luciferase expression cassette, the pmiRGlo™ Dual-Luciferase miRNA Target Expression Vector (Promega Corporation) was employed (Figure 2.3). This plasmid encodes transcription for both Renilla and Firefly luciferase with a miR binding site downstream of the Firefly mRNA. The pmiRGlo™ vector was linearized using XhoI and XbaI restriction enzymes (Promega Corporation) for three hours at 37 °C. This reaction was inactivated by heat at 65 °C for 25 minutes and purified using Qiaquick® PCR purification kit (Qiagen).
PCR primers (Eurofins MWG GmbH) sharing homology (underlined) with sticky ends of the XhoI and XbaI linearized pmiRGlom vector and 23 bases (bold) complementary with hsa/rno/mur-miR-29B; Sense: 3’-tacgctggtaaatctgtcactgatcgccggcgat-5’ and Antisense: 3’-cactgcggcgatctttaactataaaggatagt-5’, were annealed and ligated into the linearized plasmid using T4 DNA ligase (Promega Corporation) overnight at 4 °C.

DH5-α competent cells (Invitrogen) were transformed with the resultant plasmid (pmiR-29B) and unmodified plasmid (pmiR-Control), and selected twice in antibiotic containing LB broth and on LB agar plates. Plasmid expansion was performed as recommended in the Maxi-Prep™ (Qiagen) protocol and isolated using that kit. Plasmid purity was confirmed by UV spectroscopy (NanoDrop™ ND1000 Spectrophotometer, Thermo Scientific), agarose gel electrophoresis and sequenced for verification. All protocols for construction of the miR reporter plasmid are available in Appendix G. Sequencing of this plasmid to verify correct ligation is also included in Appendix G.

2.2.12 Cell Extraction

Primary rat cardiac fibroblasts were chosen as the target cell type in this study as they express the CD90 antigen (towards which, the antibody fragments bind) and are a relevant cell type to target due to their somatic lineage and given that they are a primary cell type. These were isolated from neonatal pups at day three as previously described and available in detail in Appendix I.8. Briefly, neonatal rat ventricle myocytes were isolated from the cardiac ventricles three day old Sprague-Dawley pups. Hearts were removed from the thoracic cavity and placed in a tube containing cold 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution (20 mm HEPES, pH 7.4). Ventricles were separated from cardiac tissue using scissors and minced into several pieces. Subsequently, cardiomyocytes and fibroblasts were detached from the extracellular matrix by repeated incubation in collagenase, supplemented with 2 mg/ml trypsin and 20 µg/mgDNase. Cells were collected by centrifugation and tissue clumps were removed by filtration. Subsequently, cells were pre-plated in cell culture dishes in 50 ml Dulbecco Modified Eagles’s Minimum Essential Medium (DMEM)/F12 (50:50) medium with 5% fetal calf serum (FCS) for 45 minutes. During this period, most non-cardiomyocyte cells (mainly fibroblasts) attached to the dish, whereas cardiomyocytes remained in solution. Fibroblasts were subsequently cultured in DMEM medium containing 10% FCS.

2.2.13 Transfection Studies

For all transfection and silencing studies, cardiac fibroblasts up to the second passage were seeded at a density of 10,000 cells/well in a clear 96 well plate. Cells were left for 24 hours to attach to the plates prior to the transfection studies. Three co-transfection experiments were performed. In all
Figure 2.2: Michael type addition of antibody fragment to pD-ν-P/DA using available free vinyls. (a) Antibody fragment with heavy and light chains highlighted and reduced disulphide bond to create free thiol termination. (b) Michael type addition of thiol terminated antibody fragment to free vinyls using base donor solution to donate electrons for covalent linkage of thiol to double carbon bond of pD-ν-P/DA. (c) Final reaction product.
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experiments, the media was removed from all cells (including controls) and 1 μg of pmiRGLO™ pDNA, complexed at a 9:1 w/w ratio with SuperFect™ (Qiagen) for 30 minutes, was added to each well. Plasmid complexes were incubated with cardiac fibroblasts for four hours with serum free media and removed.

In the first experiment, the validity of pmiRGLO™ as a reporter system, and the ability of the miR-29B to be a specific silencer of this system was tested. pmiR-29B and pmiR-control were added to wells and following this miRs were added; miR-29B mimic (5’-UAGCACCAUUUGAACAGUGUU-3’) (Qiagen) and a scrambled control (5’-GTGCTCTCAATTAACGTAAATTGA-3’) (Qiagen). A total mass of 330 ng miRs was delivered to each well complexed with Dharmafect™ (Thermo Scientific Corporation, USA), a commercially available lipid based transfection reagent, at a w/w ratio of 2:1. Cells were cultured in 75 μl of media containing 10 % FCS. To elucidate the efficacy of silencing, Dual-Glo® Luciferase Assay System (Promega Corporation) was employed. Forty-eight hours following administration of interfering RNA, 75 μl of luciferase reagent was added to each well and allowed to react for ten minutes in the dark. Luminescence of each well was read, indicative of Firefly Luciferase activity. After luminescence reading, 75 μl of Dual-Glo® Stop and Glo® reagent was added to each well and mixed. After ten minutes, luminescence of each well was determined which is indicative of Renilla Luciferase expression. The ratio of firefly luminescence to Renilla luminescence for each experimental sample was normalized to the ratio of firefly luminescence to Renilla luminescence for the control and expressed as a percentage of normalized transfection (see Equations 2.1).

Equations 2.1:

\[
Ratio = \frac{\text{LUMINESCENCE}_{\text{RENILLA}}}{\text{LUMINESCENCE}_{\text{FIREFLY}}}
\]

\[
\text{Relative Inhibition} \quad RI = \frac{(Ratio_{\text{sample}} - Ratio_{\text{negative control}})}{(Ratio_{\text{positive control}} - Ratio_{\text{negative control}})}
\]

In the second experiment, miR-29B mimic was delivered at varying w/w of miR-29B: pD-P/P/DA with the weight of the miR mimic kept constant at 330 ng per well. Forty-eight hours after transfection with miR-29B, the cardiac fibroblast cultures were assessed for Firefly and Renilla Luciferase activity as described above. miR-29B delivered in naked form and miR-29B complexed with Dharmafect™ were used as comparative controls.
Figure 2.3: Overview of reporter luciferase knockdown. (A) miR-29B sequence for silencing protein expression. (B) pmiRGlo vector construction. The firefly and Renilla luciferase genes are driven by human phosphoglycerate kinase (PGK) and simian virus 40 (SV40) promoters, respectively. A binding site of miR-29B was cloned into the pmiR-Glo vector downstream of the firefly gene (pmiR-29B) using a multiple cloning site (MCS). (C) Sequence details of pmiR-29B with target sequence in purple, a NOT-I digestion site in Blue and restriction enzyme sites XbaI and XhoI in red.
Concurrently, an additional plate, subjected to the same experimental conditions as this second experiment, was created in order to assess the effect of each condition on cellular metabolic activity via reduction of alamarBlue® as a oxidation reduction indicator which is dependent on both the glycolytic and oxidative metabolism of glucose within the mitochondria of eukaryotes. Oxidation reduction within the mitochondria of active cells effects a calorimetric change in alamarBlue®, and relative metabolic activity can be extrapolated from known controls. Absorbance values were normalized to the control cells (untreated cells), so any decrease from that of the control cells is a loss of metabolic activity. After the incubation time (48 hours), alamarBlue® working solution (10% alamarBlue® in Hanks Balanced Salt Solution, HBSS) was added to each well incubated for one hour. The alamarBlue® solution in each well was transferred to a fresh flat bottomed 96 well plate for absorbance measurements at 550 nm and 590 nm. Relative metabolic activity was followed as per protocol and control cell values normalized to untreated cells on tissue culture plastic. All values (including standard deviation) were subsequently normalized and plotted. See Appendix H for a detailed description of this protocol. In the third, final experiment, two cell types were employed; primary rat cardiac fibroblasts, isolated and cultured as described above; and human umbilical vein endothelial cells (HUVECs, Lonza Basel, Switzerland) cultured in Endothelial Basal Media, EBM-2™ (Lonza) supplemented with EBM-2 SingleQuots™ (Lonza), and 1 % v/v penicillin/streptomysin. All cells were seeded at 10,000 cells/well in a 96 well plate. In this experiment, miR-29B was complexed with either pD-γ-P/DA or Fab’- pD-γ-P/DA at a w/w ratio of 8:1. Dharmafect™ was used as a comparative control, with no miR-29B being used for normalization of the relative Renilla/Firefly luciferase expression. Experimental samples and controls were analyzed at 48 and 96 hours.

2.2.14 Statistical Analyses

Statistical analyses were performed using GraphPad Prism® (v.5 GraphPad Software, San Diego, CA, USA). D’Agostino and Pearson omnibus normality tests were used to verify normal distribution. Where normal distribution was evident, one-way ANOVA was performed, followed by Tukey’s post-hoc test. p values < 0.05 were considered to be statistically significant.

2.3 Results and Discussion

Linear pDMAEMA was synthesized via ATRP. This linear pDMAEMA acted as a macro initiator for deactivation enhanced atom transfer radical polymerization of PEGMEA and PEGDA to produce a PEG based copolymer with a hyperbranched structure (Figure 2.1). ATRP deactivation/activation equilibrium was tuneable by L-ascorbic acid (L-AA) that facilitated controlled chain growth. Gel permeation chromatography demonstrated the controlled nature of de-ATRP, which delays gelation,
allowing the branching of the PEGDA unit (Figure 2.4). Degree of branching and chemical structure was evaluated using \(^1\)H NMR, (Figure 2.5).

The ability of the block copolymer to complex miR was examined by acrylamide gel electrophoresis. miR mimics were reacted with increasing w/w ratios of the pD-b-PDA/ miR (Figure 2.6). Weight ratios are quoted for increased accuracy over the commonly used N/P quotation due to possible variations arising in M\(_n\) values across GPC instruments/calibration. Electrophoretic mobility of the complex shifted between a w/w ratio of 4:1 and 5:1. This shift in electrophoretic mobility is very apparent, and in w/w ratios lower than 4:1 there was an indication of some complexes beginning to be neutrally/positively charged, which is evident by some RNA migrating much slower down the lanes compared to the naked RNA control with no pD-b-PDA present. Therefore, at a w/w of five, all miR mimics have bound to the pD-b-PDA causing a shift in the net charge of the complex.

The ability of pD-b-PDA to conjugate SH groups was verified using two methods. Both methods employed Michael type addition reaction. In this reaction, base-catalyzedthio-acrylate coupling occurs whereby movement of electrons from the thiolate ion (Michael-donor) to the carbon-carbon double bond of the acrylate (Michael-acceptor) and back to the base catalyst to form the Michael adduct (See Figure 2.2 for schematic of this reaction). 0.1 mM PBS, pH 8.0, 1mM EDTA was used as the base catalyst in both reactions. In the first experiment, L-cysteine hydrochloride monohydrate was employed as the Michael-donor and reacted with pD-b-P/DA (Michael acceptor). Poly (ethylenimine) (PEI) was used as a control. Available thiols remaining during the reaction were indirectly assayed using Ellman’s reagent. This assay is a colorimetric reaction between Ellman’s reagent (5, 5′-dithiobis (2-nitrobenzoic acid), DTNB) and free SH groups. It was seen that a significantly greater consumption of thiols occurred after 30 minutes using pD-b-P/DA than what was seen using PEI control (Figure 2.7). This consumption plateaued which demonstrates that all accessible thiols had been conjugated to the pD-b-P/DA. Conversely, minimal consumption of thiols was observed using PEI, and any reduction in thiols can be attributed to oxidation/di-sulphide formation between thiols.

The reaction was maintained for one hour, after which selected samples were transferred for SDS-PAGE separation under both non-reducing and reducing conditions (via incubation with β-mercaptoethanol which reduces disulphide linkages namely the Fab’ heavy and light chain). Conjugation of the antibody fragment is evident from micrographs of SDS-PAGE gels (Figure 2.8). Under non-reduced conditions, a delay in migration of Fab’s indicated that their molecular weight increased due to conjugation to pD-b-P/DA. Under reduced conditions, breakage of the di-sulphide linkage of the heavy and light chains occurs. Typically, both the heavy and light chains should show as distinct bands at approximately 25kDa; however, there is a retardation of the heavy chain and this
Figure 2.4: Representative GPC trace showing the controlled growth of \( \text{pD}_{b\text{-}P/DA} \) over time. A increase in retention time (indicated by a shift in the curve to the left) demonstrates controlled increase in molecular weight of \( \text{pD}_{b\text{-}P/DA} \) and the unimodal distribution of the curves demonstrates an appropriate poly dispersity index (PDI).
Table 2.1: GPC peak analysis showing percentage monomer conversion during de-ATRP using linear pDMAEMA as a macroinitiator and L-AA to facilitate chain growth. Reaction took place at 50 °C, with Butanone as the reaction solvent.

<table>
<thead>
<tr>
<th>Time point (h)</th>
<th>$M_n^{[a]}$ (g/mol)</th>
<th>$M_w^{[b]}$ (g/mol)</th>
<th>PDI$^{[c]}$</th>
<th>% Polymer$^{[d]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7100</td>
<td>7400</td>
<td>1.04</td>
<td>0.01</td>
</tr>
<tr>
<td>6</td>
<td>7500</td>
<td>9500</td>
<td>1.27</td>
<td>10.7</td>
</tr>
<tr>
<td>24</td>
<td>9700</td>
<td>15624</td>
<td>1.62</td>
<td>31.4</td>
</tr>
</tbody>
</table>

[a] $M_n$ = Number average molecular weight  
[b] $M_w$ = Weight average molecular weight  
[c] PDI = Poly dispersity index  
[d] % polymer conversion calculated by measuring the peak area conversion.
Figure 2.5: $^1$H NMR spectra of pD-b-P/DA used to confirm the branching structure of pD-b-P/DA synthesized from a linear pDMAEMA (acting as a macro initiator) by in situ DE-ATRP. From this spectra, the structure pD-b-P/DA was evaluated by integrating the peaks present in the spectra and applying these integrals to Equations 2.2.
Equations 2.2

DMAEMA Component \( (n) = \frac{g}{2} \)

PEGMEA Component \( (m) = \frac{i}{3} \)

PEGDA (free vinyl) Component \( (x) = \frac{d + e + f}{3} \)

Branching PEGDA Component \( (y) = c - (2n + 4x + 2m) \)

Total Content \( = n + m + x + y \)

Therefore, the percentage of each component within the polymer structure is a percentage of the Total Content, for instance:

\[
\% \text{ DMAEMA} = \frac{n}{n + m + x + y}
\]

Table 2.2: Polymer composition as calculated from \(^1\text{H}\) NMR peak area by Equations 2.1.

<table>
<thead>
<tr>
<th>Polymer Composition by (^1\text{H}) NMR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMAEMA</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>68</td>
</tr>
</tbody>
</table>
Figure 2.6: pD-γ-P/DA binding ability of miR mimic at increasing w:w ratios (0.5:1 → 20:1) demonstrated by representative acrylamide gel electrophoresis; vertical arrow indicates direction of charge. (a) Negatively charged RNA migrates towards the anode (- negative) whereas binding with pD-γ-P/DA facilitates electrostatic interaction causing a net positive charge of RNA and the RNA travels towards the cathode (+ positive). Complete shift in electrophoretic mobility begins at a w:w ratio of 5:1 however there is RNA present towards the cathode at lower w:w ratios but complete binding does not occur at the lower w:w ratios. Verification of complexation observed in (a) was performed by reversing the charge terminals in (b) and the same observations occur.
Figure 2.7: pD\textsubscript{b}-P/DA significantly consumes cysteine compared to PEI (control) after 30 minutes as measured indirectly using Ellman’s assay. There is a slight reduction in μmoles of cysteine after 30 minutes, however this can be attributed to possible oxidation of the cysteine or formation of di-sulphide bonds between the cysteine and itself. Consumption occurs through Michael-type addition of thiols with the double carbon bond present in pD\textsubscript{b}-P/DA. Data presented is the mean ± standard deviation, n = 4.
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Figure 2.8: SDS acrylamide gel of pD-b-P/DA conjugated with CD90 antibody Fab`\'s at molar ratios of pD-b-P/DA reacted with moles thiol from antibody Fab`\'s (determined using Ellmans assay), under non-reduced conditions and reduced conditions (treatment with β-mercaptoethanol to separate the heavy (CH) and light (CL) of the antibody fragments). Non-reduced conditions reveal an increase in molecular weight of the Fab`\'s due to conjugation with pD-b-P/DA due to the slower migration of the bands which is most evident at a 2:1 molar ratio. Additionally, under reduced conditions, the heavy chain (CH) of the antibody fragment also has an increased molecular weight due to the conjugation of pD-b-P/DA.
Delivering Exogenous miRNA

Relative Inhibition: \[ \text{RI} = \frac{(\text{Ratio}_{\text{sample}} - \text{Ratio}_{\text{negative control}})}{(\text{Ratio}_{\text{positive control}} - \text{Ratio}_{\text{negative control}})} \]

Figure 2.9: Verification of reporter system with pmiR-29B (plasmid encoding firefly luciferase with miR-29B target). miR-29B delivery effectively knocks down Firefly luciferase in target pmiR-29B transfected cardiac fibroblasts compared to pmiR-Control (unmodified plasmid) and delivery of scrambled miR. Data presented is the mean ± standard deviation, n = 6. * indicates a statistically significant knockdown compared to all other groups, p < 0.05.
Figure 2.10: Optimisation of Dharmafect™ to miR-29B mimic w/w ratio by evaluating relative inhibition using reporter luciferase plasmid (left y-axis) and cellular metabolic activity through alamarBlue® metabolic oxidative reduction (right y-axis). The most significant knockdown of normalized expression occurs at a w/w ratio of 2:1 while maintaining appropriate cellular metabolic activity. A constant weight of 300 ng of miR-29B mimic was used in this experiment. Higher w/w ratios result reduced metabolic activity and also less relative inhibition. Data presented is the mean ± standard deviation, n = 4. * indicates a statistically significant knockdown compared to all other groups, p < 0.05.
Figure 2.1: Optimisation of Dharmafect™ complexed miR-29B dose in terms of μg RNA delivered at a w/w of 2:1 Dharmafect™: miR mimic. Relative inhibition was evaluated using reporter luciferase plasmid (pmiR-29B, left y-axis) and cellular metabolic activity through alamarBlue® metabolic oxidative reduction (right y-axis). The most significant relative inhibition occurs at a dose of 0.3 and 0.35 μg while maintaining appropriate cellular metabolic activity. Higher miR-29B doses result in a reduction in metabolic activity toxicity and also an increase in relative inhibition which is due to the increased Dharmafect™ that the cells are subjected to. Data presented is the mean ± standard deviation, n = 4. * indicates a statistically significant knockdown compared to all other groups, p < 0.05.
Figure 2.12: Relative inhibition of reporter luciferase (left y-axis) in response to naked miR-29B, miR-29B complexed at varying w/w ratios with pD-b-P/DA and controls. Also presented is the effect of miR-29B complexed with pD-b-P/DA and controls with on cellular metabolic activity (right y-axis). Data presented is the mean ± standard deviation, n = 4. * represents a statistically significant difference at p< 0.05.
Figure 2.13: Antibody fragment conjugation does not affect the complexation of pD$_{b}$-P/DA with RNA. Representative agarose gel electrophoresis of immunopolyplexes (decorated with antibody fragments). Each lane is labelled according to the w/w ratio of pD$_{b}$-P/DA: miR mimic. Negatively charged, uncomplexed miR mimic migrates towards the anode (- negative) terminal whereas miR mimics that are complexed with pD$_{b}$-P/DA undergo a change in electrophoretic mobility as they become positively charged and travel towards the cathode (+ positive). There is a separation at w/w ratios of 2:1 and 4:1 (i.e. two distinct bands in the same lane) as some, but not all miR mimics have become complexed with pD$_{b}$-P/DA and therefore the free negative RNA migrates towards that anode and the positive pD$_{b}$-P/DA complexed RNA travels towards the cathode.
Figure 2.14: Relative inhibition of reporter luciferase from cardiac fibroblasts (white bars) and HUVECS (black bars) pre-transfected with pmiR-29B and subsequently treated with miR-29B in different formulations. Data presented is the mean ± standard deviation, n = 4. * indicates a statistically significant difference at 48 hours compared to other groups at that time point (p < 0.05).
Figure 2.15: Cellular metabolic activity derived from alamarBlue® reduction of cardiac fibroblasts (white bars) and HUVECS (black bars) pre-transfected with pmiR-29B and subsequently treated with miR-29B in different formulations. Data presented is the mean ± standard deviation, n = 4. No significant difference was detected between any of the groups.
Delivering Exogenous miRNA

together with slower migration of Fab` above demonstrates that conjugation of Fab`s with pD-γ-P/DA has occurred, thereby increasing the molecular weight of the heavy chain of the Fab`. To monitor the efficacy of this system in silencing a gene of interest by delivering exogenous miRs, a dual-luciferase miR target expression vector (pmiRGLO™, Promega Ltd., UK) was employed. pmiRGLO™ was digested, linearized and ligated with a target sequence of rodent miR-29B. To confirm digestion, the original plasmid, and the modified plasmid (pmiR-29B) were incubated with target (miR-29B) and control mismatched sequences (miR-Scram) (Figure 2.10). Significant silencing was observed when miR-29B was delivered to cells pre-transfected with pmiR-29B. Furthermore, the pmiR-29B was sequenced (see Appendix G.9 for sequencing results) and verified to contain the ligated sequence. The conditions of the Dharmafect™ preparation were optimized beginning with optimization of w/w ratio and following this the quantity of complexes that could be delivered. This optimization was assessed by evaluation of silencing (normalized transfection) and cellular metabolic activity. The appropriate w/w ratio was first determined to be 2:1 (Figure 2.11). Following this, the least toxic w/w ratio was tested to determine the most effective dose without compromising cellular viability. The most significant silencing of the normalized luciferase expression occurred at masses of 300 ng and 350 ng of miR mimic complexed at a w/w ratio of 2:1 with Dharmafect™ (Figure 2.12).

Based on this data, it was decided to use a mass of 330 ng of miR mimic complexed at a w/w ratio of 2:1 with Dharmafect™ in all subsequent studies. Using this reporter plasmid, the optimum w/w ratio for delivering miR with pD-γ-P/DA was determined by measuring and normalizing the knockdown of Firefly Luciferase (miR target) compared to Renilla Luciferase in primary neonatal rat cardiac fibroblasts. Significant normalized transfection (knockdown) occurred at w/w ratios of 8:1 and 10:1 (pD-γ-P/DA/miR) compared to controls (no treatment and naked miR-29B), which was comparable to knockdown achieved using a commercially available control (Dharmafect™). Analysis of cellular metabolic activity using alamarBlue® assay showed a reduction in metabolic activity (consistent with the use of pD-γ-P/DA, regardless of weight used) but this activity was significantly less than cytotoxicity induced by the use of commercially available Dharmafect™ (Figure 2.10).

Finally, pD-γ-P/DA conjugated with Fab`s was complexed with miR-29B at a w/w ratio of 8:1. To verify the efficiency of knockdown using this platform, rat neonatal cardiac fibroblasts were again subjected to the reporter transfection assay. Human umbilical vein endothelial cells (HUVECs) were subjected to the same experimental controls as they have a minimal expression of the CD90 antigen (towards which the antibody fragment binds) and are not responsive to an antibody directed towards rat. Significant knockdown occurred at 48 hours with Fab` decorated complexes compared to pD-γ-P/DA with no Fab`s and also compared to the commercially available control (Dharmafect™) in rat cardiac fibroblasts. However, this effect was not seen at 96 hours. As all complexes remain in each
well during the duration of a study, it is hypothesized that in the first time point (48 hours) the Fab\(^{-}\) decorated complexes have adhered quicker to their target cell and have been internalized before those with no Fab\(^{-}\). Therefore silencing occurs quicker. However, at the 96 hour time point it is probable that all complexes have adhered to the cells due to the charge interaction of the complexes with the cell membrane and therefore no statistically significant difference can be detected between Fab\(^{-}\) decorated complexes and non-Fab\(^{-}\) decorated complexes. No effect of Fab\(^{-}\) decorated complexes was observed in HUVECs (Figure 2.15). Additionally, no significant difference was observed between any of the groups at 48 hours or between any of the groups at 96 hours with regard to cellular metabolic activity with addition of the complexes (Figure 2.16).

### 2.4 Conclusion

The development of non-toxic, efficient, and clinically relevant delivery systems remains an important challenge for the clinical applications of interfering RNA-based therapeutics. In this study synthesis of a hyperbranched multi-vinyl polymer was achieved by controlling ATRP in deactivation enhanced mode by employing L-AA as a reducing agent and using a macro-initiator (in this case pDMAEMA) achieving a co-polymer system using DE-ATRP. This reaction was monitored using GPC and the structure of the synthesized pD-v/PDA was evaluated using \(^1\)H NMR. In this study, the efficacy of a miR mimic delivery platform that is based on a cationic interaction of pDMAEMA tertiary amines and phosphates present in the backbone of the miR was demonstrated. This interaction was monitored and verified using agarose gel electrophoresis. The presence of double carbon bond sites as potential acceptor units in a relatively mild but highly efficient Michael type addition (as described in Figure 2.2) enabled the conjugation of antibody fragments to this system, which was demonstrated using SDS-PAGE and enabled improved delivery and efficacy of miRs (as verified by knockdown in normalized transfection in cardiac fibroblasts at 48 hours). This method opens up a plethora of options where this platform can be employed and tuned toward functionalization strategies. Realistically, any SH-terminated moiety can act as a Michael donor for conjugation to the pD-v/PDA synthesized and characterized in this chapter. This encompasses any monoclonal antibody reduced to form Fab’s, peptides engineered to have cysteine terminations, and also scFvs selected and amplified using phage display technology \(^{20}\) to produce effective targeting molecules. Presented here in this chapter, is demonstrative data proving the efficacy of this system which will be tested towards a functional application in the proceeding chapters.
2.5 References


Chapter Three

An Injectable Scaffold Delivery System

The majority of this chapter is due to be submitted for publication in:

3.1 Introduction

Biomaterial based scaffolds have played a central role in regenerative medicine and tissue engineering and several key requirements for scaffolds have been identified. It is desirable that scaffolds fabricated from a range of natural and synthetic materials be biodegradable to obviate the need for a removal procedure. In tandem, predictable biodegradation of the scaffold can facilitate controlled release of biomolecules embedded within the scaffold. Degradation of the scaffold can create a path for tissue ingrowth and this can be further facilitated by a highly porous scaffold which can also allow for the initial transport of oxygen and nutrients as well as for the removal of metabolic waste and degradation products.

The current paradigm of tissue engineering incorporates the use of biomolecules, which can include growth factors, pharmaceutical agents, or mediators of gene therapy. Scaffolds can act as reservoirs in the delivery of RNAi. RNAi delivery from a scaffold enables localized treatment, as the scaffold, acting as a reservoir of RNAi facilitates enhanced delivery of this therapeutic molecule than would be the case with intravenous delivery strategies which include systemic delivery in unprotected formulations. Targeting a cell population or anatomical location by injection or systemic delivery is complex and poses many challenges; direct delivery of a therapy from a scaffold, however, can surmount these barriers. Cells in a target tissue, surrounding a scaffold acting as a reservoir, become exposed to the therapeutic load within the scaffold limiting unwanted exposure in other areas. In addition, scaffold-based delivery has the potential to maintain effective levels of payload and nucleotide bioactivity for extended periods which broadens the opportunity for cellular internalization and increases the likelihood of transfection. Delivery from most scaffolds occurs by means of a combination of therapeutic payload (in this case RNA) interaction with the scaffold and subsequent release through degradation of the scaffold, with the payload and material properties having a significant influence on these interactions.

The focus of this chapter is with miR-29B of the miR-29 family. This class of miRNAs has been specifically associated with the regulation of fibrosis in a number of tissues including renal, bone, pulmonary, hepatic and cardiac tissue. miR-29B has also been elucidated to have a significant role in remodeling extracellular matrix tissue, possessing a significant relationship with collagen production.

A key requirement associated with the encapsulation of RNAi and non-viral vectors is that the scaffold fabrication method must be compatible with the biomolecules and vector integrity.
Methods to achieve this fabrication can involve high temperatures, organic solvents and the
generation of free radicals or shear stresses that may damage the payload. Even if the nucleic acid
(pDNA or RNA) is stably encapsulated, it can still be damaged by the degradation products. The
release kinetics of encapsulated RNAi from scaffolds is also reliant upon a number of factors such
as the concentration of the scaffold, its degree of crosslinking, and the scaffold material which can
make its degradation responsive to pH, temperature, cellular enzymatic products and/or
hydrolysis. This degradation rate can influence the time-course release of the embedded RNAi
as the scaffold loses volume and becomes assimilated by surrounding tissue. Although the
advent of RNAi-based therapy is relatively recent, there have, nevertheless, been numerous efforts
to deliver this therapy via scaffolds and hierarchical structures. The use of scaffolds as a reservoir
of RNAi has been illustrated in many studies because of their potential as an injectable system for
therapeutics which assume the shape of irregular spaces and defects.

In order to improve stability and mechanical properties of scaffolds, cross-linkers such as
 glutaraldehyde and carbodiimide have been used to modify the chemico-physical properties of
these scaffolds. However, these molecules have been shown to be highly toxic, limiting their use
in implantable scaffolds. Consequently, different approaches using non-toxic chemical cross-
linkers have been developed and in particular the use of poly (ethylene glycol) ether tetra-
succinimidyl glutarate (4S-StarPEG) has been advocated.

Herein, a number of concepts within one platform for the effective silencing of protein using non-
viral double stranded miRs are presented in this chapter. A method to formulate an atelocollagen
type I scaffold crosslinked using a 4S-StarPEG which is non-toxic and gels in situ is described.
The use of scaffolds for the viral and non-viral delivery of therapeutic genes is well established in
the literature. However, using a scaffold as a reservoir of exogenous miRs has not, been
previously reported. Therefore, it is hypothesized that an atelocollagen type I scaffold crosslinked
with 4S-StarPEG can act as a reservoir of miR-29B in naked form, miR-29B complexed with a
poly (2-(dimethylamino) ethyl methacrylate) (pDMAEMA) based polymer (pD27-P/DA-
developed in Chapter Two) and miR-29B complexed with PEI. This scaffold/miR platform will
effectively silence ECM proteins; namely collagen type I and collagen type III.

Therefore, the objectives of the work presented in this chapter are to:

i. Develop an atelocollagen type I scaffold and characterise the effect of crosslinking that
can be achieved using 4S-StarPEG by determining the amine content of the scaffolds
using a trinitrobenzenesulphonic acid (TNBSA) assay, the effect on the degradation profile
of the scaffolds using collagenase degradation and the effect on mechanical properties using rheology.

ii. Investigate the effect of crosslinker density on the release profile of interfering RNA from scaffolds and the effect of complexing the RNA with a complexing agent on the release of the RNA from the scaffolds, spectrophotometrically, using Cy™3 labeled siRNA.

iii. Investigate the ability of this scaffold acting as a reservoir in vitro of miR-29B, characterising its release profile and the subsequent effect of silencing collagen type I and type III expression in vitro.

3.2 Materials and Methods

3.2.1 Materials

All solvents were of analytical or HPLC grade and were obtained from Sigma Aldrich Chemical Company (Dublin, Ireland) unless otherwise stated. All oligonucleotides and primers were purchased from Eurofins MWG GmbH (Ebersberg, Germany). 4S-StarPEG (\(M_w = 10,000\)) was purchased from JenKem Technology Co. Ltd. (Texas, USA).

3.2.2 Collagen Scaffold Preparation

Atelocollagen was isolated as described elsewhere \(^{(22)}\) (Appendix D). Nine parts of collagen solution (3.5 mg/ml w/v) was gently and thoroughly mixed with one part of 10X phosphate buffered saline (PBS). The solution was neutralised by the drop-wise addition of 2 M sodium hydroxide (NaOH) until a final pH of 7–7.5 was reached and kept in an ice bath to delay hydrogel formation. 4S-StarPEG was then added to final concentrations of 0.125 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM but always in a volume of 50 μl. 0.625% glutaraldehyde (GTA) was used as a positive control. The solutions were incubated for one hour at 37 °C in a humidified atmosphere to induce gelation. See Appendix D.3 for detailed protocol.

3.2.3 TNBSA Assay

The primary amine groups of type I atelocollagen scaffolds were determined using 2, 4, 6-Trinitrobenzenesulfonic acid (TNBSA) detection assay as previously described\(^{(23-24)}\). Briefly, after crosslinking and hydrogel formation, the scaffolds were incubated in 0.1 M sodium bicarbonate pH 8.5. 0.01 % of TNBSA was added to the samples and incubated for two hours at 37 °C. The reaction was stopped using 10 % sodium dodecyl sulphate (SDS) and 1 M hydrochloric acid (HCl). The scaffolds were then incubated at 120 °C for 15 minutes. Absorbance of each sample
was read at 335 nm and the free amine groups quantified by interpolating values from a linear standard curve of known concentrations of glycine. See Appendix D.4 for detailed protocol.

3.2.4 Degradation by Collagenase

Resistance of the scaffolds to enzymatic digestion was evaluated using collagenase assay. Briefly, scaffolds were incubated for one hour in 0.1 M Tris–HCl (pH 7.4), containing 50 mM calcium chloride (CaCl₂) at 37 °C. Subsequently, bacterial collagenase type IV (770 units/mg, extracted from Clostridium histolyticum), reconstituted in 0.1 M Tris–HCl at a concentration of 10 units/mg collagen type I, was added. After incubation for 48 hours at 37 °C, the enzymatic reaction was stopped by the addition of 0.25 M EDTA. After vacuum dehydration, the remaining mass of the scaffolds was weighed and normalised to the remaining mass of GTA cross-linked scaffolds. See Appendix D.5 for detailed protocol.

3.2.5 Rheological Evaluation

In order to identify the gel time of the scaffold as a function of cross-linking, rheological measurements were performed at 37 °C using a Haake Modular Advanced Rheometer System™ (MARS) rheometer (Thermo Haake, Germany) previously described. Briefly, type I atelocollagen, 10 X PBS and 1 M NaOH alone, with 0.625% GTA, or with different concentrations of 4S-StarPEG (0.5 mM, 1 mM and 2 mM), were added to the plate at 37 °C. The rheometer was equipped with a circulating water bath to accurately control the temperature. To minimise the influence of water loss on mechanical behaviour, samples were coated with paraffin oil. Dynamic frequency sweep experiments were carried out to determine the storage (G’) and loss (G’”) moduli as a function of time at 37 °C. The measurements of the storage (G’) and loss (G’”) moduli during the gelation were recorded as a function of time for five different frequencies (a, b, c, d and e rad/s) using multi-wave facilities. The gel point was defined as the time that G’ equaled G’”.

3.2.6 Complexation

miR-29B mimic and negative control scrambled miR mimic (miR-scram) were obtained from Qiagen with the sequences for rno-miR-29B: 5’-uagcaccuuugaacagugu-3’; and a control scrambled mimic: 5’-gtgcctcattaacgtaatga-3’. miR mimics were mixed individually with pD-P/DAand poly (ethylenimine) (PEI; 25 kDa) at w/w ratios of 8:1 and 2:1 respectively. The components were mixed and complexes allowed to form at room temperature for 60 minutes in serum free media. Complexation was analysed by acrylamide gel electrophoresis (see Appendix K for detailed protocol). For monolayer culture experiments, 333 ng of miRs were complexed and added to each well of a 96 well plate in a volume of 50 μl. For scaffold delivery of miRs from a
Figure 3.1: Type I atelocollagen and 4S-StarPEG reaction. Succinimidyl glutarate is an NHS-ester which binds with amine groups and therefore the succinimidyl groups react with the amine groups present on the molecules of type I atelcollagen at 37 °C.
collagen type I scaffold crosslinked with 4S-StarPEG at a molar ratio of 1:1, 2 μg of miRs were complexed and mixed evenly to the crosslinked scaffold solution on ice before gelation occurred.

### 3.2.7 Fab` Conjugation

Antibody fragments (Fab’s) were derived from monoclonal antibodies as described in Chapter Two. Known molar concentrations (deduced from Ellman’s assay) of these Fab’s were reacted with pD-b-P/DA in a reaction buffer of 0.2 mM NaH₂PO₄ with 1 mM EDTA at pH 8.0 (purged prior to reaction with argon for 30 minutes). Briefly, reactions were performed in which molar ratios of Fab’ thiol concentration (from Ellman’s assay) and moles of free vinyls (calculated from ¹H NMR data presented in Chapter Two) were reacted at a ratio of 1:1.

### 3.2.8 Elution Studies

The characterization of the 4S-StarPEG collagen type I scaffold’s release profile of interfering RNA was performed using siRNA as a model oligonucleotide labeled with Cy™3. Previous experiments using intercalating staining of oligonucleotides with PicoGreen® proved unreliable as the use of the complexing agents hindered the binding of this agent to the nucleic acid, an observation which is noted in pDNA release studies ²⁷. siRNA was labeled using Silencer siRNA Labeling Kit Cy™3 (Ambion®/Life Technologies, Dublin, Ireland) according to the manufacturer’s instructions (Appendix V for detailed protocol). Release of the miRNA complexes was evaluated using the fluorescence from the control siRNA, which was labeled with Cy™3. Briefly, the scaffolds were prepared as described above, in a 48-well plate with the additional step of adding 2 μg of Cy™3 labeled siRNA. Three groups were investigated in this study: naked siRNA, siRNA complexed with pD-b-P/DA and siRNA complexed with PEI. In addition the effect of crosslinking density on the release profiles of siRNA from the scaffolds was investigated using three conditions: a crosslinking density of 1 mM, 0.5 mM and 0.05 mM. The loaded scaffolds were incubated for two hours at room temperature to allow the complexes to associate with the scaffold and for complete gelation to occur. Thereafter, the scaffolds were individually removed and transferred to the bottom of a 24-well well plate. To this, an equal volume of tris (hydroxymethyl) aminomethane-n/ethylenediaminetetraacetic acid (Tris–EDTA) buffer (10 mM Tris–HCl and 1 mM EDTA, pH = 7.5) was added. At each time point, this process was repeated. At the end of the experiment, the siRNA content of the solutions was quantified by measuring the fluorescence with a Varioskan Flash® spectral scanning multimode reader (Thermo Scientific, Vantaa, Finland) and the cumulative release of siRNA complexes from the scaffold was calculated following comparison with a standard curve.
3.2.9 Evaluating Matrix Binding of RNA complexes

To further understand the mechanism of how RNA complexes are held within the scaffold the electrophoretic kinetics of RNA complexes within the scaffold were evaluated using agarose gel electrophoresis. 1 % (w/v) agarose was heated in tris-acetic acid-Ethylenediaminetetraacetic acid (EDTA) buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA respectively) and a 10,000 fold dilution of Syber® Safe dye was added prior to gel casting. Non-crosslinked atelocollagen scaffolds or scaffolds crosslinked with 4S-StarPEG, to a volume of 10 μl, were deposited into the running wells of this gel with 330 ng of naked RNA, RNA complexed with pD-b-P/DA, or complexed with PEI. Naked RNA and scaffolds containing no RNA were applied as controls. Additionally, naked RNA mixed with an indicative loading buffer was run to monitor the migration of the RNA on the gel. However, this was not applied to the other samples to avoid any interference with the kinetics of the scaffolds. A voltage of 50 V was applied to the agarose gel and the current was set to ‘auto’. After 20 minutes the loading buffer indicated sufficient migration to observe the electrophoretic mobility of the complexes under UV light.

3.2.10 Cell Extraction

Rat cardiac fibroblasts were isolated from neonatal pups as previously described (Appendix I.8). Briefly, neonatal rat ventricle myocytes were isolated from the cardiac ventricles of three to five days old Sprague-Dawley pups. Hearts were removed from the thoracic cavity and placed in a tube containing cold (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES solution (20 mM HEPES, pH 7.4). Ventricles were separated from surrounding tissue using scissors and minced into several pieces. Subsequently cardiomyocytes and fibroblasts were detached from the extracellular matrix by repeated incubation in collagenase, supplemented with 2 mg/ml trypsin and 20 µg/ml DNase. Cells were collected by centrifugation and tissue clumps were removed by filtration. The cells were then pre-plated in cell culture dishes in 50 ml Dulbecco's Modified Eagle Medium (DMEM) /F12 (50:50) medium with 5% fetal bovine serum (FBS) for 45 minutes. During this period, most non-cardiomyocyte cells (mainly fibroblasts) attached to the dish, whereas cardiomyocytes remained in solution. Fibroblasts were subsequently cultured in DMEM/F12 medium containing 10% FBS.

3.2.11 Monolayer Silencing Study

Primary rat cardiac fibroblasts were seeded on a six-well plate at a density of 1 x 10⁶ cells per well. After one day incubation at 37 °C, 5% CO₂, to ensure adherence and acclimatization, miR-29B or miR-scram; uncomplexed, complexed with pD-b-P/DA or complexed with PEI were added to each well in a total volume of 250 μl with a concentration of 0.5 μg of miR. After ten minutes,
the total volume in the wells was brought to 1 ml with the addition of DMEM/F12 containing FBS at a concentration of 5%. The experiment was maintained for 48 hours after which samples were processed for gene or protein analysis via RT-PCR and Western blot respectively.

3.2.12 Scaffold Delivery Silencing Studies

Silencing studies were performed on six-well well plates seeded with $1 \times 10^6$ cells per well. After one day incubation to ensure adherence and acclimatization, scaffolds were placed onto the cells. In total 250 μl of scaffold solution was applied to each well. The scaffolds were applied directly to ensure direct contact with cells and also to bring about a direct interaction between the cells and the scaffold. The use of a 250 μl volume applied in a six-well enabled a very thin scaffold to be produced which maintained diffusion of nutrients to maintain the viability of the cells.

3.2.13 RNA Extraction

RNA extraction was performed at 7, 14, and 21 days. One mL of TRI Reagent® (Applera Ireland, Dublin, Ireland) was added to each construct and incubated for five minutes at room temperature. Scaffolds were mechanically disrupted using a sterile pipette tip. Phase separation was performed by adding chloroform (Sigma-Aldrich), and total RNA was purified using an RNeasy™ kit (Qiagen), according to the supplier’s recommended procedure (Appendix S).

3.2.14 Real-time Reverse Transcription Polymerase Chain Reaction

Total RNA quantity and purity were determined using spectrophotometry at 260 and 280 nm using an ultraviolet spectrophotometer (NanoDrop™ ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was checked electrophoretically using the RNA 6000 Nano LabChip™ kit with an Agilent Bioanalyzer 2100 (Agilent Technologies, Cork, Ireland). Reverse transcription (RT) was performed using the ImProm-II™ RT system according to the manufacturer’s protocol (Promega, Southampton, United Kingdom, Appendix T). Gene transcription was examined using real-time RT polymerase chain reaction (PCR). Reactions were performed and monitored using an ABI 7000® sequence detection system (Applied Biosystems, Foster City, CA) using TaqMan® Real-time Gene Expression Mastermix (TaqMan, Applied Biosystems) and specific primers which are detailed in Table 3.1. The primers were designed and their specificity checked using primer-BLAST (available freely from www.ncbi.com) and their efficiency determined by RT-PCR on ten-fold serial dilutions of template cDNA. Gene transcription was inferred from calibration samples and normalized in relation to transcription of the housekeeping gene: glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The $2^{ΔΔCt}$ method was used to calculate relative gene expression for each target gene (Appendices T and U).
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<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’→3’)</th>
<th>Reverse (5’→3’)</th>
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<tr>
<td>Rat Collagen 1A1</td>
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<td>ACACACAAAGACAAGAAGCAGG</td>
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<tr>
<td>Rat Collagen 3A1</td>
<td>GCCTCCCCAGAACATTACATACC</td>
<td>ACTGTCTTGGCTCCATTCACC</td>
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<tr>
<td>Rat GAPDH</td>
<td>AAGAAGGTGGTGAAGCAGG</td>
<td>CAAAGGTGGAAGAATGGGAG</td>
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</tbody>
</table>

3.2.15 Western Blot Analysis

The effect of miR-29B complexes on collagen type I and collagen type III was assayed by Western blotting. In brief, 96 hours after delivery of miR-29B, cardiac fibroblasts were washed twice with PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (Pierce, Rockford, IL, USA) at 4 °C for two hours. The lysates were pipetted up and down approximately 25 times to shear cell fragments and the protein was separated from cellular debris by centrifugation (10,000 × g, 15 min at 4 °C). Protein concentrations were determined using Bradford reagent. Equal amounts of proteins (25 μg) were boiled in sample buffer (2% SDS, 10% glycerol, 100 mM dithiothreitol (DTT), 60 mM Tris–HCl pH 6.8 and 0.001% bromophenol blue) for five minutes and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Appendix K). Protein bands on the gel were electrically transferred onto polyvinylidene fluoride (PVDF) membrane (Hybond, GE Healthcare, Buckinghamshire, UK) using a constant voltage of 30 V at 4 °C for 16 hours. The membrane was blocked (5% w/v bovine serum albumin (BSA), 0.02% sodium azide and 0.2% Tween 20 in PBS) for one hour at room temperature under rocking conditions, and incubated with anti-collagen type I (1 μg/ml diluted 1:1000, Abcam Plc, Cambridge, UK) and anti-collagen type III (Abcam 1:500) overnight at 4 °C under gentle rocking. Subsequently, the membrane was washed three times for 15 minutes each with Tris-buffered saline (TBS)–Tween (20 mM Tris–HCl pH 7.6, 137 mM NaCl and 0.1% Tween 20), and incubated for one hour at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (diluted 1:500, Invitrogen Molecular Probes, Dun Laoghaire, Ireland). After washing in TBS–Tween for 30 minutes, immunoenzymatic antigen detection was performed using diaminobenzidine (DAB) as the enzyme substrate (Amresco, Solon, OH, USA). See Appendix L for a detailed description of protocol.

3.2.16 Statistical Analysis

All samples were tested in triplicate and all experimental groups were analysed in triplicate. Graphpad PRISM™ software (v.5 GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Verification of normal distribution was determined using the Anderson-
Darling test. Analysis of variance (ANOVA) was used to determine statistical significance between groups. All graphical data is presented as mean ± standard deviation. p values of < 0.05 were considered statistically significant. The existence of outliers was determined using Grubb’s test, however no outliers were detected.

3.3 Results and Discussion

3.3.1 Scaffold Characterization

The potential of scaffolds as delivery platforms of non-viral therapeutics has been previously documented both in vitro and in vivo, with tangible beneficial outcomes. Specifically the in vitro effects of 4S-StarPEG crosslinked collagen scaffolds have been investigated as a delivery platform for delivering adipose derived mesenchymal stem cells. In the study described in this chapter, collagen type I was crosslinked using 4S-StarPEG via free amine groups available in collagen type I. Due to the four-arm structure of 4S-StarPEG, theoretically one mole of 4S-StarPEG crosslinks with four moles of free amines available in collagen type I (essentially a 1:4 molar ratio). With this in mind, the range of crosslinking densities was based around this theoretical value. Determining the extent of crosslinking via TNBSA quantification of free amine groups indicated a decrease in free amine groups which was inversely proportional to crosslinker density (Figure 3.2) which reached a maximum decrease to 9.5% free amines available after 1 mM crosslinking density. Although there is no significant difference between the most effective crosslinking concentration (1 mM and 2 mM), there is a trend towards an increase in free amines at

As previously discussed, efforts at using intercalating dyes in this project proved inefficient due to generation of standard curves that have unacceptable correlation co-efficients and therefore readings could not be extrapolated as a percent release of nucleic acid. This is due to the strong binding of cationic complexing agents to the nucleic acid which hinders intercalating dyes from binding with complexed nucleic acid appropriately. This observation has been observed in many pDNA release studies and similarly applies to other nucleic acids such as the RNA employed in this study. Based on this, the siRNAs were labelled using Cy™3 and produced standard curves with reliable correlation co-efficients which can be extrapolated.

The concentration of 4S-StarPEG had a significant effect on the release profiles of siRNA (Figure 3.4). This could be due to both an interaction of the siRNA with the crosslinker and the altered, crosslinker-dependent degradation profile of the collagen scaffold. As RNA has amine groups present in its backbone it is possible that the crosslinker interacts with these free amines. Such an interaction, however, will be minimal as Cy™3 labelling of the RNA occurs via interaction of the

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Figure 3.2: Quantification of free amine groups after crosslinking with 4S-StarPEG. * indicates statistical significance between 1mM crosslinking compared to 0.25 mM and 0.125 mM crosslinking, ** compared to all other groups as in the case of glutaraldehyde (GTA); % remaining amine groups is statistically decreased when compared with all other groups. There exists an inversely proportional relationship between the concentration of 4S-StarPEG and the % of free amines remaining in scaffolds after crosslinking. Analysis performed using one-way ANOVA (n = 4, p < 0.05).
Figure 3.3: Degradation by collagenase quantified by mass remaining after 48 hours. Non-crosslinked control (0) has completely degraded after 48 hours and therefore no mass is represented. Glutaraldehyde (GTA) was used as a positive control. * indicates a statistically significant difference using one-way ANOVA compared to all other data sets presented in the graph (n = 3, p < 0.05).
Table 3.2: Rheological evaluation of crosslinked scaffolds.

<table>
<thead>
<tr>
<th></th>
<th>0.5 mM</th>
<th>1 mM</th>
<th>2 mM</th>
<th>GTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelation Time</td>
<td>18 ± 9</td>
<td>7 ± 1.1</td>
<td>8 ± 1.3</td>
<td>Instant*</td>
</tr>
<tr>
<td>Storage Modulus</td>
<td>201 ± 165.1</td>
<td>260 ± 81.7</td>
<td>192 ± 27.8</td>
<td>4934 ± 518*</td>
</tr>
<tr>
<td>Storage Modulus (15 Days)</td>
<td>45 ± 28.6*</td>
<td>307 ± 68.4</td>
<td>402 ± 257.7</td>
<td>4014 ± 223.2*</td>
</tr>
</tbody>
</table>

Data presented is n = 3, mean ± standard deviation. * indicates a statistically significant difference when using GTA compared to crosslinking with 4S-StarPEG, p < 0.05. # indicates a statistically significant difference in storage modulus at 15 days when using 0.5 mM of 4S-StarPEG when compared to the other groups.
Cy™3 dye with the free amines available in the RNA, although the other two conditions employing complexing agents; pD-b-P/DA and PEI both possess tertiary and secondary amines respectively. However in all conditions the crosslinker is added to the collagen solution first, and only afterwards is the RNA added in order to minimise this interaction. As crosslinker density has an effect on the degradation profile of the scaffold, it is very apparent, especially at 0.05 mM crosslinking density, that this degradation plays a key role in the release profile of the RNA. At three days, the entire release of the Cy™3 labelled RNA has occurred, although it is worthy to note that at this time point and at this concentration complete degradation of the scaffold had occurred.

The effect of crosslinker concentration on biomolecule release has been reported in several studies. For instance, Zhu et al. 33 crosslinked gelatin microspheres with various concentrations of glutaraldehyde and investigated the release profile of basic fibroblast growth factor (bFGF) over a period of 14 days. In accordance with the results presented in this chapter, a definitive dependence of growth factor’s release profile with crosslinker concentration was observed. A conflicting study, but with the release of pDNA from gelatin spheres crosslinked with glutaraldehyde (10 nM and 40 nM in this case, compared with 5, 10 and 20 nM in the study reported by Zhu et al.), concluded that pDNA release was not dependent on crosslinker density but on the degradation rate of the complexing agent 32. However, in this study, the carrier was crosslinked prior to the loading of pDNA. Chun et al. employed various durations of UV photo-crosslinking on pluronic scaffolds after pDNA had been embedded within the scaffolds, so that in effect, the pDNA was subjected to the crosslinking process 34. In this study, it was found that the duration of UV crosslinking did have an effect on the release profile of pDNA from the scaffold wherein pDNA release from the scaffolds was inversely proportional to the degree of photo-crosslinking.

The results presented in this chapter investigated the interaction of nucleic acid with the scaffold matrix and the subsequent release profile. The nature of the RNA (i.e. whether as an unprotected formulation or complexed with a complexing agent) in the study had an effect on the release profiles. RNAi can be encapsulated within nanoparticles 35, liposomes 36 or cationic agents 29 to protect against RNAses, enhancing cellular delivery and provide intracellular endosomal buffering. Here, the release profile of Cy™3 labelled RNA in unmodified form, or complexed was characterised. In this case two complexing agents were investigated separately; pD-b-P/DA and PEI. Naked RNA was released first from the scaffold whereas PEI complexed RNA was released more slowly.

These observations were attributed to charge interaction between the therapeutic molecules and the scaffold. Nucleic acid naturally occurs as a negatively charged molecule. The collagen in this
study has been crosslinked using the free amine groups (NH₂) present in its backbone and not the negatively charged carboxyl (COOH) groups which offers the collagen a net negative charge. Therefore nucleic acid, in this case double stranded RNA, cannot interact electrostatically with the collagen scaffold and therefore is released much quicker from the scaffold. pD-β-P/DA possesses a net positive charge (albeit a weak one) attributable to the tertiary amines present on DMAEMA. Based on this, there exists a slightly slower release of pD-β-P/DA complexed RNA from the scaffold. Finally PEI possesses a strong net positive charge when complexed with RNA and therefore has a significant electrostatic interaction with the negatively charged collagen scaffold. This strong electrostatic interaction causes a significantly slower release profile of RNA from the scaffold. This is also reflected in the electrophoresis data present in Figure 3.5. Non-crosslinked scaffolds containing RNA (naked or complexed with pD-β-P/D) present a net positive charge. However, when the scaffolds are crosslinked with 4S-StarPEG, they lose their ability to bind with RNA in its naked form or complexed with pD-β-P/DA. This is extremely interesting and strengthens the findings from the release studies in Figure 3.4. Additionally, RNA complexed with PEI has an electrophoretic shift towards the cathode indicating a net positive charge. This is unaffected even if the PEI/RNA complexes are placed in a non-crosslinked scaffold or a scaffold crosslinked with 4S-StarPEG. Based on this, it can be postulated that the PEI/RNA complexes leave the scaffold as intact PEI/RNA complexes, however complexation with pD-β-P/DA within a scaffold suggests the RNA leaves the scaffold and in turn leaves the pD-β-P/DA behind as it is not as tightly bound as PEI/RNA complexes. Similar observations have been observed in other studies reported in the literature. For instance, Chew et al. [32] monitored the release profile of naked pDNA loaded gelatin microspheres which were incorporated into a porous poly (propylene fumarate (PPF) scaffold. Additional groups investigated in this study include pDNA incorporated into the same scaffold and complexed with hydrolytically degradable branched triacrylate/amine polycationic polymers (TAPPs); P-AEPZ and P-DED synthesized with amine monomer 1-(2-aminoethyl)piperazine (AEPZ) or N,N-dimethylethlenediamine (DED). Complexation of the pDNA caused a significantly lower initial burst release (within the first 24 hours). The authors of this study also attributed the release profiles of the pDNA to the nature in which the pDNA was embedded within microspheres and the charge interaction between them. siRNA release profiles from scaffolds have also been studied. Krebs et al. [12] found that siRNA embedded within a photocrosslinked alginate hydrogel had a much quicker release and increased burst release compared to a collagen scaffold, which can be explained due to the negatively charged polysaccharide, alginate. Modulation of alginate scaffolds by combining with chitosan or PEI resulted in decreased burst release from the hydrogels which is due to an electrostatic interaction between the nucleotides and the positively charged polymers mixed with the alginate.
3.3.2 Silencing Collagen Type I and Collagen Type III

To remove any ambiguity regarding the specificity of miR-29B to silence collagen type I and collagen type III, RT-PCR was performed on rat cardiac fibroblasts treated with miR-29B and non-targeting, scrambled controls in vitro (Figure 3.6). As expected the scrambled control did not alter any significant silencing of collagen type I and collagen type III mRNA in vitro. This non-alteration eliminates the possibility of global, non-specific silencing due to the use of double stranded RNA. While it is true that there are targets of miR-29B that are not validated in this thesis and have yet to be discovered; there are no noticeable effects on cellular viability or proliferation detected in response to miR-29B specifically in the studies presented here. The use of miR-29B in a naked unprotected form did not exhibit effective silencing of collagen type I (1.11 relative expression, $2^{\Delta \Delta Ct}$) or collagen type III mRNA (1.14 relative expression) which suggests the requirement for a complexing agent when performing miR delivery to cells in 2D culture (Figure 3.6). Complexation of miR-29B with PEI did show effective silencing of collagen type I and type III mRNA expression (0.51 and 0.55 relative expression, $2^{\Delta \Delta Ct}$ respectively). Although PEI is an effective carrier of nucleic acid to cells, it poses drawbacks as it is toxic; an important consideration if silencing is to be maintained over an extended period of time. The effect of miR-29B on collagen type I and collagen type III protein production was also investigated using western blot analysis (Figure 3.7). The bands support the observations of the RT-PCR data in Figure 3.6, such that the use of a complexing agent significantly increases the efficacy of miR-29B and thereby decreases production of collagen type I and collagen type III. However, for full appreciation of this repetition of western blot is needed with the inclusion of appropriate loading control immunoblotting for a housekeeping protein such as GAPDH or β-actin.

Exogenous miR-29B mimics, identical to those reported in this chapter, have been previously reported to silence collagen type I expression in vitro and all such studies report the employment of a complexing agent (in the majority of cases commercial agents such as Lipofectamine™ 2000) to deliver such mimics. Such agents are warranted as the administration of naked miRs in cell media are inefficient on cell monolayers in vitro at such small doses (72 ng and 361 ng) which is reflected in the results presented in this chapter with a dose of 333 ng of miR-29B mimic. However, commercial agents such as those employed in previous studies, are marketed as diagnostic and research tools; as their compromised toxicity and concerns of regulatory agencies limits their therapeutic potential.
Figure 3.4: Release profiles of Cy™3 labelled RNA from scaffolds crosslinked with varying concentrations of 4S-StarPEG. Release of Cy™3 labelled RNA was determined spectrophotometrically and extrapolating from standard curves. Naked RNA is uncomplexed Cy™3 labelled RNA whereas PEI complexed and pD-b-P/DA complexed indicates that the Cy™3 labelled RNA has been complexed with these agents. Data presented is the mean ± SD, n = 4. * indicates statistically significant release profiles using a 0.05 mM 4S-StarPEG crosslinker concentration compared to concentrations of 1mM and 0.5 mM, p < 0.05.
Figure 3.5: Agarose gel electrophoresis to indicate the binding of RNA complexes to the collagen 4S-StarPEG scaffold. The components in each lane are indicated by the grid below the picture of the gel. Bands towards the bottom of a lane indicates that the RNA has a net negative charge and is migrating towards the anode (-) whereas bands still within the loading lane or migrating upwards have a net positive charge and are migrating towards the cathode (+). In general naked RNA migrates towards the anode whereas pDγ-P/DA and PEI complexed RNA migrates towards the cathode indicating a positive charge. A non-crosslinked scaffold offers the naked RNA a positive charge whereas a crosslinked scaffold minimises the interaction of the scaffold with naked RNA. A similar trend is observed when RNA is complexed with pDγ-P/DA indicating interaction with the collagen and the crosslinker (4S-StarPEG) and pDγ-P/DA. PEI has no apparent interaction with either a non-crosslinked collagen scaffold or a crosslinked scaffold and migrates from the scaffold when subjected to electrophoresis.
Figure 3.6: qRT-PCR data demonstrating the effect of miR-29B in silencing collagen type I and type III mRNA in vitro in rat cardiac fibroblasts compared to scrambled control 48 hours after delivery of miR mimics. * indicates a statistically significant difference compared to the other treatments, p < 0.05. Data is presented as the mean ± SD, n = 3.
Figure 3.7: Western blot analysis of extracted protein investigated for collagen type I and collagen type III content in cardiac fibroblast cultures 96 hours after miR-29B treatments were applied. Two lanes were used for each treatment group and the above data presents the same blot stained first for collagen type I, stripped and then re-stained for collagen type III. Type I collagen bands occur at a molecular weight of ~130 kDa (predicted value) and collagen type III collagen bands occur at a molecular weight of ~140 kDa (predicted value is 138 kDa). Samples are displayed in duplicate.
Figure 3.8: qRT-PCR data demonstrating the effect of miR-29B in silencing collagen type I mRNA in rat cardiac fibroblasts when delivered from a 1 mM 4S-StarPEG crosslinked collagen scaffold. Knockdown was determined by comparing the collagen type I Ct value to the housekeeping GAPDH Ct value of rat cardiac fibroblasts treated with a 1 mM 4S-StarPEG collagen scaffold alone (empty control). * indicates a statistically significant difference compared to the use of a 1 mM 4S-StarPEG crosslinked collagen scaffold alone (all data relative to this group), p < 0.05. Data is presented as the mean ± SD, n = 4.
Figure 3.9: qRT-PCR data demonstrating the effect of miR-29B in silencing collagen type III mRNA in rat cardiac fibroblasts when delivered from a 1 mM 4S-StarPEG crosslinked collagen scaffold. Knockdown was determined by comparing the collagen type III Ct value to the housekeeping GAPDH Ct value of rat cardiac fibroblasts treated with a 1 mM 4S-StarPEG collagen scaffold alone (empty control). * indicates a statistically significant difference compared to the use of a 1 mM 4S-StarPEG crosslinked collagen scaffold alone (all data relative to this group), p < 0.05. Data is presented as the mean ± SD, n = 4.
3.3.3 Functional miR-29B Delivery from Scaffold

Using a collagen type I scaffold crosslinked using 4S-StarPEG as a delivery reservoir of miR-29B proved effective in delivering RNA in both naked and complexed form. Previous studies have employed collagen as a non-viral delivery vector\textsuperscript{12, 40} which accords some agreement with the \textit{in vitro} scaffold delivery silencing studies presented in this chapter. Delivery of naked miR to monolayer cultures did not produce effective silencing of collagen type I and collagen type III (Figure 3.5), however, when embedded within a collagen type I scaffold, naked miR had a significant silencing effect (Figures 3.8 and 3.9). Also, a more profound silencing occurred with collagen type I mRNA, (0.66, 0.59 and 0.59 at days 3, 7 and 14 respectively) than collagen type III when compared to an empty control of a 1mm 4S-StarPEG crosslinked gel alone.

Collagen has proved to be an effective delivery vector of pDNA and siRNA and therefore the observations obtained in this study are consistent with those reported\textsuperscript{2, 29, 40}. The embedding of siRNA within a collagen scaffold can offer greater protection from RNAses and limits degradation. Furthermore, cells interact favourably with collagen and this can enable enhanced uptake of a therapeutic payload. The silencing of collagen type I and collagen type III mRNA from a scaffold carrying naked miR-29B, miR-29B complexed with PEI, miR-29B complexed with pD-b-P/DA, and miR-29B complexed with pD-b-P/DA which was decorated with an antibody fragment was present up to the 14 days investigated in this study. However, collagen type III silencing was not as pronounced up to 14 days in all groups investigated (Figure 3.9), and only miR29B complexed with PEI significantly reduced collagen type III mRNA relative expression (0.77) when compared to cultures incubated with scaffolds containing no miR-29B.

The functionalization of miR-29B/pD-b-P/DA complexes with antibody fragments to improve cellular uptake had no significant effect on silencing of collagen type I or collagen type III in a scaffold system. This could be attributed to the choice of antigen target and it is possible that more profound silencing could be achieved with a target that is internalising rapidly into the cell. Most likely, a scaffold negates the effect of a targeting antibody as the platform is in a stationary (although still metabolically active) environment and the bulk of interaction of the surrounding environment is with the scaffold (in this case a collagen type I/4S-StarPEG scaffold). However, a scaffold is warranted in the context of this thesis to provide functional support to a treated area and effect delivery from a biomaterial platform. Antibodies and/or targeting moieties have been used previously in scaffolds \textit{in vivo}, but with the intention of recruiting circulating cells\textsuperscript{41} or using antibodies as therapeutic molecules\textsuperscript{42}. Therefore, in agreement with Chapter Two, it can be deduced that the full efficacy of miR-29B/pD-b-P/DA complexes decorated with antibody
fragments can only be realised in a bioreactor system in vitro or via systemic delivery in vivo which would be appropriate miR-29B was envisaged to be delivered systemically.

3.4 Conclusions

4S-StarPEG proved to be an effective crosslinker of type I atelocollagen and allowed the formation of stable scaffolds. The physico-chemical properties of these scaffolds can be varied according to crosslinker density. This amount of crosslinking was verified by quantification of free amine groups, resistance to enzymatic degradation and by assessment of mechanical behaviour. It was demonstrated that the release of a therapeutic agent from the scaffold was dependent on the crosslinker density (through scaffold integrity and degradation profile) and also the electrostatic interaction of the therapeutic within the scaffold. miR-29B was shown to specifically silence collagen type I and collagen type III in monolayer culture, but only when delivered using a complexing agent (down to 51 and 55% respectively). Using a 4S-StarPEG scaffold as a delivery platform of these miR-29B complexes, efficient silencing, of both collagen type I and collagen type III mRNA, up to a period of up to 14 days was achieved and knockdown of collagen type I miRNA was higher than that of collagen type III. Furthermore, naked miR-29B within the scaffold was effective at silencing collagen type I and collagen type III mRNA, but the functionalization of miR-29B/pD-γ-P/DA complexes with antibody fragments did not result in a significant improvement in efficiency when delivered in a scaffold platform.
3.5 References


34. Chun KW, Lee JB, Kim SH, Park TG. Controlled release of plasmid DNA from photo-cross-linked pluronic hydrogels. Biomaterials. 2005;26:3319-3326


Chapter Four

Evaluation of miR Delivery In Vivo

The majority of this chapter is submitted for publication in

4.1 Introduction

A complex cascade of events follows injury aiming to repair the wound in sequential and overlapping phases \(^1\). This first phase begins with haemostasis in which platelets aggregate at the injury site to form a fibrin clot. Histamine, released by ruptured cell membranes, enables blood vessels to become dilated and porous which facilitates the infiltration of inflammatory cells such as polymorphonuclear neutrophils (PMNs) and helper T cells into the wound site from the bloodstream \(^2\). In the following/overlapping inflammatory phase, bacteria and debris are phagocytised and removed. Following this, monocytes are recruited and replace PMNs in the wound and mature into macrophages which further phagocytise bacteria and necrotic tissue. This debris is then degraded by protease release \(^3\). These macrophages also secrete a number of factors such as growth factors and other cytokines which attract cells involved in a proliferation stage of healing to the area. The proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialisation, and wound contraction. By the end of the first week, fibroblasts become the predominant cell in the wound and are responsible for laying down the collagen matrix at the wound site. Formation of this granulation tissue begins in the wound during the inflammatory phase and continues until the wound bed is covered \(^1\). The granulation tissue consists of new blood vessels, fibroblasts, inflammatory cells, endothelial cells, myofibroblasts, and components of a new provisional extracellular matrix. In a final maturation and remodelling phase, collagen is remodelled and realigned along tension lines, and cells that are not needed are removed by apoptosis as there are reduced stress forces due to the accumulation of ECM components produced by myofibroblasts \(^4\). TGF-\(\beta\) \(^1\) plays a significant role in matrix remodelling following injury whereby it stimulates ECM production \(^5\) and distinct role of miR-29B is in post-transcriptional silencing of ECM fibrillar components stimulated by TGF-\(\beta\) \(^6\-8\). Matrix turnover, i.e. the enzymatic remodelling of ECM is an important consideration in healing wounds and indeed remodelling components are significantly dysregulated. Notably, MMP-8 is a collagen cleaving enzyme which functions to degrade type I, II and III collagens \(^9\) and in the context of wound healing; MMP-8 has been shown to be the predominant collagenase in healing wounds and non-healing ulcers \(^10\). TIMP-1 complexes with MMPs and irreversibly inactivates them by binding to their catalytic zinc co-factor and, furthermore, TIMP-1 is a direct inhibitor of MMP-8 \(^11\).

Adverse wound healing, such as excessive scar tissue formation, wound contraction, or non-healing wounds represent a major clinical issue in healthcare today. A biomaterials approach can be used to modify wound healing, acting as either matrices to support and promote tissue organization, act as
Figure 4.1: Schematic depiction of the platform described in this chapter applied as an injectable therapeutic. (a) Initial insult to dermis by tissue injury, (b) topical application of treatments discussed in this chapter and (c) wound healing response to degradation of scaffold and therapeutic release of agents embedded within the scaffold.
Evaluation of miR Delivery In Vivo

barriers to limit wound contraction \(^{12}\) and scar tissue formation \(^{13-19}\) and/or incorporate the delivery of bioactive substances such as cytokines \(^{20}\), growth factors \(^{21}\), living cells \(^{22}\), and with nucleic acids—specifically anti-sense oligonucleotides \(^{23-25}\) (see schematic; Figure 4.1). Collagen is the most abundant structural protein in the dermal extracellular matrix (ECM) \(^{26}\) and maintains a highly conserved amino acid sequence. Appropriately, most commercially available skin substitutes are collagen-based, ranging from acellular dressings such as BioBrane\(^{®}\) (Smith & Nephew) or Integra\(^{®}\) to autologous keratinocyte-loaded constructs such as Apligraf\(^{®}\) (Novartis) and OrCell\(^{®}\) (Ortec International Inc.) \(^{27, 28}\). Current limitations of such implants include poor vascularisation, poor healing times, compromised mechanical strength and the introduction of a relatively inert skin replacement \(^{29}\).

In the previous chapter, the in vitro evaluation of an atelocollagen type I scaffold crosslinked using poly (ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG) was described. Scaffold characterisation was performed to verify the effect of crosslinking using 4S-StarPEG on the physiochemical and rheological properties of the scaffold. It was concluded that the most effective crosslinker concentration for the atelocollagen type I using 4S-StarPEG occurred at 1 mM. This crosslinking significantly improved enzymatic resistance of the scaffold and increased storage modulus when compared to a non-crosslinked scaffold. The complexing agent, pD-\(b\)-P/DA, synthesized and characterised in Chapter Two, was also tested in Chapter Three in combination with the scaffold to deliver miR-29B as an exogenous regulator of collagen type I and collagen type III mRNA expression. Notably, it was observed that the scaffold with miR-29B was efficient in silencing collagen type I and collagen type III mRNA in cardiac fibroblasts for a period of up to 14 days in vitro. However, from the results of Chapter Three, it was concluded that the inclusion of an antibody fragment (Fab\(^{'}\)) on the surface of the pD-\(b\)-P/DA/ miR-29B complex did not significantly increase the silencing effect compared to scaffold with pD-\(b\)-P/DAmiR-29B complexes that had no Fab\(^{'}\)s. Therefore this additional functionalization was not included in subsequent studies.

In this chapter, in vivo evaluation of the scaffold functionalised with miR-29B and its effect on wound healing, specifically the effect on ECM remodelling following injury was studied. The hypothesis tested was that the scaffold functionalised with miR-29B, when applied to a rat excisional wound model will modulate the wound healing response by reducing collagen type I expression and beneficially increase the collagen type III/I ratio (indicating a less stiff, more compliant tissue) in the remodelling dermis. The second hypothesis tested was that miR-29B in combination with the scaffold will work synergistically towards an improvement in wound healing through modulation of MMP-8 and TIMP-1 expression.
Therefore, the objective was to investigate the effect of the components in this platform (miR-29B, 4S-StarPEG-collagen scaffold, pD₃₅-P/DA) on a number of key parameters involved in wound healing, using a number of techniques. Specifically:

i. Evaluate, using histological analysis, wound contraction and granulation tissue formation following full thickness excisional skin wounding in a rat model.

ii. Quantify collagen type I and III deposition by polarised light microscopy and qualitative immunohistochemistry for collagen type III within the wound bed.

iii. Employ an antibody labelled membrane protein array to elucidate the up/down regulation of factors associated with apoptosis, inflammation and ECM remodelling.

iv. Use enzyme linked immunosorbent assays (ELISA) to quantify the expression of TGF-β₁, TIMP-1, MMP-8 and determine the ratio of MMP-8: TIMP-1.

v. Determine the effect of the components of this platform on genes associated with wound healing using a rat wound healing PCR Array.

4.2 Materials and Methods

4.2.1 Materials

All solvents were of analytical or HPLC grade and were obtained from Sigma Aldrich Chemical Co. (Dublin, Ireland) unless otherwise stated. All oligonucleotides and primers were purchased from Eurofins MWG GmbH (Ebersberg, Germany). 4S-StarPEG was purchased from JenKem Technology USA (Allen, TX, USA).

4.2.2 Complexation

miR-29B mimic was obtained from Qiagen (Hilden, Germany) with the sequences for rno-miR-29B: 5'-uagcaccauugaaaucaguguu-3'; miRNA mimics were mixed individually with pD₃₅-P/DA at a w/w ratio of 8:1 in 1 X phosphate buffered solution (PBS) as previously optimised in Chapter Two. The components were mixed and complexes allowed to form at room temperature for 60 minutes.

4.2.3 Atelocollagen/ Poly (ethylene glycol) Ether Tetrasuccinimidyl Glutarate Scaffold Preparation

Atelocollagen was isolated as described elsewhere and the procedure is outlined in detail in Appendix D. Nine parts of collagen solution (3.5 mg/ml w/v) was gently and thoroughly mixed with one part 10 X PBS. The solution was neutralised by the drop-wise addition of 2 M sodium hydroxide (NaOH) until a final pH of 7–7.5 was reached and kept in an ice bath to delay gel formation. 4S-StarPEG was then added at a final concentration of 1 mM in a volume of 200 μl. The solutions were incubated for one hour at 37 °C in a humidified atmosphere to induce gelation (Appendix D.3).
4.2.4  In Vivo Rodent Skin Excisional Wound Model

All procedures performed were conducted under animal license no. B100/4342 granted by the Irish Department of Health and approved by the Animals Ethics Committee of the National University of Ireland, Galway. In addition, animal care and management followed the Standard Operating Procedures of the Animal Facility at the National Centre for Biomedical Engineering Science (NCBES). Fourteen Lewis female rats obtained from Harlan Laboratories (Bicester, UK) were allowed to acclimatize to housing conditions for at least seven days prior to use. The animals ranged between 200 and 250 g of body weight. The agents used to anesthetise the animals were intraperitoneal xylazine and ketamine (Xylapan and Narketan, Vetoquinol Ltd., Buckingham, UK) at a dose rate of 100 and 10 mg/kg, respectively. The experimental design included one time period of 28 days, which is an appropriate timepoint to evaluate the effect of treatments on extracellular matrix remodelling and composition of the maturing wound 31. The animals were anaesthetized, shaved and the dimensions of the wounds marked with a permanent ink marker. The surgical field was disinfected prior to surgery using iodine. Four full thickness one cm² wounds were placed at least one cm apart on the back of each rat. All procedures were performed under standard general practice principles of asepsis. Analgesia was administered subcutaneously up to four days post surgery and antibiotic therapy if necessary. The positioning of the four treatments was randomised and recorded. The treated wounds were covered with transparent polyurethane dressing (Opsite®, Smith & Nephew, Hull, UK). Numbered jackets were used on all the animals with the intention of preventing wound disturbance and facilitating the identification of experimental groups. Extra measures were taken to minimise wound disruption by housing all the animals individually for the duration of the study which was 28 days following surgery (See Appendix O for detailed description of surgery). Animals were monitored throughout the study and scored according to a behaviour scoring sheet (Appendix O).

A number of parameters were investigated in this in vivo study. The 4S-StarPEG collagen scaffold and miR-29B were independently and jointly investigated. Furthermore, the efficacy of pD-P/DA as a vehicle of miRs was investigated when embedded into a scaffold. To investigate the optimal dose delivery regimen through a scaffold, two dosing regimens were employed: a low dose (0.5 μg) and a ten-fold higher dose (5 μg). The higher dose of 5 μg was chosen as there exist reports of topical siRNA delivery in the ranges of 1 μg 32, 7 μg 33 and 8.7 μg 34. Furthermore, 0.5 μg was chosen as a low dose as it is a ten-fold decrease and is also within the range of siRNA doses employed in vitro (Chapter Two). Specifically; the groups investigated are detailed in Table 4.1 as follows:
Table 4.1: Groups investigated in an *in vivo* model of full thickness dermal wounds.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAi Dose</td>
<td>No</td>
<td>No</td>
<td>0.5 μg</td>
<td>5 μg</td>
<td>5 μg</td>
</tr>
<tr>
<td>Complexed</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Scaffold</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

4.2.5 *Harvesting and Processing of Tissue*

Animals were sacrificed by CO₂ asphyxiation at the post-implantation time period (28 days). Jackets and dressings were carefully removed and the wounds photographed prior to explantation. Samples were divided into half and the other half again further divided resulting in a total of one half and two quarters of tissue. The half portion of the explant was fixed in 10% neutral buffered formalin for 12 hours to be subsequently embedded in paraffin and was sectioned perpendicularly to the wound surface in 3 μm consecutive sections (see Appendix Q for detailed protocol). For general morphological analysis serial sections were stained with haematoxylin–eosin (H&E) stain (see Appendix R.1 for detailed protocol). A modified Movat pentachrome stain used (Russel–Movat–Pentachrome-stain kit, Mastertechs, Lodi, CA, USA) to stain ECM components (see Appendix R.2 for detailed protocols).

4.2.6 *Normalised Wound Contraction*

Wound closure was determined by comparing the distance between the wound boundaries at the initial time of surgery with the distance between the wound boundaries at day 28. Attempts were made at determining gross wound closure using tracing paper and photographs on the wounds at day 0 and day 28 but no significant difference between any of the groups could be detected using this method (Appendix Figure O.2). Therefore wound closure was evaluated using histologically as a function of wound contraction. To account for shrinkage effects due to dehydration and paraffin embedding, wounds were excised at day zero and processed in an identical fashion to those at day 28. Normalized Wound Contraction Index was calculated using the following formula:

\[
\text{Normalized Wound Contraction Index: } = \frac{W_0 - W_t}{W_0} \quad (\text{eqn. 4.1})
\]

where \(W_0\) is the distance of the wound bed at day 0 and \(W_t\) is the distance of the wound bed at day 28.

4.2.7 *Volume Fraction of Granulation Tissue*

Granulation tissue resulting from the wound healing process was readily distinguished using Russel-Movat’s Pentachrome staining, in which granulation tissue stained blue/cyan in contrast to a mature
yellow dermis. Micrographs were obtained at a magnification of 5 X; in each case the epithelium was horizontal to the image plane. The area of the granulation area was determined using ImageJ imaging software (National Institute of Health, USA) and the volume fraction of granulation tissue calculated using the following formula:

\[
\text{Volume Fraction of Granulation Tissue (\%) = \frac{A_{Gt}}{A_t} \times t \times 100}
\]  

(eqns. 4.2)

where \(A_{Gt}\) is the area of the granulation tissue on the slide, \(A_t\) is the area of total tissue present on the slide and \(t\) is the thickness.

4.2.8 **Ratio of Collagen III/I**

Quantitative polarised microscopy was performed on paraffin embedded sections stained with sirius red dye (Biocolor Ltd., Carrickfergus N. Ireland), a bifringence enhancer of collagens \(^{36}\). The slides were counterstained using Weigerts Haematoxylin for eight minutes. The sirius red dye enhancement of bifringence was used to differentiate between collagen type I and type III. Collagen type I-like fibres were identified as yellow and red and collagen type III-like fibres in green \(^{37}\) (see Appendix R.3 for detailed description of protocol). In the visualisation of the images, a polarising attachment BX-POL (Olympus, South End-on-Sea, UK) was used in a bright field microscope. This device consists of a polariser (U-POT) inserted in the illumination path and an analyser (U-ANT) oriented orthogonal to the polarised beam. Images were taken at 200 X magnification in the newly formed tissue within the wound bed and analysed (Appendix R.3). This analysis was performed using ImageJ threshold functions and the ratios calculated by dividing the output of collagen type III by collagen type I fibre analysis.

4.2.9 **Collagen Type III Immunohistochemistry**

Collagen type III production by the infiltrated fibroblasts in the wound area was elucidated using immunohistochemistry of representative sections. Hydrated sections were subjected to heat-induced antigen retrieval in pH 6.0 citrate buffer in a pressure cooker. The antibody used in the identification was a mouse monoclonal antibody to collagen type III (Abcam Plc, Cambridge, UK; dilution: 1:50); applied overnight at 4°C following blocking with goat-block. Endogenous peroxidase was blocked using hydrogen peroxide (DakoCytomation, Stockport, UK) for five minutes. An anti-goat horse radish peroxidase (HRP) labeled secondary antibody (DakoCytomation) was applied for 30 min followed by addition of 3,3′ Diaminobenzidine (DAB) chromagen (DakoCytomation) and the samples were counterstained with Mayer’s Haematoxylin. Three images were taken per slide and six slides per
treatment. The location of analysis was medial and the magnification used was × 400. Volume fractions of collagen type III were analysed using a grid size of 2.5 μm² (Appendix R.4).

4.2.10 Simultaneous Detection of Rat Protein Expression Using Membrane Array for 90 Proteins

Explanted tissue was frozen at -80 °C until ready. The tissue was thawed slowly on ice, chopped into small pieces and approximately 5 mg was incubated in a lysis buffer containing a cocktail of protease inhibitors for five minutes. The tissue was mechanically disrupted using a bead mill homogeniser which oscillates a stainless steel bead through the tissue (TissueLyserLT, QIAGEN, Hilden Germany) for five minutes at least twice until tissue was completely homogenised and centrifuged at 15,000 g for 15 minutes. The protein fraction of the centrifuged sample was extracted, aliquoted and stored at -80 °C until further use. The protein content of the samples was determined using a protein quantification assay kit (BioRad, Hercules, CA. Appendix M.1) and probed for 90 proteins simultaneously using a biotin label-based rat antibody array (RayBio®, Norcross, GA, USA) according to the manufacturer’s instructions. Briefly, four samples were pooled according to the treatment group to a total of 1 mg/ml and dialysed overnight to remove remaining lysis buffer and the protein membranes were blocked using a provided blocking buffer. The proteins were labelled with biotin using amines present in proteins and incubated with the antibody labelled membranes overnight. Following this, HRP-conjugated streptavidin was reacted with the membranes and afterwards treated with the incubation buffer provided. Membranes were exposed using a Kodak™ Image Station 4000MM Pro (Kodak, Japan). Micrographs of the exposed membranes were digitally evaluated using ImageJ software plug-ins. The images obtained were imported into ImageJ and analysed using a protein array analyser plug-in by normalising to the given positive control signals on the membranes. For relative comparison, all samples were normalised to healthy native skin which was not wounded (see Appendix M for details of all procedures).

4.2.11 TGF-β1, MMP-8 and TIMP-1 Quantification

Three enzyme linked immunosorbent assays (ELISAs) were performed using protein isolates which had been aliquoted from the treatment groups. The ELISA analytes included one for transforming growth factor (TGF)-β1 (Abcam Plc), matrix metalloproteinase (MMP)-8 (Abcam Plc), and tissue inhibitor of metalloproteinase (TIMP)-1 (R&D Systems, Minneapolis, MN, USA). As previously mentioned; TGF-β1 plays a significant role in matrix remodelling following injury whereby it stimulates ECM production. A distinct role of miR-29B is in post-transcriptional silencing of ECM fibrillar components stimulated by TGF-β1, therefore quantification of TGF-β1 expression was essential in this study. Matrix turnover, i.e. the enzymatic remodelling of ECM is an important consideration in healing wounds and indeed remodelling components were revealed to be
significantly dysregulated in the protein membrane array. Notably, MMP-8 is a collagen cleaving enzyme which functions to degrade type I, II and III collagens and in the context of wound healing; MMP-8 has been shown to be the predominant collagenase in healing wounds and non-healing ulcers. TIMP-1 complexes with MMPs and irreversibly inactivates them by binding to their catalytic zinc co-factor and, furthermore, TIMP-1 is a direct inhibitor of MMP-8.

An equal amount and concentration of each protein sample was added to each well for each ELISA plate which was determined using a protein assay kit (BioRad, Hercules, CA). ELISAs were performed according to the manufacturer’s instructions (Appendices Q.4, Q.5 and Q.6).

4.2.12 RNA Extraction

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

4.2.13 Wound Healing RT-PCR Array

Contaminant DNA was eliminated from RNA preparations using DNase I. The yield and quality of total RNA was determined according to the ratio of spectrophotometric absorbance values at wavelengths of 260 and 280 nm. cDNA synthesis was performed using DNase-treated RNA and random decamer primers using an RT² First Strand Kit (Qiagen). The cDNA generated was used as a template for quantitative real-time PCR. A mastermix was prepared using a RT² SYBR Green Mastermix. This mixture was added to 384 wells in an RT² Profiler™ PCR Array, Rat Wound Healing Array (PARB-0121Z; Qiagen). This PCR array contained RT² qPCR Primer Assays for a set of 84 cytokines which are detailed in Appendix U, Table U.5. The standard cycling conditions were as recommended by the PCR array supplier. Data were collected at the end of the annealing step. Fold changes in gene expression between the affected and control groups were calculated using the ΔΔCt method in the PCR array data analysis template. An examination of Ct value consistency for the housekeeping genes indicated that normalization was performed adequately. A similar evaluation of the built-in RNA controls verified an absence of genomic DNA contamination and inhibitors of either the reverse transcription or PCR (Appendix U.2).

4.2.14 Statistical Analysis

All samples were tested in triplicate and all experimental groups were analysed in triplicate. GraphPad Prism® (v.5 GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Analysis of
variance (ANOVA) was used followed by Tukey’s post-hoc test to determine statistical significance between groups. ANOVA was performed assuming normal distribution of the data which was tested and verified using the Anderson-Darling test. All graphical data is presented as mean ± standard deviation of mean. p values of < 0.05 were considered statistically significant. Outliers were calculated using Grubb’s test and eliminated from the results to be analysed. Where appropriate, Pearson’s test for correlation was performed where significance was set at p < 0.05.

4.3 Results and Discussion

4.3.1 Gross Wound Contraction

In the evaluation of gross wound contraction, the use of sterile tracing papers was attempted as an objective method for measuring wound area fractions. However, this method proved inaccurate and did not provide a statistically significant output (see Appendix O, Figure O.2 for these results). Furthermore, post-excisional wound photographs were used as visual aids in the assessment of wound healing but were not considered an option for quantification purposes. Measurements cannot be performed accurately on photographs of non-flat surfaces and, furthermore, another major difficulty in the evaluation of wound closure by this method was to determine an objective visual boundary between wounded and healed areas. Assessment of epithelialisation showed that all wounds were fully healed and therefore no difference between treatment groups can be detected using this parameter. The rationale for using a rat excisional model in this study was due to the established data of miR profiling in rodents and also the commercial availability of antibody and protein tools that can be applied to this species. Additionally, this rodent model is an established model of cutaneous wound healing. Other models such as a rabbit ear ulcer model could not be used due to the limited availability of primary antibodies, ELISAs and miR profiling.

4.3.2 Normalized Wound Contraction

Through histological analysis of the wounds at day 28 compared to day 0, application of scaffolds to the wounds significantly reduced the contraction of the wounds which was evaluated by the distance of original wound edges at day 28 (Figure 4.2). Such a response with regard to wound contraction after treatment with biomaterial scaffolds has been conclusively demonstrated by others. The percentage of wound contraction, as measured histologically when comparing the wound width at day 28 against initial wound width at day 0, was reduced significantly by 10 % in the scaffold only treatment group (Figure 4.2). Contraction is also significantly reduced when miR-29B is incorporated into the scaffold. Incorporation of 0.5 μg or 5 μg of miR-29B significantly reduced contraction by 15 % when compared to no treatment control. However, incorporating 5 μg of miR-29B complexed with
Figure 4.2: Effect of treatments on wound contraction (normalized to wound width at day 0). Wound contraction was evaluated sterologically using pictures such as the representative images in Figure 4.3. Normalised wound contraction is the relative decrease in wound margin width at day 28 compared to day 0. Data presented is the mean ± standard deviation (n = 6) analysed by one-way ANOVA and Tukey’s post-hoc test. * indicates statistical significance when compared to indicated groups; p < 0.05. # indicates statistical difference compared all other groups presented p < 0.05.
Figure 4.3: Representative Russel-Movat’s Pentachrome staining of wound bed sections at day 28 with blue/cyan representing the field of granulation tissue and yellow being the original skin collagen which indicates the wound margins. Scale bar indicates 100 μm.
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Figure 4.4: Granulation Volume Fraction was evaluated sterologically using pictures such as the representative images in Figure 4.3; n = 6. Granulation volume fraction is indicated by the cyan/blue granulation tissue presented centrally in the images of Figure 4.3 between the wound margin boundaries and the epithelium. Data subjected to Grubbs outlier test to remove outlying data. Data presented is the mean ± standard deviation analysed by one-way ANOVA and Tukey's post-hoc test. * indicates statistical significance between the groups indicated, p < 0.05.
Figure 4.5: Ratio of collagen type III-like fibers to collagen type I-like fibers, within the wound bed, determined from sections staining with Picrosirius Red (representative images in Figure 4.7). n = 6. Data presented is the mean ± standard deviation analyzed by one-way ANOVA and Tukey’s post-hoc test. * indicates statistical significance when compared to healthy un-wounded skin, p < 0.05. # indicates statistically significant difference between the groups indicated, p < 0.05.
Figure 4.6: Polarised light microscopy of Picrosirius stained wound bed sections. Green stained fibres demonstrate the presence of collagen type III-like fibres and yellow and red stained fibres demonstrate the presence of collagen type I-like fibres. Collagen type-I like fibers are highlighted with red arrows within some sections and collagen type-III like fibres are highlighted with green arrows. Sections are counterstained with Weigerts Haematoxylin (purple). Scale bar indicates 100 μm.
Figure 4.7: Micrographs of collagen type III immunohistochemical staining of wound bed sections, counterstained with haematoxylin. Scale bar indicates 100 μm. Black arrows highlight collagen type III deposition which is stained brown.
Figure 4.8: (a) Negative control for collagen type III immunohistochemistry. Micrograph presents tissue not incubated with the primary collagen type III antibody, but still subjected to all other steps, including incubation with the DAB chromagen and counterstaining with haematoxylin. Scale bar indicates 100 μm. (b) 100 X micrograph to magnify the visualisation of collagen type III immunostaining in samples stained with the collagen type III antibody. Arrow indicate the presence of collagen type III, sample counterstained with haematoxylin. Scale bar indicates 20 μm.
pDb-P/DA in a scaffold compromised this effect on wound contraction. Overall, the incorporation of uncomplexed 5 μg miR-29B into the scaffold produced the greatest reduction in wound contraction and this effect was significant when compared to the no treatment control, the use of a scaffold alone and also the treatment with 5 μg of complexed miR-29B in the scaffold. This improvement when using a scaffold with uncomplexed 5 μg miR-29B is corroborated when measuring the volume fraction of granulation tissue which revealed a significant increase in volume fraction of granulation tissue when compared to no treatment (55 % versus 30 %, Figure 4.5), an effect that was statistically significant when compared to all other groups. This improvement when using a scaffold with uncomplexed 5 μg miR-29B is corroborated when measuring the volume fraction of granulation tissue which revealed a significant increase in volume fraction of granulation tissue when compared to no treatment (55 % versus 30 %, Figure 4.5), an effect that was statistically significant when compared to all other groups. This is significant considering the effect of contracture on scar formation. Cutaneous wounds heal through a combination of epithelial migration from the wound margins and contraction of the wound bed to bring these wound margins closer together. This wound contraction is a vital part of the wound repair process and a highly evolved strategy to reduce the size of the area of wound exposed to the external environment, which subjects the patient to potential infection and fluid loss. Wound contraction and scarring, which occur after large injuries can, however, lead to substantial loss of function and poor aesthetic appearance. Therefore therapeutic strategies that reduce contraction may significantly improve the quality of the life of patients recovering from substantial cutaneous injuries. Wounded areas that risk restriction due to contracted scars will potentially benefit from scaffolds that resist contraction forces and therefore skin tension until healing is complete.

Polarised light microscopy was employed to obtain simultaneous imaging of collagen type I like fibres and collagen type III like fibres in the same field of view (Figure 4.7). These images were analysed using ImageJ with threshold functions for quantifying the volume fraction of collagen type I-like fibres and collagen type III-like fibres and ultimately the ratio of one to another. When compared with native, unwounded skin, all groups, with the exception of the scaffold with 5 μg uncomplexed miR-29B had a statistically significant reduced collagen type III: collagen type I ratio. This result suggests that the miR-29B, at this dose (5 μg) delivered from a scaffold, has a significant effect in restoring the natural balance of collagen type III: collagen type I ratio which otherwise becomes dysregulated following traumatic injury. Immunohistochemical staining of collagen type III expression and deposition showed that collagen type III expression was increased in wounds treated with the scaffold and a high dose of uncomplexed miR-29B. Evaluation of collagen deposition (Figures 4.6, 4.7 and 4.8) may perhaps have been premature as collagen fibres are still at early stages of remodelling, though if the time periods in the animal model were prolonged the identification of wounded areas could have become an issue and the wound model would be adapted accordingly (bigger wounds or wound frames to counteract wound closure by contraction).
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4.3.2 Proteomic Profiles from Treatments

In order to obtain an overview of the proteins dysregulated following cutaneous injury in this model, and following application of the platform developed in this study, a biotin label based rat antibody array, capable of detecting the expression levels of 90 rat proteins was employed. This array provided the most extensive range of proteins that could be assessed in this model and encompassed many key proteins pivotal in ECM remodelling. The array, which consisted of a membrane blot array, was analysed using a protein array plug-in on Image J (see Appendix M.2 for details of how this analysis was performed). The membranes were normalised according to internal controls specified in the array, and all membranes were analysed in comparison with tissue obtained from the dermis of an untreated (and unwounded) animal. Following this, the array of proteins dysregulated in the control group which received no treatment to an incurred wound was considered the standard from which all the treatments were compared. For simplicity and ease of understanding the main findings of this array (i.e. proteins that are upregulated and those that are downregulated) are represented in Figure 4.9 in a Venn diagram where each treatment parameter is represented by a circle. The findings illustrate a global effect as they are representative of pooled samples and cannot be subjected to discrete quantification. The proteins affected have been clustered for transparency; for instance; growth factors such as VEGF, EGFR; apoptotic factors such as Fas Ligand, FADD; ECM remodelling proteins Activin, TGF-βs, TIMPs, MMPs; and inflammatory cytokines; IL-1α, IL-1β, MCP-1.

Apoptosis, is a morphologically and biochemically distinct process of cell death which is a crucial control mechanism for the development of organs during embryogenesis, and also for the maintenance of tissue homeostasis in mature organisms. Apoptosis has been identified as a key player in the transition between granulation tissue and the formation of definitive scar after soft tissue injury. The apoptosis of fibroblasts, endothelial cells, and pericytes appears to affect cell types in successive waves after wound closure. When a wound closes and evolves into a scar, there is a dramatic decrease in cellularity and, in particular myofibroblasts.

In Figure 4.9, the proteins associated with apoptosis, namely Fas TNFSF6 and Fas Ligand TNFSF6 are shown to be downregulated when a scaffold is applied. This suggests that the transition between granulation tissue and formation of a definitive scar has not evolved. Indeed, this is in agreement with the previous observations in which wound contraction was reduced when using a 4S-StarPEG collagen scaffold (Figure 4.2). However, when incorporating miR-29B into the 4S-StarPEG collagen scaffold at the high dose of 5 μg of miR-29B, uncomplexed or complexed with pD-b-P/DA, apoptotic factors are upregulated. This is extremely significant for a number of reasons. It demonstrates that the miR-29B is having an effect on the upregulation of apoptotic proteins and this effect is also dose dependent. The role of miR-29B on apoptosis has only recently been elucidated.
For instance, miR-29B is known to have many targets, one of which is the anti-apoptotic protein McI-1. Therefore, increased presence of miR-29B can elicit increased apoptosis. This is somewhat in agreement with the data presented from the protein array in which an upregulation of apoptotic proteins is present when delivering 5 μg of miR-29B (uncomplexed or complexed with pD-b-P/DA). However, this contradicts the association of apoptosis with the transition of granulation tissue to the formation of a definitive scar. While the underlying mechanisms may remain unclear, one needs to take into consideration the other targets and effects of miR-29B. Additionally, the in vivo environment present here has increased expression of TGF-β1, and TGF-β1 has often been reported to offer apoptotic resistance to cells. The inflammatory response is regarded as the first of a number of overlapping processes that constitute wound healing. In skin repair, the infiltrating leukocytes, monocytes (which later become macrophages) and neutrophils are the principal components of the inflammatory response. They not only act as effector cells combating pathogens but are also involved in tissue degradation and tissue formation. Ultimately, the inflammatory response dictates the quality of the healing response. There are several situations that provide evidence that the inflammatory phase during repair is intimately linked to the extent of scar formation. In contrast to post-natal repair, wound healing in early foetal skin exhibits scarless regeneration of dermal architecture which is intrinsically linked with the lack of inflammatory response. In support of this, scar formation has been demonstrated to be accelerated when inflammation is initiated in foetal wounds. From the results of the protein membrane array, a number of observations can be made on the up/down regulation of proteins involved in the inflammatory response. For instance, application of the 4S-StarPEG collagen scaffold resulted in a knockdown of the inflammatory cytokines. This suggest that the 4S-StarPEG collagen scaffold elicits a minimal host response as it is partially composed of atelocollagen, a form of collagen without the terminal peptides, which has extremely low antigenicity due to the elimination of antigenic regions by proteolytic processing. The incorporation of miR-29B into the scaffold elicits the upregulation of some inflammatory cytokines (IL-1α, IL-1β) and this pattern exists for both 0.5 μg and 5 μg of uncomplexed miR-29B. However, when 5 μg of miR-29B complexed with pD-b-P/DA is delivered with the 4S-StarPEG collagen scaffold there is an upregulation of multiple inflammatory cytokines. This is due, in part, to the presence of pD-b-P/DA in the scaffold. The higher dose of miR-29B chosen in this study, 5 μg, equates to 40 μg of pD-b-P/DA when delivered in complexed form. pD-b-P/DA is relatively non-toxic in vitro due to its highly PEGylated structure; however this characteristic is dose-dependent. The degradation of pD-b-P/DA in vivo most likely occurs through hydrolysis of the ester links in all of its components; pDMAEMA, PEGDA and PEGMEA. The PEG component of both PEGDA and PEGMEA are linear esters which are susceptible to hydrolytic degradation. This degrades the complexing agent into smaller components which can be gradually cleared by the tissue and excreted.
Figure 4.9: Venn diagram summarising the proteins that are upregulated (red) and downregulated (blue). Proteins are clustered into various pathways such as those indicative of remodelling, growth factors, inflammatory markers and ligands of apoptosis.
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via renal filtration. Acrylates and methacrylates (which are components of PEGDA and PEGMEA respectively) are widely used in the formulation of polymeric materials for medical and dental applications, but there are reports on the local toxicity of these monomers. The degradation products of pD-P/DA will contain some acrylates and methacrylates as PEGDA and PEGMEA were used as branching and backbone structures in the complexing agent. However, considering that 3% of vinyl groups are free in this structure (as determined in Chapter Two), local toxicity due to acrylate products should be negligible. There also exists a concern regarding the presence of copper chloride which was used in the ATRP reaction to synthesise pD-P/DA. Although filtration and dialysis were performed, it is possible that trace amounts of copper may still be present. This could account for the upregulation of inflammatory cytokines present in the group which includes pD-P/DA due to its degradation products in vivo.

Based on the observations from the biotin label based rat antibody array, three ELISAs were performed to quantify and determine a statistically significant relationship between the treatments and the affected protein. These ELISAs measured TIMP-1 (Figure 4.10), MMP-8 (Figure 4.11) and TGF-β1 (Figure 4.12). The observations detected in the membrane protein array with regard to TGF-β1, TIMP-1 and MMP-8 expression are, for the most part, in agreement with the ELISAs. Any inconsistency between the two representations can be attributed to samples which may have high levels of a particular protein causing deviation in the ELISA results and possible saturation during the pooling of sample for the membrane protein array. TGF-β1 is dysregulated (in this case downregulated) in the 4S-StarPEG collagen scaffold group (based on protein array data and ELISA quantification). This is reflected in the same group by the downregulation of MMP2 and MMP13 (in the membrane protein array) which are known to be stimulated by TGF-β1. However, the membrane protein array was performed on pooled samples and it could be possible that one sample could be masking/saturating the true levels of TGF-β1. The TGF-β family is a key mediator of ECM production and this has been the basis upon which TGF-β1 has been delivered with biomaterials in order to enhance ECM production. For instance, Pandit et al. delivered TGF-β using a collagen scaffold in a rabbit ear ulcer model and found an increased contraction rate when compared to a collagen scaffold alone. However, this treatment also resulted in a significantly increased ultimate tensile strength of the wounds when compared to an untreated wound suggesting a stronger healed wound. Regardless, TGF-β1 is beneficial to increase the healing rates of wounds that do not need to be aesthetically appealing, yet the ideal scenario of tissue regeneration needs to allow for controlled healing through which there is not exaggerated deposition of fibrillar ECM proteins that contribute to unsightly, compromised scar tissue. It is established that miR-29B acts post-transcriptionally on many of the ECM proteins that are stimulated by TGF-β1, however there are many conflicting reports.
Figure 4.10: Fold change of TIMP-1 expression in excised tissue at day 28, data normalised to healthy un-wounded skin. No Treatment indicates a wound only control. Data presented is the mean of \( n = 4 \pm \text{standard deviation} \) analysed by one-way ANOVA and Tukey's post-hoc test. * indicates statistical significance between the groups indicated (\( p < 0.05 \)).
Figure 4.11: Fold change of MMP-8 expression in excised tissue at day 28, data normalised to healthy un-wounded skin. Data presented is the mean of n=4 ± standard deviation analysed by one-way ANOVA and Tukey’s post-hoc test. * indicates statistical significance when compared to a treatment of scaffold alone (p < 0.05).
Figure 4.12: Fold change of TGF-β1 expression in excised tissue at day 28, data normalised to healthy un-wounded skin. Data presented is the mean of n=4 ± standard deviation analysed by one-way ANOVA and Tukey's post-hoc test. * indicates statistical significance when compared to a treatment of scaffold with 0.5 μg uncomplexed miR-29B (p < 0.05).
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Figure 4.13: Ratios of MMP-8 to TIMP-1 expression in excised tissue at day 28, data normalised to healthy un-wounded skin. Data presented is the mean of n=4 ± standard deviation analysed by one-way ANOVA and Tukey's post-hoc test. * indicates statistical significance between the groups indicated (p < 0.05).

No Treatment 0 0.5 5 5
μg miR-29B delivered

With Scaffold

With Scaffold and pD-b-P/DA Complexation

**Figure 4.13** Ratios of MMP-8 to TIMP-1 expression in excised tissue at day 28, data normalised to healthy un-wounded skin. Data presented is the mean of n=4 ± standard deviation analysed by one-way ANOVA and Tukey's post-hoc test. * indicates statistical significance between the groups indicated (p < 0.05).
### Table 4.2: Pearson’s correlation co-efficients between parameters measured for no-treatment group.

<table>
<thead>
<tr>
<th></th>
<th>Gran</th>
<th>Col III/I</th>
<th>MMP-8</th>
<th>TIMP-1</th>
<th>TGFβ1</th>
<th>Ratio</th>
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Abbreviations: *Gran*: granulation tissue volume fraction, *Cont*: normalized wound contraction, *Col III/I*: collagen type III to collagen type I ratio, *MMP-8*: matrix metalloproteinase-8, *TIMP-1*: tissue inhibitor of matrix metalloproteinase-1, *TGFβ1*: transforming growth factor-β1, *Ratio*: ratio of MMP-8: TIMP-1. * indicates a statistically significant correlation, p < 0.05.

### Table 4.3: Pearson’s correlation co-efficients between parameters measured for scaffold + 0 μg miR-29B group.

<table>
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<th>TIMP-1</th>
<th>TGFβ1</th>
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Abbreviations: *Gran*: granulation tissue volume fraction, *Cont*: normalized wound contraction, *Col III/I*: collagen type III to collagen type I ratio, *MMP-8*: matrix metalloproteinase-8, *TIMP-1*: tissue inhibitor of matrix metalloproteinase-1, *TGFβ1*: transforming growth factor-β1, *Ratio*: ratio of MMP-8: TIMP-1. * indicates a statistically significant correlation, p < 0.05.
Table 4.4: Pearson’s correlation co-efficients between parameters measured for scaffold + 0.5 μg miR-29B group.

<table>
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<th>TIMP-1</th>
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Abbreviations: Gran: granulation tissue volume fraction, Cont: normalized wound contraction, Col III/I: collagen type III to collagen type I ratio, MMP-8: matrix metalloproteinase-8, TIMP-1: tissue inhibitor of matrix metalloproteinase-1, TGFβ1: transforming growth factor-β1, Ratio: ratio of MMP-8: TIMP-1. * indicates a statistically significant correlation, p < 0.05.

Table 4.5: Pearson’s correlation co-efficients between parameters measured for scaffold + 5 μg miR-29B group.

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Abbreviations: Gran: granulation tissue volume fraction, Cont: normalized wound contraction, Col III/I: collagen type III to collagen type I ratio, MMP-8: matrix metalloproteinase-8, TIMP-1: tissue inhibitor of matrix metalloproteinase-1, TGFβ1: transforming growth factor-β1, Ratio: ratio of MMP-8: TIMP-1. * indicates a statistically significant correlation, p < 0.05.
Table 4.6: Pearson’s correlation co-efficients between parameters measured for scaffold + 5 μg miR-29B/ pD-α-P/DA group.

<table>
<thead>
<tr>
<th></th>
<th>Gran</th>
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<th>MMP-8</th>
<th>TIMP-1</th>
<th>TGFβ1</th>
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Abbreviations: Gran: granulation tissue volume fraction, Cont: normalized wound contraction, Col III/I: collagen type III to collagen type I ratio, MMP-8: matrix metalloproteinase-8, TIMP-1: tissue inhibitor of matrix metalloproteinase-1, TGFβ1: transforming growth factor-β1, Ratio: ratio of MMP-8: TIMP-1. * indicates a statistically significant correlation, p < 0.05.
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regarding the direct crosstalk between miR-29B and TGF-β1. TGF-β1 has not been established to be a target of miR-29B. On the contrary, TGF-β1 has been suggested to downregulate endogenous miR-29B expression. Winbanks et al. found that TGF-β1 can attenuate the differentiation of myogenic cells by increasing the expression of histone deacetylase 4 (HDAC4), a key inhibitor of myogenic commitment. Down-regulated expression of miR-29B which acts as a translational repressor of HDAC4 was the main determinant. A similar effect has been noted in renal fibrosis where Smad3 mediated TGF-β1 has downregulated miR-29B by binding to the promoter of miR-29. MMPs and their inhibitors; TIMPs, specifically MMP-8 and TIMP-2, play important roles in the degradation and regeneration of wounded tissue. MMPs are inactivated by TIMP-1, TIMP-2, TIMP-3, and TIMP-4 which act by forming a 1:1 complex with the catalytic zinc in the MMPs site. It has been suggested elevation of TIMP-1 may be a surrogate marker for increased ECM turnover. The scaffold with 5 µg miR-29B had the lowest level of TIMP-1 compared to the other samples, and this decrease was statistically significant in comparison with the no treatment control and treatment with 5 µg of miR-29B complexed with pD-b-P/DA in a scaffold. TIMP-1 is a tissue inhibitor of MMP-1 and MMP-8 which in turn breaks down collagens type I, II and III in tissue. MMP-8 was chosen for ELISA analysis as it is established as a predominant collagenase in healing wounds. MMP-8 expression was increased in the group treated with a scaffold, containing both uncomplexed miR-29B and miR-29B complexed with pD-b-P/DA in the membrane protein array. Conclusively, the ELISA results show that a scaffold alone had the lowest MMP-8 expression, which was statistically significant when compared to a scaffold with a 5 µg of uncomplexed miR-29B.

Considering the effects of the treatment groups on the ratio of MMP-8 to TIMP-1 (Figure 4.13) brings further understanding to the results of this study. The use of a scaffold with 5 µg miR-29B resulted in a significantly higher MMP-8: TIMP-1 ratio when compared to all other groups analysed in this study. This suggests a number of things. Firstly, ECM remodelling is still ongoing as a ratio of 1:1 would suggest that the MMP-8: TIMP-1 ratio is balanced but with the application of a scaffold with 5 µg miR-29B; this ratio is ~1.4. Although this ratio is not excessively high, it is in much contrast to all the other groups, the greatest of which has a MMP-8: TIMP-1 ratio of 0.5. Pearson's correlations were obtained in order to assess whether the wound healing parameters correlated when compared to the controls, which gave an indication of the state of healing for each treatment group (Table 4.2, 4.2, 4.4, 4.5 and 4.6) and some significant correlations were detected between wound healing events in each group. For instance, the no-treatment group had a significant inverse relationship between granulation volume fraction and collagen type III/I ratio which suggests that an increased granulation volume fraction correlates with a decreased collagen type III/I ratio, which suggests more collagen type I present in the wound bed. Additionally, a statistically significant correlation was detected between...
wound contraction and the parameters TGF-β₁ fold change and the MMP-8: TIMP-1 ratio. This is in agreement with studies that associate increased TGF-β₁ expression with faster wound healing. Furthermore, granulation volume fraction in the no treatment group had a statistically significant inverse relationship with TIMP-1 fold change which is reflected in a strong correlation between granulation volume fraction and MMP-8: TIMP-1 ratios. This indicates that remodelling and proliferation is still quite active in this group.

Multiple regression analyses were carried out to determine whether any of the output parameters can be predicted by measurement of another parameter. In this analysis, all the parameters that were measured (normalised wound contraction, granulation tissue volume fraction, ratio of collagen type III to collagen type I, TIMP-1 fold change, MMP-8 fold change, TGF-β₁ fold change and the ratio of MMP-8: TIMP-1) were cross-compared with each other. However, no statistically significant regression trend was detected (p < 0.05). Following this, linear regression analysis was performed to elucidate if there was a significant trend between the input parameters and the output parameters investigated, and revealed a statistically significant correlation between uncomplexed miR-29B dose (only when delivered through a scaffold) and the MMP-8: TIMP-1 ratio (Figure 4.13, Pearson’s coefficient r = 0.9996, p < 0.05). This suggests that the employment of a scaffold with miR-29B results in a modulation of wound healing in which the granulation tissue is still being remodelled, one which is dose dependent.

The wound healing PCR array analysis revealed that a number of genes were upregulated and downregulated which were in agreement with results from the protein membrane array and ELISAs. Four select samples which showed significant results from the membrane protein array and ELISAs were investigated, namely; the treatment with the scaffold alone, the treatment with 5 μg uncomplexed miR-29B in a scaffold, the no-treatment control for comparison and healthy skin to base the relative expression of genes. B2M, HPRT1, RPL13A, GAPDH were chosen as internal housekeeping controls within each sample group. For ease of representation, the genes which were upregulated and also downregulated are presented in the form of Venn diagram in Figure 4.15; a cut off of a fold change of five was chosen. The Venn diagrams present the number of genes that are upregulated or downregulated independently by a treatment, those that are commonly upregulated or downregulated between two groups, and those that are commonly upregulated or downregulated by all three groups. Gene expression analysis of the scaffold alone versus the scaffold with 5 μg uncomplexed miR-29B was conducted to investigate further the mechanism underlying improved collagen type III/I ratio when miR-29B is incorporated into the scaffold. Interestingly, this analysis yielded some results that had minor discrepancy with the protein expression study results. The most likely explanation for the opposing observations is that, in this study, wound healing gene expression...
Figure 4.14: An example heat map of gene expression data obtained from wound healing RT-PCR array. Data presented is the upregulated (red) and downregulated (green) genes after 28 days when compared with healthy skin. Treatments analysed include No Treatment (wound only) and treatment of wounds with a 1 mM 4S-StarPEG crosslinked collagen scaffold alone, or a 1 mM 4S-StarPEG crosslinked collagen scaffold with 5 μg of miR-29B.
Figure 4.15: Overview of number of genes altered in the different comparisons studied in the PCR array analysis with fold change cut off being five. Data presented is the upregulated (red) and downregulated (blue) genes after 28 days when compared with healthy skin. Treatments analysed include No Treatment (wound only) and treatment of wounds with a 1 mM 4S-StarPEG crosslinked collagen scaffold alone, or a 1 mM 4S-StarPEG crosslinked collagen scaffold with 5 μg of miR-29B.
regulation is not just occurring at the mRNA level. Gene expression can be controlled at the post-transcriptional level by modulating the degradation rates of mRNA (as is the function of miRs) and thereby increasing the number of proteins translated per mRNA molecule. It is possible that there are other post-transcriptional controls that are still being unravelled, such as processes that can increase/decrease the affinity between the desired mRNA and ribosomes. It has been observed that protein and mRNA transcript levels do not consistently correlate and that it is not valid to assume that correlation implies causation in this context. Based on this, it would be appropriate to perform individual immunoblots such as Western blots (including appropriate loading controls such as GAPDH) in future studies. Regardless, multiple genes were deregulated when evaluated using PCR Array. The data is summarized in Figure 4.14 in a heat map format. Notably, col1a1, col1a2, col3a1, col4a1 and col4a3 were downregulated in the scaffold with 5 μg uncomplexed miR-29B compared to the scaffold alone. This is in agreement with numerous reports that document a decrease in ECM gene transcription following delivery or overexpression of miR-29B. TGF-β1 gene expression remained unchanged between the samples and would normally be expected that unchanged TGF-β1 expression correlates with an unchanged col1a1, col1a2 and col1a3 gene expression, however, in the scaffold with 5 μg uncomplexed miR-29B these genes were downregulated. This is because miR-29B silencing of these genes occurs post-transcriptionally. Notably, the five-fold downregulation of the col1a1, col1a2 and col3a1 gene expression was unique to the scaffold with 5 μg uncomplexed miR-29B.

4.4 Conclusions

In conclusion, multiple aspects of the remodelling response were evaluated in this study and from these evaluations there was a significant impact when excisional wounds were treated with a scaffold alone, a scaffold with a dose of 0.5 μg uncomplexed miR-29B, 5 μg miR-29B; uncomplexed or complexed with pD-b-P/DA. Any one of the treatments; scaffold alone or scaffold with a dose of 0.5 μg miR-29B, or 5 μg miR-29B resulted in a significant reduction in wound closure. Granulation volume fraction was greatest in the scaffold with 5 μg of uncomplexed miR-29B treatment. This treatment did not have a significantly lower collagen type III/I ratio when compared to native skin, and also had a statistically significant higher MMP-8:TIMP-1 ratio when compared to all other treatments. The dose of miR-29B in the scaffold had an effect in the majority of the parameters investigated. Two doses were investigated in this study; uncomplexed 0.5 μg, and uncomplexed 5 μg, both uncomplexed, and both incorporated within a scaffold. The use of the complexing agent; pD-b-P/DA, did not improve the effects of miR-29B in wound remodelling, when assessed by the collagen type III/I ratios, granulation volume
fraction and wound closure. Indeed the use of pD-β-P/DA complexed with 5 μg miR-29B in a scaffold caused an upregulation of inflammatory cytokines when compared to 5 μg of uncomplexed miR-29B in a scaffold. Notably, there was a significant correlation between the dose of uncomplexed miR-29B when delivered through the scaffold and the ratio of MMP-8:TIMP-1 which indicates that not only is the combination of these parameters important, but the dose of miR-29B also has a significant effect on the parameters investigated. Through all the investigations of this chapter it can be concluded that although a scaffold alone is beneficial towards reduced wound contraction, incorporation of miR-29B in a dose dependent manner (in this case 5 μg) ameliorates the wound healing process through modulation of MMP-8 and TIMP-1, collagen type I degradation, and the post-transcriptional inhibition of ECM proteins which are being stimulated by TGF-β1.
4.5 References


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68. Leivonen SK, Chantry A, Hakkinen L, Han J, Kahari VM. Smad3 mediates TGF-β-induced collagenase-3 (MMP-13) expression in human gingival fibroblasts. Evidence for cross-talk between Smad3 and P38 signaling pathways. J. Biol. Chem. 2002;277:46338-46346


Chapter Five

Summary and Future Directions

A portion of this chapter has been previously published in:


5.1 Introduction

Regenerative medicine aims to restore damaged tissue to its native structural and functional state. While this goal may be overly optimistic, progress towards this goal will likely improve the outlook for patients in a variety of disease and injury states. Simply reducing the extent of fibrotic scarring will reduce the pathology in a number of conditions. This is unquestionably true in the case of remodelling of cardiac tissue after myocardial infarction (MI). Gene therapy and biomaterials have each been proposed for the treatment of damaged heart tissue in a variety of applications, ranging from reducing inflammation, inducing angiogenesis, for functional reprogramming of cells, and to mechanically augment the damaged tissue. The overall goal of this research was to develop a biomaterial RNAi delivery system which inhibits fibrosis in a region of interest and thereby improving the functional outcome in a pathological setting with the future objective being towards a therapeutic option following MI.

5.2 Summary

5.2.1 Phase I- Delivering Exogenous miRNA (Chapter Two)

The objective of the first phase of this work was to develop a non-viral delivery vector that enabled effective complexation of microRNAs (miRs) and also conjugate antibody fragments to enable improvement in vector delivery. The proposed system was a linear poly (dimethylamino) ethyl methacrylate (pDMAEMA) structure synthesized using atom transfer radical polymerization (ATRP) and used as a macro-initiator to grow a hyperbranched poly (ethylene glycol) methyl ether acrylate (PEGMEA) based unit with a poly (ethylene glycol) diacrylate (PEGDA) block in a deactivation enhanced ATRP reaction to PEGylate and attribute functionalisation. This system was optimized and characterized in vitro as described in Chapter Two and given the nomenclature pD-b-P/DA.

The development of pD-b-P/DA included growth and characterization of this polymer to a molecular weight of 14,000 kDa, the controlled growth of which was verified using gel permeation chromatography (GPC). Proton nuclear magnetic resonance (1H NMR) determined this structure to be highly branched and PEGylated and to have vinyl groups (3 % content) present for conjugation of thiol terminated moieties. Optimization of the miR- pD-b-P/DA complexes was then conducted. This involved characterization using agarose gel electrophoresis, transmission electron microscopy (TEM), and UV NanoDrop™ spectroscopy to verify and optimise the complexation of pD-b-P/DA with miR mimics. A miR: pD-b-P/DA ratio of 1:8 was found to be optimal for the reporter gene; a dual plasmid of Firefly and Renilla Luciferase which was designed, constructed and verified as part of this thesis. A lipid based transfection agent; DharmaFect™ was optimized in order to serve as a comparative control.
when silencing reporter genes encoded by a luciferase-based plasmid transcribing in primary rat cardiac fibroblasts.

Antibody fragments (Fab’s), receptive to the CD90 antigen, were generated using hybridoma technology to generate monoclonal antibodies. Thiol terminated fragments were generated using pepsin digestion followed by reduction of the di-sulphide bond present in antibodies using dithiothreitol. pD-β-P/DA was tested for its ability to bind thiols using two methods. The first test entailed the use of cysteine mono-hydrochloride as a model of thiol terminated fragments. pD-β-P/DA was able to effectively consume thiols within 30 minutes which was determined via Ellmans assay. Thiol conjugativity was also demonstrated using SDS-PAGE of Fab’s with pD-β-P/DA. Using a custom luciferase reporter of knockdown; improved silencing was observed when delivering miR mimics complexed pD-β-P/DA decorated with Fab’s at 48 hours after delivery compared to miR/pD-β-P/DA complexes with no Fab’s; however this effect disappeared at 96 hours.

5.2.2 Phase II- An Injectable Scaffold Delivery System (Chapter Three)

Phase II aimed to develop a scaffold using collagen type I and obtain non-toxic in situ crosslinking using a PEG-based crosslinker, which in this case was poly (ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG). It was hypothesized that this scaffold will be non-toxic to cells, have improved mechanical and chemical stability due to its crosslinking, and will enable the delivery and release of miR-29B complexes. Phase II of this project, which is described in Chapter Three realises all of these objectives. Crosslinking was achieved using 4S-StarPEG, and this was verified using quantitative chemical absorbance of the free amine groups remaining after crosslinking. The optimal crosslinking concentration was determined to be a 1 mM concentration of 4S-StarPEG which had only 9.5 % of free amines remaining after crosslinking (relative to a non-crosslinked collagen scaffold). Crosslinking using 4S-StarPEG also improved resistance to enzymatic degradation of the scaffolds and hydrogels crosslinked with concentration of 1 mM 4S-StarPEG had a remaining mass of 85 % whereas a non-crosslinked hydrogel had completely degraded at 48 hours. Furthermore, a plateau in crosslinking occurred in which a concentration of 1 mM crosslinker offered the maximum crosslinking density. Investigation of the release profiles of RNA from the scaffolds in vitro showed a crosslinker concentration dependent release, which was attributed to two reasons. Firstly, that the more crosslinked the scaffold, the slower degradation occurs which delays the release of RNA. Furthermore, due to the possible interaction of the crosslinker with amine groups present in the RNA, the crosslinker is likely to have crosslinked the RNA into the scaffold. In essence, the higher the crosslinker density (in this case the highest used was 1 mM) the more retarded the release from the scaffold. The lowest crosslinker density (0.05 mM) had the quickest release, in fact, 100 % release had
occurred after three days, but this was also due to the fact that the scaffold had completely degraded at this point. An additional parameter investigated, that of RNA carrier, was deemed to also have a significant effect on the release profile for RNA from the scaffold. Poly (ethylenimine) (PEI) which is highly cationic due to an abundance of primary amine groups had the slowest release from the scaffold. This is attributed to a charge interaction of the positive amine groups of PEI interacting with the negatively charged collagen in the scaffold and also possible crosslinking with the 4S-StarPEG which reacts with amine groups. pD-β-P/DA has only a slightly positive charge due to the presence of tertiary amines and therefore had less charge interaction with the scaffold. Due to this, its release profile was much quicker than that of PEI and was similar to that seen with naked RNA.

The next component of in vitro testing in Phase II required the investigation of miR-29B as an inhibitor of collagen type I and collagen type III. Previously, in Chapter Two, delivery of exogenous miRs resulted in a demonstrated knockdown in a reporter Luciferase protein. It was sought to evaluate this in both a monolayer culture model and scaffold delivery model of knockdown. miR-29B was shown to specifically silence collagen type I and collagen type III in monolayer culture, but only when delivered using a complexing agent (down to 51 and 55% respectively). Then, using a 4S-StarPEG scaffold as a delivery platform of these miR-29B complexes, efficient silencing, of both collagen type I and collagen type III mRNA, up to a period of up to 14 days was achieved and knockdown of collagen type I mRNA was more profound than that of collagen type III. Furthermore, naked miR-29B within the scaffold was effective at silencing collagen type I and collagen type III mRNA, but the functionalization of miR-29B/pD-β-P/DA complexes with antibody fragments did not result in a significant improvement in efficiency when delivered in a scaffold.

5.2.3 Phase III- System Evaluation in a Rat Excisional Wound Model (Chapter Four)

The penultimate phase of testing of this system occurred in vivo, as described in Chapter Four. The goal in Phase III was to evaluate the scaffold and miR-29B and its effect on wound healing in vivo, specifically the effect on ECM remodelling following injury in a rat dermal excisional wound model. It was hypothesized that the scaffold functionalized with miR-29B, when applied to a rat excisional wound model will modulate the wound healing response by reducing collagen type I production and ameliorate the ratio of collagen type III/I in the remodelling dermis. It was also the hypothesis of Chapter Four that functionalization of the scaffold with miR-29B will work synergistically towards an improvement in the ratio of collagen type III/I and ameliorate the wound healing process through reduction in wound contraction and modulation of matrix metalloproteinase-8 (MMP-8) and its tissue inhibitor; TIMP-1. There was a significant impact when excisional wounds were treated with a scaffold alone, a scaffold with a dose of 0.5 μg uncomplexed miR-29B, 5 μg miR-29B; uncomplexed
or complexed with pD-b-P/DA. Any one of the treatments; scaffold alone or scaffold with a dose of 0.5 μg miR-29B, or 5 μg miR-29B resulted in a significant reduction in wound closure. Granulation volume fraction was greatest in the scaffold with 5 μg of uncomplexed miR-29B. This same group was also the only group which did not have a significantly lower collagen type III/I ratio when compared to native skin, and this group also had a statistically significant higher MMP-8: TIMP-1 ratio when compared to all other groups. The dose of miR-29B in the scaffold had an effect in the majority of the parameters investigated. Two doses were investigated in this study; uncomplexed 0.5 μg, and uncomplexed 5 μg, both uncomplexed, and both incorporated within a scaffold. The use of the complexing agent; pD-b-P/DA, did not improve the effects of miR-29B in wound remodelling, when evaluated through the collagen type III/I ratios, granulation volume fraction and wound closure. Indeed the use of pD-b-P/DA complexed with 5 μg miR-29B in a scaffold caused an upregulation of inflammatory cytokines when compared to 5 μg of uncomplexed miR-29B in a scaffold. Notably, there was a significant correlation between the dose of uncomplexed miR-29B when delivered through the scaffold and the ratio of MMP-8: TIMP-1 which signifies that not only the combination of these parameters, but the dose of miR-29B also has a significant effect on the parameters investigated.

Further analysis of phase III warrants quantitative analysis of collagen type I and collagen type III expression within the wound bed through employment of ELISAs, additional immunohistochemistry and immunoblots (including appropriate loading controls). Additionally, labelling the miR-29B with radio-labelling agents or the Cy™3 labelling agent, employed in phase II for evaluation of the release profiles, would facilitate an analysis of the penetration of the miR-29B therapeutic into the surrounding tissue. Live imaging could also be employed if suitable experimental constraints were applied (such as minimising light exposure to the wound).

### 5.3 Limitations

#### 5.3.1 Phase I- Delivering Exogenous miRNA (Chapter Two)

The choice of transfection reagent is critical, as every available agent has both advantages and disadvantages. Choosing to use a non-viral approach reduces the potential concerns associated with viruses but dramatically reduces the efficiency of transfection. Within the non-viral set of transfection reagents, other trade-offs must be made. The use of pD-b-P/DA offered adequate complexation of miRs and its PEGylation enabled the delivery of a higher dose, without a significant impact on cellular viability. More importantly, it was envisaged that the resultant transfection reagent would facilitate apt conjugation of antibody fragments to improve delivery.
The complexing polymer described; pD-b-P/DA, efficiently complexed miR mimics and conjugated antibody fragments. However, it is not without its drawbacks. pD-b-P/DA could only compete with the commercially available controls on a ‘dose basis’, which essentially means that although it poses minimal toxicity and therefore can be delivered in higher doses than the commercially available controls, it is less effective in transfection. That being said, it does provide good transfection, and effective silencing \textit{in vitro}. pD-b-P/DA also facilitated the conjugation of antibody fragments via a Michael-type addition chemical reaction. Conjugation of a CD90 antibody fragment effected a significant knockdown in the reporter luciferase system when compared to the use of pD-b-P/DA with no antibody fragment at 48 hours. Additionally, the effect was shown to be cell specific when comparing to HUVECs which have minimal expression of CD90 and are cells from another species. Knockdown was maintained at 96 hours, however the use of pD-b-P/DA conjugated with the CD90 antibody fragment had no statistically significant difference between any of the other groups, including pD-b-P/DA with no antibody fragment attached. This is hypothesized to be due to saturation at this point (Figure 2.15). Many non-viral delivery agent are tested in systems where they are in contact with cells for a limited amount of time (four hours) and then removed, also sometimes in serum-free conditions (which can reduce the exposure of exogenous RNA/DNA to serum proteases and enzymes). As a potential therapeutic delivery agent, pD-b-P/DA was not removed from cells in culture. The use of ‘targeting ligands’ is a controversial area which, following the results in Phase I and II, can only be demonstrated in flow/systemic delivery environments. In this study, all conditions were in static environments. Furthermore, the CD90 antigen targeted by the antibody fragment in this platform; in hindsight, may have not been the most appropriate one. Monoclonal antibody engineering is an arduous and perhaps archaic process and the identification of appropriate, rapidly internalising antigens with which to target is warranted. Furthermore, a more specific, targeted ligand is needed which could be achieved using phage display technology for more accuracy.\footnote{1}

The \textit{in vitro} transfection system was optimal for the initial testing of pD-b-P/DA based miR delivery. The reporter Luciferase transfection analysis of silencing was robust and enabled reliable testing. In the experiments described in Chapter Two, all testing was conducted on monocultures that received nutrients via cell culture media. This was an ideal environment for comparing an array of formulations as there were minimal ethical considerations and the conditions in which transfection occurred could be tightly regulated. However, \textit{in vitro} systems, especially two-dimensional cell monolayers, do not represent \textit{in vivo} conditions.
Summary and Future Directions

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<th>Phase II: In Vitro Characterization</th>
<th>Phase III: In Vivo Characterization</th>
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Outcomes

- Synthesis of pDMAEMA based co-block PEGDA/PEGMEA complexing agent (pD-\(b\)-P/DA)
- Complexation of miR mimics (1:8 ratio)
- Conjugation of antibody fragments
- Silencing of target proteins via miR-pD-\(b\)-P/DA
- Transfection system using Luciferase Reporter System

Outcomes

- Atelocollagen type I scaffold crosslinking with 4S-StarPEG (1 mM concentration optimal)
- miR release is dependent on crosslinking density (0.05 - 1 mM) and complexation of miRs
- Silencing of collagen type I and type III is achievable up to 14 days using scaffold/miR-29B platform

Outcomes

- Atelocollagen type I/4S-StarPEG scaffold improves miR delivery in a rat excisional wound model
- ECM remodelling and MMP-8:TIMP-1 ratio are dependent on miR dose
- pD-\(b\)-P/DA does not make miR delivery more efficient in vivo

Figure 5.1: Schematic representation of the elements explored in this project and the subsequent outcomes according to each phase.
5.3.2 Phase II- An Injectable Scaffold Delivery System (Chapter Three)

Even as the lack of a perfect transfection reagent represented as a limitation in the first phase of this work, the nature of the miR- pD₃₋₅P/DA complexes, or complexes represented a major limitation to the second phase of the work. The scaffold employed has huge potential as an injectable system in situ. However, in the scope of this PhD, each gel was prepared individually using standard pipetting techniques in the laboratory. There exists a variation in collagen sources as to the volume of NaOH required to neutralise the solution, however, within all of the experiments of this thesis only one source was used. Additionally, gels could not be made in large batches from which aliquots could be used to make smaller gels as larger volumes had a negative impact on the self-assembly kinetics of the 4S-StarPEG crosslinked hydrogels. A robust, automated dual syringe system, such as is the case in fibrin platforms, is appropriate for the mixing and injection which would minimise any possible batch to batch variation in scaffold, and allow for efficient injection of scaffolds, perhaps even incorporating a simultaneous delivery of therapeutic agents such as DNA, RNA, cells etc.

The scaffold’s physico-chemical properties were investigated in vitro; however these tests cannot fully imitate the complex interactions that are presented in vivo. For instance, although crosslinking of the scaffold offered resistance to degradation against collagenase produced in clostridium histolyticum, the true degradation of this atelocollagen scaffold in vivo can never be completely elucidated in vitro. The same limitations apply to the release profiles of RNA in its various complexed/uncomplexed forms. As mentioned in the limitations of Phase I, Phase II cell experiments were performed on monocultures that received nutrients via cell culture media. Although an ideal environment for comparing the array of formulations, two dimensional sheets do not truly represent in vivo conditions.

A final limitation in phase II is that of collagen type I and collagen type III knockdown. Granted, knockdown of collagen type I and collagen type III expression was demonstrated. However, in the evaluation of collagen type I and collagen type III production by cardiac fibroblasts, the western blot presented in Figure 3.6 can only be used as a guide and future studies require repetition of this with the inclusion of appropriate loading controls such as GAPDH or β-actin. Such a study would require the analysis of protein at additional time points to monitor silencing over time.

5.3.3 Phase III- System Evaluation in a Rat Excisional Wound Model (Chapter Four)

The ideal model for all of the in vivo work is a model of myocardial infarction. However, as only a single treatment can be tested per ischemic heart, and very little information existed in the
literature regarding optimal doses, scaffold mediated miR delivery or miR persistence \textit{in vivo}, the excisional wound model presented in Phase III was selected as a method to narrow the focus of a possible future myocardial infarction study, develop analysis techniques and to determine answers to key questions present from the outset of this study. However, the clinical significance of this model in the context of myocardial infarction is limited. True, it presents a wounded environment, with aspects of inflammation and extracellular matrix remodelling; however, it does not represent an ischemic environment and the unique cascade of inflammatory events that follows it. The excisional wound model did, however, have one primary similarity to the cardiac model; in that the scaffold would be in direct contact with a region of ECM after acute insult. While there may be diseases where such a model could have clinical relevance, they were not investigated in this study. Furthermore, as there was minimal ischemia associated with the delivery of these scaffolds, the miR-29B as an inhibitor of fibrosis, may have been less significant in this wound model.

A further limitation is the model itself. A rabbit ear model of hypertrophic wounding was considered; however, antibodies and complete miR sequencing of this species is incomplete and could render the study limited if further mechanistic and investigatory probes were to be employed. The rodent excisional wound healing model has been extensively used to study wound healing, cutaneous regeneration, stem cell and tissue transplantation and immune rejection.\(^3\) Despite its convenience and frequent use, the model has marked limitations due to the differing wound healing characteristics of rodents when compared with humans.\(^4\) In rodents, whose skin is mobile, contraction accounts for a large part of wound closure; in humans, where the skin is tethered to subcutaneous tissues, wounds are healed by the generation of new tissue. Moreover, contraction of the rodent skin around the wound is affected by factors such as animal posture and motion, as well as wound dressing, and it thus causes considerable variations in wound closure.\(^5\) Efforts were made in order to normalise the epithelialisation rate to the degree of contraction which has been performed previous studies\(^6,7\), yet at a 28 day time point this consideration revealed no significant impact on the results as complete epithelialisation and wound closure had occurred at this time point, which was in agreement with previous studies\(^6,7\). The rat excisional wound model has been previously reported\(^6\) and its limitations stressed from the onset, however, it does provide a model to apply a number of treatments in a resilient animal. Furthermore, it allows four separate conditions to be created on the dorsum of each rat.

\textbf{5.4 Future Directions}

Each aspect of the phases described in this thesis can be further investigated by addressing their limitations and discussing the various avenues of exploration which have been highlighted
through this research. This section discusses five potential future projects that follow. Briefly, the first discusses the immediate future direction of this work; that being intramyocardial injection of miR-29B mimics embedded within a collagen scaffold. The second describes alternative methods of harnessing the endogenous miRs but through the approach of miR inhibition via antagomiRs. The third potential future project describes the incorporation of therapeutic stem cells and reprogramming these stem cells with the platform described in this thesis towards a myocardial application. The fourth proposes the option of delivering pre-miR through various strategies, and finally the fifth discusses additional therapeutic applications of the described platform developed in this thesis. The future directions that can be explored are summarized schematically in Figure 5.2.

5.4.1 Delivery of miR-29B using 4S-StarPEG/Collagen Scaffold via Intramyocardial Injection

An important characteristic of using injectable biomaterials for cardiac repair is their potential ability to provide a minimally invasive approach, which will decrease the damage incurred to the targeted and surrounding tissues during delivery. Furthermore, exposure of non-target tissue to the embedded therapeutic will be substantially minimized. Therapeutic delivery of injectable scaffolds includes epicardial, intracoronary, and endocardial injection. In the instance of using the atelocollagen type I/4S-StarPEG system described in this thesis, a double-barrel syringe is ideal to support delivery if this system were to ever reach the clinic, yet no such system for double-barrel driven cardiac catheter technology exists presently. Direct epicardial and catheter-based endocardial delivery can be applied via thoracic puncture and injection into the wall of the heart. An alternative approach is to utilize the coronary artery, via its leaky vasculature following MI, to deliver the scaffold, in liquid form before gelation has occurred, into the tissue avoiding direct puncturing of the tissue. Single bolus injections are common in small animal models 2, 8-12, however, multiple injections are typically necessary to adequately distribute the material in large animal models where the size of the heart is closer to that of human patients 13-15. Furthermore, each animal model has a range of injected volumes that is scaled to the relative size of the animal. A further important consideration is the time point of injection which can range from immediate, to one week, to two months following MI. This time point is a crucial parameter for designing new treatment strategies due to the dynamic processes post-MI. The stages following ischemic injury involve cardiomyocyte necrosis, a cascade of inflammatory cells, fibrotic scar formation, and cardiomyocyte slippage followed by LV dilation. A different population of cells is impacted depending on the time of administration, and some processes such as LV dilation cannot currently be reversed. Thus, the optimal time post MI needs to be carefully considered and whether the therapy is intended to prevent or reverse negative LV remodelling.
Figure 5.2: Schematic based on da Vinci’s Ventrivian Man. Schematic is arranged such that the five corners represent the basic themes/aspects on which this project was based. The legs represent the phases of the project. The text in the spaces beside the torso show the plausible lines in which each phase can progress. The arms represent the possibility of employing this platform in future projects and in other diseases. This is by no means a comprehensive depiction, rather only the most direct and important aspects are represented to demonstrate cohesion. Further details are discussed in section 6.4.
Several *in vivo* studies have implemented immediate injections of their material post-MI, but this procedure does not properly mimic clinical restrictions as patients would not normally receive a therapy for several hours post-MI. Scaffolds have previously been injected independently into the infarcted myocardium and have resulted in improvement in ventricular function due to ventricular thickening \(^1^2\) and therefore the platform developed here can be applied. Biopolymers that have already been used as injectable biomaterials into the infarcted myocardium include fibrin \(^2,^11,^16,^17\), collagen \(^10,^11\), alginate \(^8,^9,^16\), hyaluronic acid \(^13,^18,^19\), chitosan \(^20,^21\), decellularized extracellular matrix \(^12,^22\) and, more recently, keratin \(^23\). Although the functional measurements and histological investigations vary between the studies, a clear consensus is emerging that the injection of scaffolds into the myocardium post-MI results in functional improvement following MI and presents as a therapeutic option following MI. Clinical trials with alginate as an injectable material treating MI have now been reported. This alginate scaffold was originally developed and tested by BioLinRX as BL-1040, and the study yielded positive results in the first-in-man trial according to unpublished reports \(^24\). The pilot study, which ended in January 2012, exhibited preliminary efficacy and safety of the material after implantation in 27 patients \(^25\).

The progression of the injectable scaffold developed in this thesis could include evaluation in large animal models with extended time points. Appropriate controls such as systemic administration of current clinical pharmacological interventions generally prescribed follow MI, empty treatment control groups and an open-chest intervention procedure are warranted in such a study. A consistent parametrical standard to identify a successful outcome for injectable biomaterials is lacking in the field. The parameters of wall thickness, LV volumes, fractional shortening, and ejection fraction have been relied as important indicators of recovery; yet in the clinic, ejection stroke volume is the best predictor of survival and re-hospitalization events post-MI \(^26,^27\).

### 5.4.2 Using Oligonucleotides to Silence miRs

As previously discussed in this thesis, several approaches have been adopted to hijack the endogenous regulation of genes via the miRNA pathway. Another approach is using antisense oligonucleotides targeted toward miRNAs, referred to as antagomiRs (Figure 5.3, black pathway and Figure 5.4). These antagomiRs target the mature miRNA sequence and inhibit the silencing of oligonucleotides and can be conjugated to cholesterol to improve cellular uptake facilitating discrimination between single nucleotide mismatches of the targeted miRNA. Reduction of target miRNA levels has been achieved in multiple tissues for an extended period when antagomiRs are designed to bind to pathogenic or abnormally expressed miRNAs \(^28\). These molecules can be embedded within the scaffold developed in this project. There are several key requirements for
Figure 5.3: Schematic illustrating non-viral RNAi delivery mechanisms. Red arrows show the mechanism of siRNA and miR mimics entering the cell, uptake and activation by RISC, binding to target mRNA and inhibiting translation. Blue arrows indicate the delivery of pDNA encoding shRNA, which must enter the nucleus to transcribe shRNA from the nucleus. shRNA becomes cleaved by DICER, uploaded by RISC and terminates at the same endpoint as the red arrow pathway. Finally, black arrows indicate the delivery of antagomiRs, designed to bind to endogenous miRs and inhibit them from binding to their target mRNAs.

therapeutic oligonucleotides to achieve effective down-regulation of a targeted miRNA; namely: \textit{in vivo} stability, specificity and high binding affinity to the miRNA of interest. Previous research in antisense oligonucleotides has been based on these criteria to improve the efficacy of the oligonucleotides. Only a handful of modifications have been studied to date, one of which involves the linkage of chemical groups to the 2’ hydroxyl group to modify the stability and affinity of the oligonucleotides. These chemical modifications include 2’-O-methoxyethyl (MOE)-modified oligonucleotides which have better affinity and specificity to RNA than their OMe-counterparts, and locked nucleic acid (LNA)-modified oligonucleotides (LNA-antagomiR), in which the 2’-O-oxygen is joined to the 4’-position via a methylene spacer to form a rigid cyclic structure, fixed into a C3’-endo (RNA) sugar conformation. An additional chemical modification applied to regulate oligonucleotide stability is the balance between phosphodiester and phosphorothioate linkages between the nucleotides, with phosphorothioate being more resistant to nucleases than phosphodiester, thereby providing greater stability to the oligonucleotides.

The 2’–O-methylo-group (OMe) is a frequently used modification to oligonucleotides and has a limited amount of nuclease resistance but improved binding affinity to RNA compared to unmodified sequences. In 2004, Huttagner and colleagues pioneered the knockdown of let-7 function in Dropshilia using OME modified antisense oligonucleotides. Following this, Krutzfeldt et al. used antagomiRs to inhibit miR-122, a liver specific miRNA in the first mammalian \textit{in vivo} study using antagomiRs to inhibit endogenous miRNA. These chemically modified oligonucleotides were conjugated to cholesterol to improve cellular uptake facilitating discrimination between single nucleotide mismatches of the targeted miRNA. Reduction of target miRNA levels has been achieved in multiple tissues for an extended period when antagomiRs have been systemically administered via intravenous injection. However, off target results were observed; as non-predicted up-regulation of mRNAs which were not predicted targets of miR-122 were upregulated indicating that a secondary mechanism can be involved in the modulation effect of miR-122. Another oligonucleotide modification strategy, the 2’-O-methoxyethyl phosphorothioate modification (MOE) antisense oligonucleotide, has demonstrated successful miRNA inhibition in the liver. As previously discussed, 2’MOE antisense oligonucleotides are also effective \textit{in vivo} at silencing miR-122, up-regulating target-gene expression and reducing cholesterol levels in normal mice. However, the onset of action of the 2’ MOE ASO appears to be slower than 2’Ome as a similar acute dosing schedule as that applied to the 2’Ome antisense oligonucleotides did not produce cholesterol lowering and produced only ~50% reduction of miR-122 for the 2’MOE oligos.
In theory, oligonucleotides may antagonize miRNA function at all levels from transcription of the miRNA gene through processing and maturation of the miRNA to binding of the miRNA to its target mRNA. Because miRNAs are relatively small it is imperative to use high-affinity antisense chemistries to target miRNAs. So far, greater than 50 publications on the use of antagoniRs in cell culture and in vivo exist and one can conclude that 2’-O-ME, 2’-O’MOE, morpholino and LNA are probably the most efficient antagoniR strategies.

5.4.3 Combination of 4S-StarPEG Collagen Scaffold with Cell Therapy

Considering the crucial role that miRs play in development and differentiation, it seems intuitive that miRs could be used to program stem cells toward a beneficial lineage. Many transplanted mesenchymal stem cells (MSCs) are lost following delivery to the infarcted myocardium and efforts at using gene therapy to improve their survival have proven moderately successful. RNAi re-programming of these cells to improve their survival and/or modulate their paracrine effects in vivo has not been reported to date. The scaffold/miR platform presented in this thesis can be harnessed to improve MSC survival in vivo, thereby enhancing their therapeutic effect (see Figure 5.5). A possible derivative of stem cell incorporation into this platform is the reprogramming of stem cells or progenitor cells embedded within a functionalized hydrogel towards a cardiomyogenic lineage to facilitate improved ventricular recovery following myocardial infarction. For instance, cardiac progenitor cells transduced to overexpress miR-21, -24 and -221 and injected via the intra myocardial route following MI without reperfusion have led to greater functional recovery. Other researchers have demonstrated the direct reprogramming of cardiac fibroblasts to cardiomyocyte cells in vitro and in vivo using a cocktail of miR-1, -3, -208 and -499, most notably in ischemic mouse myocardium. Although this proof-of-concept study does not, as of yet, report a functional improvement with this therapy, it has huge potential considering the cardiac fibroblast is the most populous cell in the myocardium, both pre- and post-MI. Additionally, RNAi can be used to induce pluripotent stem (iPS) cells, an approach that has only, very recently, been achieved by one group using only miRs to successfully induce somatic cells to pluripotency. This approach will have a potential in directing cells that are easy to harvest into pluripotent cells ex vivo and then directing them towards a cardiomyogenic lineage in vivo again using a combination of miRs.

5.4.4 Delivery of pre-miR-29B

The miR-29B discussed in this thesis is transcribed endogenously as a cluster. The miR-29 family in humans includes hsa-miR-29A, hsa-miR-29B-1, hsa-miR-29B-2 and hsa-miR-29C. miR-29B-1 and mir-29B-2 have identical mature sequences, which are together called miR-29B. Mature 29s
A cyclic relationship exists between all considerations such as the therapeutic target and delivery method, and this will govern the functionalized scaffold delivery approach and also the RNAi intervention employed. Future research presents antagomiRs as an alternative method of gene up-regulation whereas complex hierarchical delivery devices enable a potent and tunable means of intervention.
Figure 5.5: Proposed directions of 4S-StarPEG collagen scaffold/miR platform in inducing cardiomyogenesis (a) incorporation of pluripotent stem cells (MSCs/ADSCs/iPS) within miR loaded scaffold to induce cardiomyogenesis of passenger cells towards a cardiomyogenic lineage (b) incorporation of pluripotent cells (MSCs/ADSCs/iPS) to facilitate production of therapeutic factors from passenger cells and (c) delivery of candidate miRs to reprogram resident cardiac fibroblasts, and possibly other cells surviving myocardial ischemia, towards a cardiomyogenic lineage.
share identical sequences at nucleotide positions 2-7, the seed region that plays a key role in determining which protein-coding-genes a miR would target. Therefore, predicted target genes for the miR-29 family members largely overlap. It has been confirmed that miR-29B-1 and miR-29A are transcribed together as a polycistronic pri-miR and likewise miR-29B-2 and miR-29C are transcribed together. Studies have identified several critical cis elements in the proximal region of miR-29 gene promoters which indicates that they have binding sites to transcriptional factors including a Gli binding site at -424 and three NF-κB binding sites at -561, -110, and +134 in the human miR-29B-1/A promoter, a Smad3 binding site in a highly conserved region ~22 kb upstream of miR-29B-2/C promoter, at least one Yin-Yang-1 binding site in the miR-29B-2/c promoter, a CEBP binding site located at +15 to +29 bp immediately downstream of the miR-29B-1/A transcription start site, and two TCF/LEF binding sites within the proximal promoter of miR-29B-1/A. In addition, miR-29 expression has been shown to be regulated by various transcriptional regulator and signalling pathways, including Wnt signalling. Post-transcriptional processing or stability of mature miR-29s may contribute importantly to the observed differential regulation documented in many studies.

In the experiments presented in this thesis, miR-29B was delivered as a synthetic, double stranded siRNA which mimics mature endogenous miR before being uptaken into the RNA induced silencing complex (RISC), however there are a number of other steps and formats at which miR-29B can be delivered at, and in, respectively. True, siRNAs can be readily synthesized and can quickly silence the expression of a vast number of genes both in vitro and in vivo and have shown moderate success in clinical trials; their remains however many drawbacks. In vivo delivery of siRNAs or even lipid-complexed siRNAs is inefficient and these oligonucleotides have short half-lives in vivo. One approach to overcome this is to express interfering RNA (miRs and siRNAs) intracellularly from plasmid DNA, or so called ‘short hairpin RNAs’ (shRNAs). These are generally expressed from Pol III promoters and contain perfect stems of 21-25 bp with small terminal loops. Similar to pre-miRNAs, they are exported from the nucleus by Exportin-5 where they are cleaved by Dicer to form structures analogous to siRNAs. Three approaches discussed below allow for more stable delivery of miR/siRNA sequences via the delivery of miRs as pre-miRs being transcribed from the nucleus and the preceeding pre-miR clusters that transcribe them.

5.4.4.1 Viral Delivery of pre-miR-29B

Using viral vectors, it is possible to alleviate some of the drawbacks of non-viral approaches, some of which are; inefficient in vivo delivery and transient gene silencing. A large number of in vivo studies, using mostly adeno-associated viruses (AAV) and lentivirus (LV) vectors to express
shRNAs have been performed to evaluate the effectiveness of RNAi in the treatment of cancer, cardiac disease, retinal disease, neurodegenerative disease, viral infections, and other diseases which are reviewed in the literature \(^{47-49}\). To date, these categories of viral vectors have the best safety records and pseudotyping of the vectors with different capsids (AAV) or envelope (LV) proteins permits tissue-tropic targeting. The use of AAVs for the delivery of sh/miRNA expression cassettes is interesting as these cassettes are restricted in size making them amenable for packaging into AAV vectors, which are the smallest known viral vectors. On the other hand, lentiviruses stably integrate into the host cell genome and thus allow persistent transgene expression (although this can become silenced over time). LVs are capable of transducing both dividing and non-dividing cells.

Viral delivery has been used to mimic endogenous miRs in anti-cancer therapeutics. Specific miRNAs are often overexpressed in tumor cells, but in general most miRs are down-regulated in tumor tissue \(^{50}\). In a mouse model of hepatocellular carcinoma, miR-29A is markedly reduced in human hepatocellular carcinoma (HCC) cells. Intravenous administration of a self-complementary adeno-associated (scAAV8) vector expressing miR-26A from a ubiquitous Pol II promoter, to transgenic mice that conditionally express Myc in the liver, protected the mice from tumor formation \(^{51}\). Specifically, eighty percent of vector-injected mice were either completely free of tumor or had significantly smaller tumours. Viral delivery of pre-miR-29B enables more stable expression of miR-29B or its entire linked cluster, inducing more stable expression of this anti-fibrotic molecule.

5.4.4.2 Non-viral Delivery of pre-miR-29B

5.4.4.2.1 Plasmid DNA

pDNA can be designed to transcribe pre-miRs and indeed pDNA delivery of miRs has been documented in many studies \(^{52-55}\). McAnuff et al. compared the potency of siRNA and shRNA-expressing pDNA mediated gene-silencing \textit{in vivo} by co-administration of siRNA or shRNA-expressing pDNA with pDNA encoding a target reporter gene \(^{56}\). The extent of the reduction in the target gene expression was comparable to that between siRNA and shRNA-expressing pDNA at a 10 mg dose; however on a molar basis, the shRNA was 250 fold more effective than the siRNA one-three days after administration. A concern in this study was that the expression of the reporter gene was transient, and therefore it was difficult to conclude whether these compounds were effective and, furthermore, were comparable with each other for longer than three days \(^{56}\). pDNA transient transfection of cells \textit{in vitro} exhibits gene silencing at three and five days in
rapidly dividing cell lines and, with naked siRNAs in general, achieves transient gene knockdown at three and seven days due to dilution of the siRNAs below a therapeutic level with repeated cell division. In quiescent or non-dividing cells, the silencing effect can remain at three weeks \(^{57}\) after which the siRNAs are naturally degraded \(^{56}\). *In vivo*, a similar trend was observed using rapidly dividing subcutaneous tumours vs. non-dividing hepatocytes \(^{56}\).

Although pDNA is considered safe and easy to produce in large-scale quantities, one disadvantage is the low gene transfer efficiency when compared with viral vector systems. Major focus has been placed on improving their delivery and optimizing the pDNA itself. Conventional plasmid vectors can be subdivided into a bacterial backbone and a transcription unit \(^{58}\). This transcription unit usually carries the target gene or sequence along with necessary elements. The bacterial backbone includes elements including an antibiotic resistance gene, an origin of replication, unmethylated CpG motifs which activate the innate immune system of the host by binding to the Toll-like receptor 9 (TLR9) of antigen-presenting cells \(^ {58}\). Some of these sequences are required for the production of plasmid DNA, but independently, each of them raise biological safety concerns. Additionally, regulatory agencies recommend completely avoiding the use of antibiotic resistant markers \(^ {59}\). Therefore, the bacterial backbone of conventional pDNA constitutes a significant portion of pDNA without a therapeutic effect leading to a decreased bioavailability because of the increased size of the plasmid DNA.

**5.4.4.2.2 Minicircle DNA**

To address the issue just described, minicircle DNA can be employed. These are small (~ 4kb) circular plasmid derivatives that have been freed from all prokaryotic vector parts. They are derived from full length parental plasmids from which this parent plasmid is excised into two supercoiled molecules: a replicative miniplasmid carrying the undesired backbone sequence, and a minicircle carrying the therapeutic expression unit \(^ {60}\). Conventional minicircles lack an origin of replication so they do not replicate within target cells and the encoded genes will disappear as the cell divides (which can be either an advantage or a disadvantage depending on whether the application demands persistent or transient expression). This property can be overcome however using self-replication minicircles which possess S/MAR elements \(^ {60}\). Darquet *et al.* were the first to show that minicircle DNA leads to much higher transgene expression levels in cell culture experiments than a parental plasmid or other conventional control plasmids encoding the same transgene \(^ {61}\). The same group investigated this transgene expression in a pre-clinical model in 1999 and demonstrated that minicircle DNA in a mouse cranial tibial muscle was more effective in reporter gene expression than with parental plasmids or larger plasmids \(^ {62}\).
Delivery of shRNA via minicircle plasmids has been achieved in the literature and has shown much promise in the knockdown of target proteins. For instance, Huang et al. created a minicircle vector transcribing shRNA for silencing both prolyl hydroxylase and factor-inhibiting hypoxia-inducible factor (HIF) –1α (both of which degrade HIF-1α which is cardioprotective to ischemic injury). The authors reported higher expression of angiogenic factors in the double knock-down group compared with single knockdown and a shRNA scramble control groups in vitro. In vivo, the authors demonstrated improved cardiac function in the minicircle group compared to the scrambled control and a greater recruitment of bone marrow cells to the ischemic myocardium when delivered intramyocardially in a syngeneic mouse model of MI. Minicircle DNA has yet to fully demonstrate its potential as an effective transcriber of pre-miRs as very few examples exist in the literature. Only recently, Hu et al. have shown that miR-210 delivered via a minicircle vector intramyocardially following MI in a syngeneic mouse model caused decreased apoptosis, increased neovascularisation and significant improvement in left ventricular fractional shortening compared with scrambled minicircle vectors and sham controls. Plasmid mediated miR delivery is still in its infancy, however, plasmid mediated RNA interference will be more likely to achieve clinical acceptance if vectors, such as minicircle vectors are used to eliminate elements in the nucleotide backbone that cause regulatory concern.

5.4.4.3 Delivery via Avirulent Bacteria

Avirulent bacteria with specificity for infarcted myocardium can present as an alternative delivery vehicles for RNAi molecules. The use of live, attenuated Salmonella for in vivo delivery of therapeutic molecules has proven effective in pre-clinical models in specifically targeting tumors and effecting expression of delivered genes after intravenous delivery. There has been little consideration of the delivery of functional genes/RNAi in this manner to date; yet functional studies have reported the effectiveness of using Salmonella, which specifically accumulated in tumours in a mouse model, to deliver STAT3-targeting shRNA to rescue the effects of a vaccination. The combined approach of specific accumulation and silencing of STAT3 resulted in reduced STAT3 expression and, furthermore, significantly suppressed tumour growth in an advanced melanoma tumour model. The effectiveness of this approach in silencing STAT3 indicates the potential of delivering other interfering RNAs through a shRNA approach. Additionally, Le et al. have described accumulation and selective proliferation in the infarcted myocardium of one strain of an avirulent bacterium that can be cleared using antibiotics within two days. Furthermore, protein excretion could be controlled by the administration of L-arabinose by virtue of design of the plasmid-encoded gene expression cassette. This approach has many favourable characteristics pertaining to therapeutic delivery following MI: specific localisation to
infarcted myocardium, activation of expression through a secondary activator, and optional elimination of this ‘live’ therapy through antibiotic treatment. However, there are concerns surrounding immunogenicity and possible mutagenesis of the bacteria, concerns that already exist in the field of viral gene delivery and plasmid mediate non-viral delivery.

5.4.5 Application of Platform in Other Pathological Settings

A collagen type I scaffold can be used as a reservoir of miR-29B in other pathological settings (see Figure 5.6). For instance, in Chapter 4, this system was applied in a dermal wound healing model where the scaffold was found to improve the effectiveness of miR-29B delivered. The concept was relatively simple and had a number of advantages. For example, as non-viral techniques were used, there was minimal risk of viral proteins being expressed by the subjected cells. Moreover, the use of mature miR sequences negated the requirement of nuclear entry and presented the delivery of one single miR.

It has collectively been shown that the miR-29 family members (which includes miR-29B which has been tested in this thesis) target at least 16 genes related to extracellular matrix which encode for several key proteins involved in the physiological or pathological formation of extracellular matrix including; a large number of collagen isoforms, laminin γ, fibrilin 1, elastin, matrix metalloproteinase 2, and integrin β1. This is unique to the miR-29 family as no other miR is predicted to target more than 11 of the 20 collagen genes. With regard to hind limb ischemia (HLI), Greco et al. investigated the cohort of miRs that become dysregulated in the recovering ischemic limb in a mouse model of HLI and found that miRs-29A and 29B were decreased in the ischemic tissue, whereas the expression of miR-29C was increased. miR-29B is also a key regulator in pulmonary fibrosis. Cushing et al. performed a large-scale screening for miRNAs potentially involved in bleomycin-induced fibrosis and found the expression of miR-29 family members was significantly reduced in fibrotic lungs. Additionally, Xiao et al. found that miR-29 gene transfer into diseased lung tissues in mice was capable of preventing and treating pulmonary fibrosis including inflammatory macrophage (M1) infiltration induced by bleomycin in mice. It has also been suggested that miR-29B can also prevent liver fibrosis by blocking activation of hepatic stellate cells through cell arresting mechanisms which resulted in an indirect blocking of TGF-β upregulation, which is significant as TGF-β is an important transcriptional stimulator of many extracellular matrix genes and is an important factor that downregulates miR-29B.

An important role of miR-29B in renal injury has been identified in studies using Dahl salt sensitive rat (a consomic variation of this rat; SS.13BN, which has substantially attenuated...
Figure 5.6: Overview of potential applications of the 4S-StarPEG collagen scaffold and miR-29B mimics therapy presented in this thesis.
hypertension and renal injury \(^{81}\)) where miR-29B expression in the renal medulla, was upregulated by three days of a high-salt diet. *In vivo* knockdown experiments using locked nucleic acid-modified antagomiRs concluded that miR-29B contributed to protection against interstitial fibrosis in the SS.13BN rat \(^{70}\). Further studies linking miR-29B in renal fibrosis was reported in a mouse model of obstructive nephropathy \(^{42}\). Severe tubulointerstitial fibrosis was associated with reduced expression of miR-29. Forced overexpression of miR-29B attenuated renal fibrosis in this model and a study of cultured renal tubular cells showed that miR-29B was downregulated by TGF-\(\beta_1\) via Smad3 \(^{42}\). Long *et al.* found miR-29C upregulated in glomeruli of diabetic (db/db) mice and in kidney microvascular endothelial cells and podocytes cultured in high ambient glucose \(^{82}\). Systemic delivery of antagomiRs directed towards miR-29C reduced albuminuria and mesangial matrix accumulation in db/db mice. The injurious effect of miR-29C was associated with proapoptotic effects of miR-29C on cultured podocytes and targeting of Sprouty homolog 1 \(^{82}\).

miR-29B also plays an important role in the regulation of cell proliferation, differentiation, and apoptosis, a role which has been illustrated by the role of miR-29s in cancer. Downregulation of miR-29s has been correlated with many types of cancer including leukemia \(^{83-84}\), melanoma \(^{85}\), liver \(^{86}\), colon \(^{87}\), cervical \(^{88}\), and lung cancer \(^{89}\). In many cases, downregulation of miR-29B correlated with more aggressive forms of cancer or relapse, suggesting therapeutic restoration of miR-29B could improve disease prognosis \(^{83,90-91}\). This has been explored where induced expression of miR-29A and miR-29B slowed cell growth and induced apoptosis of leukaemia cells *in vitro* and exogenous delivery of miR-29B to xenografted K562 cell tumours was effective in reducing their size \(^{92}\). One of the mechanisms by which all members of the miR-29 family suppress tumor growth is by relieving the suppression of p53. The p53 transcription factor is important in controlling expression of genes that regulate cell growth, senescence, apoptosis, and genome integrity in response to stress and suppression or inactivation of p53 is a common characteristic in many types of cancer \(^{93-94}\). All three members of the miR-29 family, including miR-29B can target p85\(\alpha\) and CDC42, which are genes that normally suppress p53 expression \(^{95}\).

The application of miR-29B therapeutics, in tandem with the delivery platform presented in this thesis has applicability in the treatment of ischemic brain injury. Shi *et al.* have found that miR-29B levels are significantly increased in rodent brains after transient middle cerebral artery occlusion and neurons after oxygen-glucose deprivation \(^{96}\). Furthermore, ectopic expression of miR-29B promoted neuronal cell deaths, whereas its repression decreased cell death. The authors linked this to the miR-29B targeting Bcl2L2 and thereby inhibiting Bcl2L2 gene expression. More importantly, Bcl2L2 overexpression rescued neuronal cell death induced by miR-29B \(^{96}\). Given
the plethora of evidence described it is feasible that the levels of miR-29B can be modulated towards therapeutic benefit in other pathological settings, which is summarized in Figure 6.4.

5.5 Conclusions

In conclusion, a scaffold functionalized with miR therapy has been developed which can be used as a delivery system capable of effectively and silencing the production of collagen type I and modulating the turnover of ECM components over time. *In vitro*, it was demonstrated that this system can effectively mediate the protein silencing of cells with both reporter genes and therapeutic genes. *In vivo*, a statistically significant effect was observed on the collagen type III/I ratio, MMP-8/TIMP-1 ratio, wound contraction and volume fraction of granulation tissue 28 days after administration of a scaffold with miR-29B to a dermal excisional wound in a rat model. These effects were most significant when a dose of 5 μg of uncomplexed miR-29B was incorporated into the scaffold. However the use of the complexing agent developed in phase I; pD_{3b}-P/DA, did not have the same significant effect when used with 5 μg of miR-29B incorporated into the scaffold.
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Summary and Future Directions


Summary and Future Directions


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Summary and Future Directions
A. Evaluation of miR-29B in a Myocardial Infarction Model

A.1. Introduction

Immediately following myocardial infarction (MI) a highly regulated process of cardiac repair/remodelling follows the necrotic loss of cardiomyocytes beginning with the activation of latent matrix metalloproteinases (MMPs) which degrade the existing extracellular matrix (ECM) and coronary vasculature 1. This proteolytic activity declines by the end of week one post-MI and is coincident with the increased expression of tissue inhibitors of MMPs (TIMPs) 2. Circulating inflammatory cells (which include neutrophils and monocytes/macrophages) arrive at the infarct site drawn by adhesion molecules and chemoattractant cytokines expressed by the endothelium of the coronary vasculature bordering the infarct site. The penultimate fibrotic phase following MI substitutes for lost parachymal cells following the initial phase of collagen degradation and begins with the activation of transforming growth factor-β1 (TGF-β1), a key mediator of fibrosis. Increased synthesis of fibrillar type III and type I collagens is present at week one post-MI and their organized assembly in the form of scar tissue becomes evident at week two which continues to accumulate over eight weeks 3. This excessive accumulation of ECM proteins in the interstitium and perivascular regions of the myocardium during cardiac fibrosis is a hallmark of maladaptive hypertrophy and heart failure 4. It causes the disruption of normal myocardial structures and increased mechanical stiffness, which together contribute to contractile dysfunction of the heart. This fibrosis can also disturb the electrical continuity between cardiomyocytes, leading to the impairment of conduction and facilitating the occurrence of arrhythmias.

Fibroblasts are responsible for the turnover of ECM components, both in the healthy heart, as well as in pathological fibrosis 5-6. In the stressed myocardium, fibroblasts differentiate and become active (termed myofibroblasts) in response to cytokines and growth factors such as TGF-β1 6. These activated cells proliferate, migrate, and remodel the cardiac interstitium by modulating the secretion of ECM components and MMPs. Signalling cascades that control ECM synthesis, ECM degradation, and fibroblast proliferation and apoptosis involve SMADs, Rho/Rock, Elk-related tyrosine kinase-mitogen activated protein (ERK-MAP), and PI3K/Akt signalling pathways 6. Thus far, four microRNAs (miRs) have been implicated in the regulation of cardiac fibrosis: miR-21, miR-29, miR-30, and miR-133.

The Chapters Two, Three and Four have detailed the development and understanding of a tissue engineering platform consisting of an atelocollagen type I hydrogel crosslinked using poly (ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG), a complexing agent; pD-ε-P/DA and miR-29B.
As previously discussed, miR-29B has been specifically associated with the regulation of fibrosis in a number of tissues including renal, bone, pulmonary, hepatic and cardiac tissue. miR-29B has also been elucidated to have a significant role in remodelling ECM tissue, possessing a significant relationship with collagen production. It has collectively been shown that the miR-29 family members target at least 16 genes related to ECM which encode for several key proteins involved in the physiological or pathological formation of extracellular matrix including; a large number of collagen isoforms, laminin γ, fibrillin 1, elastin, MMP 2, and integrin β1. This is unique to the miR-29 family as no other miR is predicted to target more than 11 of the 20 collagen genes. In cultured cardiac fibroblasts, miR-29 has been downregulated after TGF-β stimulation, suggesting that the decrease in miR-29 in the pathological remodelling of the heart is mediated by TGF-β. Van Rooij et al. were the first to show that the miR-29 family directly targets a multitude of ECM genes such as col1a1, col3a1, elastin and fibrillin. The reported downregulation of miR-29 in several cardiac pathologies suggests that this loss may actually contribute to the development of cardiac fibrosis, by relieving the repression on ECM gene expression. Knockdown of miR-29 by anti-sense oligonucleotides designed to block miR-29 (antagomiRs) in the healthy mouse heart resulted in increased expression of ECM genes at the mRNA level, but it is currently not known whether this is sufficient to induce excessive fibrosis. miR-29 is also linked to fibroblasts survival. In a screen for miRs that are able to modulate p53 activity in NIH-3T3 cells, Park et al. found that the miR-29 family induces apoptosis through targeting of CDC42 (a Rho family GTPase) and P85α (the regulatory subunit of P13K), both of which are known to negatively regulate p53. In the diabetic kidney, miR-29 was shown to directly target the miR-21 target gene, Spry1 and promote activation of Rho kinases. Together this data, coupled with conclusions from Chapter Three and Chapter Four, indicate that miR-29 acts as a regulator of cardiac fibrosis via direct repression of a multitude of ECM genes and possibly also by inducing apoptosis of fibroblasts. Therapeutically, it will be interesting to test whether overexpression of miR-29B in the heart, using miRNA mimics would be sufficient to prevent or repress pathological fibrosis.

To achieve this goal the delivery of miR-29B mimics systemically to the infarcted myocardium; three and five days following ischemia reperfusion (IR) injury of the myocardium was performed. It is the hypothesis of this approach, that miR-29B will inhibit maladaptive remodelling of the infarcted myocardium and border zone areas thereby improving the remodelling response of the injured myocardium, and the overall functional recovery of the animal. Therefore the objectives of the studies presented here are to:

i. Determine the effect of miR-29B, delivered systemically, on cardiac function at 14 days and 28 days following MI by measuring left ventricular ejection fraction and fractional shortening by echocardiography.
quantify the deposition of collagen type I, collagen type III and the ratio of collagen type III/I in the infarcted heart 28 days post-MI within the infarct, at the border zone of the infarct and at the remote myocardium and determine if systemic administration of miR-29B has an effect on this deposition.

iii. Investigate the effect of miR-29B on the expression of α-smooth muscle actin (SMA), TGF-β1, and also the effect on programmed cell death (apoptosis) in the infarct and remote myocardium using immunohistochemistry.

A.2. Materials and Methods

A.2.1 Materials

miR-29B mimic and miR-239B mimics (negative control) were obtained from Dharmacon (Waltham, MA, USA) with the sequences for mur-miR-29B: 5’-uagcaccauuauacauguuu-3’ and cel-miR-239B: 5’-uuuguacuucggcuaagugu-3’. miR-239B is expressed in C. Elegans, and, having no mammalian target is a suitable negative control. Mimics were reconstituted in 1 X of an accompanying siRNA buffer upon arrival, stored at −20 °C and thawed immediately prior to use. All other materials were obtained from Sigma Aldrich Chemical Co. (Dublin, Ireland) unless otherwise stated.

A.2.2 Animals

Specific pathogen-free wild type C57BL/6J mice were obtained from Charles River (Sulzfeld, Germany). All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996), guidelines for the use of living animals in scientific studies, and the German Law for the protection of animals. The animal studies were approved by the District Government of Tübingen.

A.2.3 Myocardial Ischemia and Reperfusion Model

Anaesthesia and surgery were performed as described previously. To induce MI, left anterior descending artery (LAD) ligation was performed for 30 minutes on male C57BL/6J mice (Appendix W). The mice were randomly assigned to receive either miR-29B mimic or miR-239B mimic by intravenous injection three and five days following reperfusion (n = 6, 50μg for each dose, suspended in 200 μl of RNA reconstitution buffer). Fourteen and 28 days after IR, left ventricular function was assessed echocardiographically (Vevo 770; VisualSonics, Amsterdam, the Netherlands) by determination of the fractional area change (FAC). Reperfusion of the ischemic area (area at risk, AaR) was estimated by Evans Blue/ triphenyltetrazolium chloride (TTC) staining immediately before
sacrifice. After re-ligation of the left coronary artery at the level marked by the suture left in place, 4% Evans Blue was injected as a negative stain for perfused regions, and the infarcted area (IS) was determined by TTC staining (Sigma Aldrich, St. Louis, MO, USA). 1% TTC added to metabolically active tissue develops a red colour whereas a white color indicates ischemic regions in the tissue. The ISs were determined by quantitative morphometric planimetry, with an image analysis software program after slicing the heart transversely from apex to base in 2 mm sections. The IS/AaR ratio indicates the area of damage occurring in the heart after myocardial infarction 16.

A.2.4 Explant Analysis

Following morphometric planimetry, the explanted tissue was fixed for 12 hours in 10% formalin, subjected to gradual serial dehydration and embedded in paraffin. The tissue was then sectioned in 3 μm thick sections. For general morphology serial sections were stained with haematoxylin–eosin (H&E) stain (cellular components, nuclei). A modified Movat pentachrome stain 17 was carried out with the Russel–Movat–Pentachrome-stain kit (Mastertechs, Lodi, CA, USA) to demonstrate ECM components (Appendix R).

A.2.5 Immunohistochemistry

Collagen type I, collagen type III, α-smooth muscle cell (α-SMC) actin and TGF-β1 expression within the cardiac tissue was revealed using fluorescent immunostaining and quantified using stereological methods in terms of volume fractions. Hydrated sections were subjected to heat induced antigen retrieval in pH 6.0 citrate buffer (for collagen III, α-SMA actin and TGF-β1 antigen retrieval) or Tris-EDTA pH 9.0 (for collagen I antigen retrieval) in a microwaveable pressure cooker. The antibodies used in the identification were mouse monoclonal to collagen I (Acris GmbH, Herford, Germany, dilution: 1:50), mouse monoclonal to collagen III (Acris GmbH; dilution: 1:50) mouse monoclonal to α-SMA (Sigma Aldrich, dilution: 1:100) and rabbit polyclonal to TGF-β1 (Abcam Plc, Cambridge, UK, dilution 1:50) which were applied overnight at 4 °C. Following this the slides were treated with goat anti-mouse FITC conjugated secondary antibody (Invitrogen, Dublin, Ireland), goat anti-mouse AlexaFluor 555 conjugated secondary antibody (Invitrogen) or rabbit anti-mouse AlexaFluor 555 conjugated secondary antibody (Invitrogen). All sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Three images were taken per section, with five slides per treatment. The location of analyses was within the infarct, the border zone of the infarct and the remote myocardium where determinable. Images were analysed with ImageJ and quantified as a measure of volume fraction (Appendix R.4).
A.2.6 Apoptosis Detection Staining

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) in a commercially available kit format (GenScript Corp, Piscataway, NJ, USA), was used to detect apoptotic cells within freshly cut paraffin embedded sections as per the manufacturer’s instructions. All sections were treated with Proteinase K (20 μg/ml) for 10 minutes prior to blocking and staining steps. The negative control was stained with the label solution without the TUNEL reaction solution to control for background fluorescence and non-specific binding. As a positive control, a sample was incubated with DNase I to degrade DNA in that sample. For staining, the sections were incubated with a TUNEL reaction mixture, treated with a streptavidin-horse radish peroxidase solution, and finally the samples were treated with a 3, 3'-Diaminobenzidine (DAB) working solution to develop the colour. All samples were counterstained with Mayer’s Haematoxylin and micrographed for stereological analysis using ImageJ. Apoptosis was quantified as the number of brown (apoptotic) cells per field of view (Appendix R.5).

A.2.7 In Situ Hybridization to Detect Delivered miR-29B

5'-Digoxigenin (DIG) labelled, LNA-modified oligonucleotide ISH probes were purchased from Exiqon for miR-1 (positive control), miR-29B, U6 spliceosome (positive control) and a scrambled LNA-modified oligonucleotide as background control. Formalin-fixed slides were hydrated in serial alcohol concentrations and treated with Proteinase-K for 10 minutes at 37 °C. Sections were dehydrated in quick incubations with various concentrations of ethanol. Sections were hybridized with the LNA probes at various concentrations at the melting temperature (T_m) for one hour. Hybridized sections were then washed with 50% formamide and 2 × SSC at the hybridization temperature. Following 1 hour of blocking in 2% sheep serum, 2 mg/ml bovine serum albumin in phosphate-buffered saline (PBS) with 0.1% Tween, the slides were incubated with anti-DIG/ alkaline phosphatase antibody/enzyme conjugate (1:800; Roche Diagnostics Ltd, Burgess Hill, UK) for one hour at 30 °C. Following successive washes in PBS with 0.1% Tween, the sections were incubated with nitroblue tetrazolium and 5- bromo-4-chloro-3-indoyl phosphate substrate (NBT-BCIP; Roche) for two hours. The reaction was stopped by washing with PBS, and nuclei were counterstained with Nuclear Fast Red.

A.2.8 Statistical Analysis

Results are depicted throughout as means ± standard deviation. Statistical analysis was performed using Prism™ (GraphPad Software, La Jolla, CA, USA) and Student’s t-test statistics to determine statistically significant differences between miR-29B treatment and the control miR-239 treatment. Statistical significance was set at p < 0.05.
A.3. Results and Discussion

The results represent the findings from a pilot pre-clinical evaluation in n = 6 (miR-29B treatment group) and n = 7 (negative control; miR-239B treatment group) animals surviving to 28 days. Given this number, it is feasible to assume that statistically significant differences could be detected if these numbers were increased to provide more power in this study. With this in mind, optimistic trends were observed in many of the parameters investigated in this study. For instance; the left ventricular ejection fraction (LVEF) % tended to be improved in the group treated with miR-29B at both 14 and 28 days (Figure A.6). No significant changes were observed in infarct size (Figure A.2) or wall thickness (Figure A.4), nor were any correlations observed between LVEF% (Figure A.6) and the morphometric or histological parameters. While functional improvements are often associated with improved histological parameters, many other studies have failed to find statistically significant effects on the morphometry of the myocardium despite observing changes in overall cardiac function. This disparity may be due to a number of factors. Firstly, histological infarct measurement techniques are subject to several limitations. Thinning of the infarcted wall can lead to underestimations of infarct volume, for example. Furthermore, induction of MI by coronary artery ligation yields highly variable infarct sizes and because these parameters can only be measured once the heart has been explanted for analysis, the initial infarct size is unknown. Thus, it is difficult to assess the change in infarct size. Conversely, LVEF %, can be assessed frequently during the course of the experiment. This allows continuous monitoring of cardiac function and comparisons between time points.

The dosing regimen delivered in this study was a tail-vein injection at three days and at five days, and a delivery of 50 μg of miR (suspended in 200 μl of RNA reconstitution buffer) at each time point. It is very difficult to ascertain the appropriate dosing amount and regime in such a study as there is a limited amount of information available in the literature with regard to therapeutic regimens of siRNA. That being said, doses are reported at between 20 μg to 2 mg per Kg weight which equates to approximately a 40 μg maximum dose in a mouse model, close to the 50 μg doses described in this study. One must take into consideration the actual amount of miRs that reach the intended target, for instance in this case; the infarcted myocardium. Studies report that systemically delivered siRNA tends to accumulate in the liver, kidneys, lungs, heart and spleen, with negligible traces of siRNA present in other tissues. The persistence of siRNAs delivered in vivo can be improved through encapsulation within nanoparticles and liposomal formulations. Most in vivo studies with naked siRNAs attempt to circumvent the problem of limited bio-distribution by administering high doses directly to the target organ by injection but in this initial study it was sought to determine the effect (if any) of miR-29B on the infarcted myocardium before embarking on local delivery of the therapeutic. Efforts were made at performing in situ hybridisation (ISH) of cardiac sections to
visualise the delivered miR-29B by probing with a digoxigenin (DIG) labelled locked nucleic acid (LNA) against miR-29B (Figure A.16). ISH was performed on freshly cut sections of myocardium for nuclear U6 spliceosomal RNA (as a method/user control), miR-1 which is highly expressed in the myocardium (as a tissue control) and miR-29B which was delivered in this study. Additionally, ISH was performed on human biopsies of cardiac tissue, cervical tissue and breast tissue as additional controls. U6 spliceosomal RNA was detected in all tissue sections, however, its intensity was lower in the mouse cardiac tissue explanted in this study when compared to the control tissues. miR-1 was not detected in the mouse cardiac explants in this study but was detected in the human cardiac biopsies. miR-29B was not detected in any tissue samples. Based on these observations in can be concluded that the RNA in the mouse explants had degraded in the processing of the tissue. U6 spliceosomal RNA was detected, as this is a highly expressed RNA and, being present in the nucleus, had an additional protection against endogenous RNases but its weaker detection when compared to the human biopsies indicates some degradation had occurred. Furthermore, detection of miR-1 was achieved in the human cardiac biopsies whereas it was not detected in the explanted mouse cardiac sections. No detection of this otherwise positive control lends further evidence that the RNA in the mouse explants has been subjected to degradation. A review of the processing techniques indicates that the RNA may have degraded during Evan’s Blue/TTC staining as the RNAse free status of these agents was not ensured and the human biopsies used as controls had never been subjected to such processing. Future studies are now ensuring that all processing steps are RNase free for future miR-29B detections and that possibly the RNA could be conjugated with a radioisotope or Cy™3 for ease of detection.

Infarct size was investigated at the day 28 time point using TTC and Evans Blue staining (Figure A.1) and there was a trend towards a smaller infarct size (when normalised to the area at risk, Figure A.2). One limitation of conventional infarct size estimates is that these estimates do not account for the composition of the infarcted area and this can be a critical parameter in predicting cardiac function. Employment of a modified Movat’s Pentachrome stain enabled the visualisation of many ECM components following myocardial infarction (Figure A.3). For instance, muscle tissue composed predominantly of cardiomyocytes stains red while mature collagens are stained yellow. There are copious amounts of ECM structures staining blue, indicative of immature fibrosis both in the infarct and remote myocardium. This tissue is most likely composed of glycoproteins such as fibronectin, proteoglycans such as heparan sulfate, immature fibrillar collagens and non-fibrillar collagens (for example, type IV collagen). Although 28 days is a standard end-point in pre-clinical models of MI it would be appropriate to repeat this study with additional sacrificial time points; one at an
Figure A.1: Representative transverse cardiac sections of miR-29B treated mice and miR-239B (negative control) treated mice showing AaR (red) and ischemic tissue (white), scale bar indicates 1 mm.
Figure A.2: Quantification of relative infarct size at 28 days following myocardial infarction with miR-239B (negative control) or miR-29B treatment. No significant difference was detected between groups at $p < 0.05$ using Student’s $t$-test. Data presented is the mean of $n = 6 \pm$ standard deviation.
Figure A.3: Representative micrographs of sections stained with a modified Movat’s Pentachrome stain from animals treated with miR-239B (negative control) or miR-29B. Muscle tissue stains red, nuclei stain purple, mature collagen bundles stain yellow, whereas immature fibrosis is stained blue/cyan and indicates the presence of proteoglycans. Scale bars indicate 1 mm.
Figure A.4: Quantification of left ventricular wall thickness at 28 days following myocardial infarction with miR-239B (negative control) or miR-29B treatment. No significant difference was detected between groups at p < 0.05 using Student’s t-test. Data presented is the mean of n = 6 ± standard deviation.
Figure A.5: Fractional shortening % results obtained from echocardiographic data of animals in groups with miR-29B or miR-239B (negative control) treatment. No significant difference was detected between groups at p < 0.05 using Student’s t-test at each time point. Data presented is the mean of n = 6 ± standard deviation.
Figure A.6: Left ventricular ejection fraction (LVEF) % results obtained from echocardiographic data of animals in groups with miR-29B or miR-239B (negative control) treatment. No significant difference was detected between groups at p < 0.05 using Student’s t-test at each time point. Data presented is the mean of n = 6 ± standard deviation.
earlier time point of 14 days and one at a much later time of 56 days which would reveal more premature and more advanced remodelling respectively. Regardless, measurements were performed on these micrographs to determine the effect of miR-29B on left ventricular wall thickness at 28 days following MI (Figure A.4). Again, no statistically significant difference was observed between animals treated with miR-29B (average wall thickness = 370 μm) and the negative control; miR-239B. However, there was a trend towards a thicker left ventricular wall the myocardium from animals treated with miR-29B (average wall thickness = 580 μm, Figure A.4) which suggests that there may be less, or even still retarded remodelling occurring in this treatment group. Furthermore, there was evidence of increased viable cardiomyocytes in the left ventricular wall of animals treated with miR-29B but this observation was not statistically significant (Figure A.3).

A decreasing ratio of collagen type III to collagen type I is associated with reduced compliance of the tissue, as observed in cardiomyopathy. When the ratio of collagen type III/I was investigated in this study it was found that while there were no significant changes in this ratio in the infarct, within the border zone and also in the remote myocardium, there was a statistically significant decrease in the collagen type I volume fraction in the group treated with miR-29B at the border zone area (Figure A.6). This is somewhat reflected (although not significantly) in the collagen type III/I ratios (Figure A.8) at the border zone where there is a trend towards an increased ratio in the miR-29B group in comparison with the miR-239B control group (1.085 vs. 0.78, p = 0.14). This implies that the infarcted tissue in these hearts was more elastic and less rigid than in the hearts treated with scaffold alone. Improved elasticity of the infarcted area could explain the improvement in cardiac function in these animals.

The infarct scar is composed of a population of fibroblast-like cells termed myofibroblasts (myoFb) due to expression of α-SMA and resultant contractile behaviour. These cells continue to turn over type I and III fibrillar collagens long after scar tissue has restored the structural integrity of the infarcted myocardium and are supported by a neovasculature. MyoFb collagen turnover is regulated by substances including angiotensin II and TGF-β1. The resultant fibrosis that appears over time at sites remote to the infarct represents the majority of connective tissue found in ischemic cardiomyopathy and is considered the major component of the adverse structural remodelling found in the failing human heart of ischemic origin. In this study, the expression of α-SMC was investigated using immunofluorochemistry to determine the effect of miR-29B on the accumulation of myoFb that may have derived from infiltrating stem cells or differentiated fibroblasts (Figure A.11). As expected, an abundance of α-SMC expressing cell volume fraction was observed in the infarct area of both the miR-239B control and miR-29B group (0.037 and 0.043) whereas less α-SMC expressing cells were detected in the remote myocardium (Figure A.12). There was a trend (although this was not
Figure A.7: Confocal micrographs of border zone, infarct and myocardium remote to the infarct immunostained for collagen type I (green) and collagen type III (red) deposition using immunofluorescent staining in cardiac sections from mice treated with miR-29B or miR-239B (negative control). Sections are counterstained with DAPI (cyan). Scale bar indicates a length of 100 μm.
Figure A.8: Volume fractions of collagen type I obtained from confocal micrographs of cardiac sections from mice treated with miR-29B or miR-239B (negative control). Data presented is the mean of $n = 6 \pm$ standard deviation. * indicates a statistically significant difference between the two groups indicated at $p < 0.05$, from Student’s $t$-test.
Figure A.9: Volume fractions of collagen type III obtained from confocal micrographs of cardiac sections from mice treated with miR-29B or miR-239B (negative control). Data presented is the mean of $n = 6 \pm$ standard deviation. No statistically significant difference between groups is detected using Student’s $t$-test, $p < 0.05$. 
Figure A.10: Ratio of volume fractions of collagen type III/I obtained from confocal micrographs of cardiac sections from mice treated with miR-29B or miR-239B (negative control). Data presented is the mean of n = 6 ± standard deviation. No statistically significant difference between groups is detected using Student’s t-test, p < 0.05.
Figure A.11: Micrographs of infarct and remote myocardium following treatment with miR-29B or miR-239B (negative control). Sections are stained for α-SMA using immunofluorescent staining (red) and counterstained with DAPI (cyan). Scale bar indicates a length of 200 μm.
Figure A.12: $\alpha$-SMC volume fractions obtained from confocal micrographs of cardiac sections from mice treated with miR-29B or miR-239B (negative control). Data presented is the mean of $n = 6 \pm$ standard deviation. No statistically significant difference between groups is detected using Student’s t-test, p < 0.05.
Figure A.13: Micrographs of infarct and remote myocardium following treatment with miR-29B or miR-239B (negative control). Sections are stained for TGF-β1 using immunofluorescent staining (green) and counterstained with DAPI (blue). Scale bar indicates a length of 100 μm.
Figure A.14: Micrographs of infarct and remote myocardium following treatment with miR-29B or miR-239B (negative control). Sections are stained using an in situ TUNEL kit for apoptosis and counterstained with Mayer’s Haematoxylin. Arrow indicates an apoptotic cell. Scale bar indicates a length of 20 μm.
Figure A.15: Quantification of number of apoptotic cells per field of view in the infarct and remote myocardium following treatment with miR-29B or miR-239B (negative control). Data presented is the mean of n = 6 ± standard deviation. No statistically significant difference between groups is detected using Student’s t-test, p < 0.05.
Figure A.16: In situ hybridisation using LNA oligonucleotides to detect miRs on formalin fixed paraffin embedded sections. U6 is detection of U6 spliceosomal RNA. Scrambled LNA is employed as a background control. Blue indicates the detection of RNA using LNA oligonucleotides which is highlighted with arrows. Sections are counterstained with Nuclear Fast Red. Rat myocardium has very low detection of miR-1 and U6 whereas human sections have a higher detection.
Appendices

statistically significant) to an even more decreased occurrence of α-SMC cells in the remote myocardium of animals treated with miR-29B compared with miR-239 control treated animals (0.006 vs. 0.01). Investigation of TGF-β₁ expression revealed no difference between the administration of miR-29B or miR-239B control (Figure A.13) neither within the infarcted myocardium nor in the remote myocardium. In this study, one intervention was investigated; the sequence specificity of the delivered miR. Therefore it can be presumed that no additional effects are observed as described in Chapter Four, due to the simplicity of the intervention here. MI will itself elicit a dynamic response, of which TGF-β₁ plays a key role. Although the administration of miR-29B and miR-239B control did not have any difference on the expression of TGF-β₁ as described in this study; it is most likely that miR-29B is silencing collagen type I (which was detected in the border zone of the infarcts, Figure A.8), the production of which is being promoted by TGF-β₁, post-transcriptionally.

Finally, it was sought to investigate the prevalence of apoptosis in cardiac tissue in response to the administration of miR-29B or miR-239B control. The role of miR-29B on apoptosis has recently been elucidated. For instance, miR-29B is known to have many targets, one of which is the anti-apoptotic protein Mcl-1. However, no significant apoptosis was noted in any of the sections stained in this study, and in fact, apoptosis was in general, extremely low in both the infarcted myocardium and also in the remote myocardium (Figure A.14 and A.15). Furthermore, apoptosis due to the event of ischemia/reperfusion would not be detectable at this point.

A.4. Conclusions

The results thus far presented are part of a pilot investigation to determine the therapeutic effect of miR-29B as a modulator of fibrosis and maldaptive remodelling following myocardial infarction. Given the study presented here, future investigations would warrant the employment of a robust delivery vector to negate any degradation or rapid clearance of the miR-29B following tail-vein injection. It has been reported that naked siRNAs and also siRNA in liposomal formulations, or mixed with cationic reagents to improve their stability in vivo, become rapidly cleared from the bloodstream by renal filtration within 5-30 minutes. To avoid this drawback, and indeed that of affecting organs not intended to be affected, a more stable localised delivery approach is desired. This could include consideration of a viral vector, such as an adeno-associated virus, which could be delivered systemically and be engineered with a cardiac specific promotor.

Unfortunately the analyses of this pilot study have revealed that the study design was not powerful to detect statistically significant differences for a number of the parameters investigated. However, some conclusions may be made on the results obtained. The administration of miR-29B had a statistically significant effect on the collagen type I deposition at the border zone of the infarcted myocardium and this was also reflective in a strong trend towards an improved collagen type III/I ratio in the border zone of the infarcted myocardium. Few conclusions can be made about the other parameters.
investigated as there exist no statistically significant differences. However, miR-29B had an apparent trend towards an improvement on LVEF % which can be attributed to improved mechanical compliance at the border zone of the infarct due to an increased collagen type III/I ratio. Finally, these results provide an insight into further parameters to be investigated in future studies and highlight the requirement of a localised delivery system to circumnavigate possible off-target effects and clearance of a systemically delivered therapeutic.

The cardiac study was somewhat limited in its applicability. For example, a mouse heart weighs less than a gram, while a human heart generally weighs more than 200 g. The difference in mass is essentially correlated in the difference in the wall thickness (~1 mm vs. ~1 cm). The cellular composition of the myocardium; however, do not differ, and thus the host response is somewhat representative of the response a human heart would have to the treatment. Additionally, the MI model in this study was created by ligating the LAD of an otherwise healthy animal. In humans, occlusion of the coronary artery is a slow process, onset by underlying atherosclerosis, and present (in the majority of cases) in individuals with sedentary lifestyles and unhealthy diets. Where possible, MI is treated by reperfusing the affected tissue, an event which was simulated in this model.

The study was powered to detect changes in left ventricular ejection fraction (LVEF %) but not in wall thickness or other histological parameters. The number of animals in each treatment group should be at least ten to provide such power. Thus, there may have been changes in the histological parameters that were not statistically significant due to the small sample size in this pilot study. It was decided to investigate first the systemic delivery of miR-29B which is reported here. Intramyocardial delivery is envisaged and is discussed in the Future Directions Section of this Thesis.
A.5. References

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B. Assessment In Vitro Using a Human Skin Equivalent Model

B.1. Introduction

In this Appendix in vitro evaluation of the scaffold functionalised with miR-29B and its effect on wound closure in an in vitro human skin equivalent model, and also its influence effect on collagen type I and collagen type III was investigated. The hypothesis tested was that the scaffold functionalised with miR-29B, when applied to a human skin equivalent model will modulate the healing response by reducing contraction of the wound and reduce collagen type I production.

Therefore, the objective was to investigate the effect of the components in this platform (miR-29B, 4S-StarPEG-collagen scaffold, pD-β-P/DA) on a number of key parameters involved in wound healing, using a number of techniques. Specifically,

i. Evaluate, using histological analysis, wound closure following injury with a biopsy punch in an in vitro human skin equivalent model.

ii. Quantify collagen type I and III deposition using immunohistochemistry.

B.2. Materials and Methods

B.2.1 Materials

All solvents were of analytical or HPLC grade and were obtained from Sigma Aldrich Chemical Co. (Ireland) unless otherwise stated. All oligonucleotides and primers were purchased from Eurofins MWG GmbH (Ebersberg, Germany). 4S-StarPEG was purchased from JenKem Technology USA (Allen, TX, USA).

B.2.2 Complexation

miR-29B mimic was obtained from Qiagen (Hilden, Germany) with the sequences for rno-miR-29B: 5′-uagcaccauuaagacagugu-3′; miRNA mimics were mixed individually with pD-β-P/DA at a w/w ratio of 8:1 in 1 X phosphate buffered solution (PBS) as previously optimised in Chapter Two. The components were mixed and complexes allowed to form at room temperature for 60 minutes.

B.2.3 Atelocollagen/ Poly (ethylene glycol) Ether Tetrasuccinimidyl Glutarate Scaffold Preparation

Atelocollagen was isolated as described in detail in Appendix D. Nine parts of collagen solution (3.5 mg/ml w/v) was gently and thoroughly mixed with one part 10 X PBS. The solution was neutralised by the drop-wise addition of 2 M sodium hydroxide (NaOH) until a final pH of 7–7.5 was reached and kept in an ice bath to delay gel formation. 4S-StarPEG was then added at a final concentration of 1
mM in a volume of 200 μl. The solutions were incubated for one hour at 37 °C in a humidified atmosphere to induce gelation (Appendix D.3).

**B.2.4 Generation of In Vitro Skin Equivalents**

A reconstructed human epidermis was prepared using dermal fibroblasts embedded in a matrix of collagen type I, isolated from rat tails, and keratinocytes. The keratinocytes differentiate during the cultivation process into a multi-layered epidermis with stratum corneum. Collagen type I was isolated directly from rat tails (University of Hohenheim) after removal of the skin. The tails were frozen in liquid nitrogen, broken, and the collagen bundles were squeezed out and cleaned after thawing of the tail pieces. The isolated collagen was incubated for one hour in 70% ethanol, washed with water and dissolved in 0.1% acetic acid for two days at 4 °C. After centrifugation, for one hour at 10,000 g, the supernatant containing the collagen was lyophilized and could be stored at 4 °C, or dissolved in 0.1% acetic acid at 4 mg/ml (gel matrix). Both the human keratinocytes and the epidermal fibroblasts were generated from foreskin. The dermis was dissected, cut into small pieces and incubated overnight with 1 U dispase in PBS at 4 °C. To isolate the keratinocytes, the epidermal tissue was removed from the dermis and incubated for 30 minutes at 37 °C with trypsin (2.5%). The reaction was halted by the addition of keratinocyte growth medium (KGM) plus 5% fetal calf serum (FCS). After resuspension in KGM+5% FCS, cells were allowed to adhere for 4 h to tissue-culture flasks coated with collagen type I. The media was changed to remove tissue debris and thereafter every third day until the keratinocytes reached 80% confluency. Epidermal fibroblasts were isolated by incubation of the human dermis for 45 minutes with 0.25% collagenase in PBS, 2 mM Ca²⁺ and 2 mM Mg²⁺, at 37 °C. Cells were harvested and resuspended in M199+10% FCS (GIBCO). Cells were allowed to adhere for 24 h; the media was then changed to remove tissue debris. The medium was changed every third day until confluency was reached.

To generate the skin model, epidermal fibroblasts from primary culture were harvested and diluted with pre-cooled gel medium (4 °C; 2x Dulbecco’s Modified Eagle Medium (DMEM) containing 100 mM HEPES) to 5 x 10⁶ cells/ml. The cell suspension was carefully mixed with an equal volume of acidic collagen solution extracted from rat tails (4 mg/ml in 0.1% acetic acid solution). Two hundred microlitres of this formulation was instantly poured into each cell-culture insert (12 mm diameter, polycarbonate membrane, 0.3 μm pore size). The gels were allowed to solidify for 15 minutes at 37 °C, 5% CO₂. After solidification, 50 μl fibronectin (5 μg/ml) was spread onto the gels. Each insert was transferred to a cavity of a 24-well plate, provided with 1 ml M199 medium (top and basolaterally) and equilibrated for 24 hours. On the second day the medium was replaced with 500 μl KGM+5% FCS basolaterally, and 500 μl KBM+5% FCS containing 1 x 10⁵ keratinocytes/gel was added to the inserts. The incubation medium was changed after the first day, thereafter every
second day, using KGM+2% FCS. After four to six days of growth, under submerged conditions, the cell layer was exposed to air (5% CO₂) (airlift culture) to allow formation of a stratum corneum. For this purpose the insert was transferred to a 6-well plate, and airlift medium (KBM, without supplements, except 1.88 mM Ca²⁺ and 0.025 M glucose) was added basolaterally only. The gels were cultivated for another 12–14 days, with daily change of medium, before use in the assay (Figure B.1).

A 2 mm diameter biopsy punch (Kai Medical GmbH, Solingen, Germany) was used to create wounds in the centre of the skin equivalents. Briefly, under a sterile environment, the punch blade was placed as central as possible to the wound equivalent, and pressed down until it had gone completely through the skin equivalent model (Figure B.2 (a)). Removal of the punch brought with it the excised portion of tissue. Following this, the treatments listed in the table below were applied.

Table B.1: List of treatments applied to wounds created in in vitro human skin equivalent.

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B.2.5 Harvesting and Processing of Tissue

Samples were divided into half and the other half again further divided resulting in a total of one half and two quarters of tissue. The half portion of the explant was fixed in 10% neutral buffered formalin for 12 hours to be subsequently embedded in paraffin and was sectioned perpendicularly to the wound surface in 3 µm consecutive sections (see Appendix Q.1 for detailed protocol). A modified Movat pentachrome stain used (Russel–Movat–Pentachrome-stain kit, Mastertechs, Lodi, CA, USA) to stain ECM components (see Appendix R.2 for detailed protocols).

B.2.6 Wound Closure

Wound closure was determined by comparing the wound area at the initial time of intervention with the wound area at the final time point (day 10) from gross micrographs.

Normalized wound area index: \[ \frac{A_0 - A_t}{A_0} \]

where \( A_0 \) is the area wound at day 0 and \( A_t \) is the area of the wound bed at day 10.
**B.2.7 Collagen Type I and Collagen Type III Immunohistochemistry**

Collagen type I and collagen type III expression within the skin equivalents was revealed using fluorescent immunostaining and quantified using stereological methods in terms of volume fractions. Hydrated sections were subjected to heat induced antigen retrieval in pH 6.0 citrate buffer (for collagen III, α-SMA actin and TGF-β1 antigen retrieval) or Tris-EDTA pH 9.0 (for collagen I antigen retrieval) in a pressure cooker. The antibodies used in the identification were mouse monoclonal to collagen I (Acris Antibodies dilution: 1:50) and rabbit monoclonal to collagen III (Acris, Antibodies; dilution: 1:50) Following this the slides were treated with goat anti-mouse FITC conjugated secondary antibody (Invitrogen) and goat anti-rabbit AlexaFluor 555 conjugated secondary antibody (Invitrogen). All sections were counterstained with 4’,6-diamidino-2-phenylindole (DAPI).

**B.2.8 Statistical Analysis**

All samples were tested in triplicate and all experimental groups were analysed in triplicate. GraphPad Prism® (v.5 GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Analysis of variance (ANOVA) was used followed by Tukey’s post-hoc test to determine statistical significance between groups. ANOVA was performed assuming normal distribution of the data which was tested and verified using the Anderson-Darling test. p values of < 0.05 were considered statistically significant.

**B.3. Results and Discussion**

This *in vitro* skin equivalent model proved not suitable to determine the effect of the treatment on wound closure. There was closure in all of the wounds, but there was no statistically significant difference between any of the groups investigated. There are a number of reasons to explain this. The human skin equivalent can act as a suitable model to elucidate in vitro the permeation of drugs and compounds through the epidermis and into the dermis. It has also been proposed as a model of skin cancer, psoriasis and infection. Its applicability in a wound model however is somewhat limited as the wound healing process in vivo includes more cell types than only fibroblasts and keratinocytes. Several approaches of inducing a wound include scratching, abrading and burning. The most commonly used instruments to induce the injury are mashers, scalpels, biopsy punches, dermatomes, liquid nitrogen and lasers. The most common model is the burn model which employs a bass wire heated to 150 °C. However, there are concerns over wound-size reproducibility in all of these methods employed. The biopsy punch employed here had a standard size of 2 mm diameter yet the application of this wound is influenced by user expertise, angle of entry employed and force applied. Standardized application of such a wound would require an automated, robotally controlled system with geospatial positioning.
Figure B.1: Schematic representation of human skin equivalent fabrication.
Figure B.2: (a) Photograph of wounded skin model with biopsy punch wound in the centre, (b) Movat’s pentachrome staining of wounded skin model after 10 days with 4S-StarPEG scaffold still present (black arrow) and (c) immunostaining of wounded skin model after 10 days. Green indicates collagen type I immunostaining, red indicates collagen type III immunostaining and blue indicates cell nucleus.
Figure B.3: Effect of treatments on skin equivalent model wound area contraction (normalized to wound area at day 0). Data presented is the mean ± standard deviation (n = 6) analysed by one-way ANOVA and Tukey's post-hoc test. No statistically significant difference is detected between the samples at p < 0.05. 29B indicates the use of 0.5 μg of miR-29B, Scram indicates the use of 0.5 μg of scrambled miR, and C indicates the use of pD-pDA.
Fibroblasts will contract hydrogels in vitro and the system developed here has no boundary to impede horizontal contraction. Additionally, this model possesses no innate immune system so the cascade of inflammation is not initiated to the degree to which that in vivo is subjected. This complex cascade of events follows cutaneous injury in sequential and overlapping phases. It initiates with haemostasis in which platelets aggregate at the injury site to form a fibrin clot. Histamine, released by ruptured cell membranes, enables blood vessels to become dilated and porous which facilitates the infiltration of inflammatory cells such as polymorphonuclear neutrophils (PMNs) and helper T cells into the wound site from the bloodstream. In the following/overlapping inflammatory phase, bacteria and debris are phagocytised and removed. Macrophages secrete a number of factors such as growth factors and other cytokines which attract cells involved in a proliferation stage of healing to the area. By the end of the first week, fibroblasts become the predominant cell in the wound and are responsible for laying down the collagen matrix in the wound site. Formation of this granulation tissue begins in the wound during the inflammatory phase and continues until the wound bed is covered. The innate immune response and also a systemic inflammatory response is lacking in the in vitro skin equivalent model presented. Therefore a true representation of wound healing and also evaluation of the treatments applied in this chapter, which aim to modulate wound healing, cannot be achieved. This could account for the absence of any statistically significant differences when wound contraction area and collagen type I and collagen type III deposition were investigated (Figure B.2). Additionally, there was strong staining of collagen type I which is due to the matrix of the skin equivalent model matrix being entirely composed of collagen type I. Based on these conclusions and results, it was decided not to perform further analysis on the models employed in this study.
Appendices

B.4. References

C. Materials and Reagents

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<td>FITC-anti-mouse antibodies</td>
<td></td>
</tr>
<tr>
<td>Horseradish peroxidase (HRP)-conjugated anti-mouse IgG</td>
<td></td>
</tr>
</tbody>
</table>
D. Re-Constitution of Collagen

1. Remove collagen (atelocollagen isolated from bovine Achilles tendon) from -20° storage.
2. Pull apart fibrous collagen to enable homogeneous freeze drying.
3. Place collagen in container suitable for freeze drying, e.g. 6 well plate/petri dish.
4. Place container in freeze drier and dry overnight.
5. Once dried, weigh out desired mass of collagen while ensuring maintenance at 4 °C.
6. Note: Consider Sircol™ assay results when weighing collagen, e.g. 1 mg may equal 0.8 mg collagen given 80 % purity. The Sircol™ assay protocol is described in Appendix D.1.
7. Prepare a solution of 50 mM hydrochloric acid (bring to 4 °C).
8. Place desired amount of collagen in the hydrochloric acid.
9. Place solution, maintained at 4 °C for 8-12 hours (overnight, for convenience) to swell the collagen.
10. Homogenise the solution with a blender/homogenizer.

Note: Kinetic effects of blending may induce heat, so ensure maintenance at 4 °C.
11. Remove excess bubbles by vacuum through Buckner flask.
12. Store re-constituted collagen at 4 °C until use.

D.1. Sircol Assay to Determine Collagen Purity

1. Adjust concentration of isolated collagen to 1 mg/ml using 5 mM hydrochloric acid. Rationale for this is that the collagen standard supplied with the Sircol™ kit is also 1 mg/ml and will thus ensure better relativity.

Note: The isolated sample does not contain 1 mg/ml of pure collagen as there are trace amounts of fat tissue and non-digested proteins. The Sircol™ assay will determine the actual collagen content within this isolate.
2. Label 1.5 ml eppendorfs appropriately for 0, 10, 20, 30, 40 and 50 µg of standard and test samples. Both standards and test samples should be prepared in triplicate. 
3. Add 0, 10, 20, 30, 40 and 50 µl of collagen standard into appropriate tubes.
4. Add 0, 10, 20, 30, 40 and 50 µl of isolated collagen into appropriate tubes.
5. There should now be a total of 36 tubes, half of which contain collagen standard and the other half containing isolated collagen.
6. Adjust the volume of all tubes to 100 µl with distilled water.
7. To each tube add 1 ml of Sircol™ Dye reagent (Sirius Red in picric acid).
8. Place tubes in an eppendorf rack and place rack on mechanical shaker for 30 minutes. The dye should bind to the collagen and precipitate out of the solution.
9. Transfer the tubes to a micro-centrifuge and centrifuge at 10,000 g for 10 minutes.
10. The un-bound dye can be removed by gently tipping the contents gently onto absorbent paper.
11. The remaining un-bound dye can be removed with a cotton bud. Be careful not to touch the pellet during this procedure.
12. Add 1 ml of alkali reagent (0.5 M sodium hydroxide) to each tube.
13. Re-cap the tubes and centrifuge the contents until the precipitate has become dissolved (may take up to 15 minutes).
14. Remove 200 µl from each tube and place in a separate well of a clear 96 well plate.
15. Place the plate in a plate reader and measure absorbance at 550 nm for 0.1 seconds per well.
16. Remove the average blank reading i.e. 0 µg collagen sample, from all other readings.
17. By plotting collagen reference samples against isolated collagen samples the collagen content can be determined. E.g. if 50 µg/ml standard gives average reading of 0.963 and 50 µg/ml test samples gives an average reading of 0.769, then the test sample contains 80% collagen (0.769/0.963).

D.2. Preparation of Non-crosslinked Hydrogel

1. Perform all actions while ensuring maintenance at 4 °C.
   
   **Note:** When using scaffolds for biological interaction, perform steps below in cell culture hood with sterile-filtered buffers.

2. Add collagen type I solubilised in 50 mM hydrochloric acid to an eppendorf to required volume.
3. Mix this at a 1:9 ratio of 10 X PBS to collagen solution to buffer.
4. Mix evenly with 1 ml pipette and ensure complete mixing.
5. Add dropwise 2 M sodium hydroxide (in 1/2 µl quantities) until pH reaches 7.2 – 7.5.
   
   **Note:** The proportion of NaOH can vary from batch to batch of collagen, depending on purity, storage conditions and solubility of collagen. Monitor pH using pH strips with a narrow range (typically 6 – 8).
6. When desired pH has been achieved, maintain at 4 °C until gelation and/or crosslinking is required.

D.3. Crosslinking of Collagen Type I Hydrogel using 4S-Succinimydyl Glutarate Terminated Poly (ethylene glycol)

1. After preparing hydrogel solution, leave at 4 °C (on ice is suitable).
2. Prepare four arm poly (ethylene glycol) succinimidyl glutarate (4S-StarPEG) solutions in 1 X PBS. **Note:** 4S-StarPEG must be stored under argon, protected from light and at -20 °C.
Figure D.1: Absorbance ranges of titrated isolated collagen and collagen standards stained with Sircol™ Reagent. Data presented is the mean of n = 3 with trendline added to denote pattern. Based on the equations of the trendlines, the % collagen of the isolated collagen, relative to the 100% collagen standard is ~80%.
3. Mix required mass of 4S-StarPEG to neutralised collagen solution. Theoretically, the 4S-StarPEG will covalently bind to free amines in the collagen. On a molar basis, calculate the required number of moles to bind at a 1:4 ratio of 4S-StarPEG to collagen free amines and determine the required mass of this.

4. Mix crosslinking solution containing required mass/moles with collagen solution. Maintain at 4 °C.

Note: Work quickly after adding crosslinker because complete gelation occurs within eight minutes. Add to well plate/eppendorf/syringe as required.

D.4. TNBSA Quantitation of Free Amines Following Crosslinking

1. Prepare a standard curve with Glycine in sodium bicarbonate pH 8.5 (0.1 M) (H₂N-CH₂-COOH) (100 nM, 50 nM, 25 nM, 10 nM, 5 nM and 0 nM).
2. Add 250 µl of 0.01% of TNBSA in 0.5 ml of each sample. Mix well.
3. Incubate at 37 °C for 2 hours.
4. Add 250 µl of 10% SDS and 125 µl of 1 M HCl.
5. Hydrolyse scaffold/hydrogel by incubating the samples at 120 °C for 15 minutes (autoclaving is also appropriate).
6. Measure the absorbance at 335 nm from 250 µl.

D.5. Enzymatic Degradation of Hydrogels Following Crosslinking

1. Incubate hydrogel for 1 hour in 0.1 M Tris-HCl (pH 7.4), 50 mM CaCl₂ at 37°C.
2. Reconstitute bacterial collagenase (extracted from Clostridium histolyticum) in 0.1 M Tris-HCl at a concentration of 10 units/mg.
3. Incubate at 37 °C. At each time point (12, 24, 48 hours), cease the enzymatic reaction by adding 0.25 M EDTA. Store and label supernatant at -20°C until further analysis.
4. Centrifuge samples at 4,000 rpm, for 5 minutes at 4°C.
5. Wash samples 3 times with distilled water and freeze-dry.

Mass loss is calculated by comparing the initial mass \( W_o \) with the mass at a given time point \( W_t \), as shown:

\[
Mass\ loss\ (\%) = \left( \frac{W_o - W_t}{W_o} \right) \times 100
\]
Figure D.2: Reaction of TNBSA with primary amine-containing molecule to produce a chromogenic derivative.
E. Synthesis of Linear pDMAEMA co PEGMEA/PEGDA Hyperbranched Polymer

E.1. Synthesis of Linear pDMAEMA

1. Add the following (in Table E.1) to a two-neck flask with a magnetic stirring bar.

Table E.1: Chemicals and monomers required for linear pDMAEMA synthesis

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>PMDETA</th>
<th>DMAEMA</th>
<th>EbrIB</th>
<th>THF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Role</td>
<td>Initiator</td>
<td>Monomer</td>
<td>Ligand</td>
<td>Reaction Solvent</td>
</tr>
<tr>
<td>Molar Ratio</td>
<td>1</td>
<td>127</td>
<td>0.5</td>
<td>n/a</td>
</tr>
<tr>
<td>Volume Required</td>
<td>73 μl</td>
<td>15 ml</td>
<td>103.8 μl</td>
<td>15 ml</td>
</tr>
</tbody>
</table>

2. THF is used to dilute the solution, typically a similar volume as the monomer used. Base all calculations on the decided volume (and subsequent moles) of monomer and base all other calculations on this. Seal the two-neck flask and de-gas the solution using argons with an inlet needle and outlet needle in each neck of the flask. De-gas for at least 20 minutes. In the mean time set the oil bath to a temperature of 50 °C.

3. Use Copper Chloride I as a catalyst for this reaction. Use the catalyst at a molar ratio of 0.25 based on the table above. Add the copper chloride as carefully and quickly as possible. De-gas briefly to minimise any oxygen that may have entered. Mix well in the solution, and begin the reaction by immersing, with stirring, into the oil bath.

4. Allow reaction to occur for approximately 40 minutes to achieve a pDMAEMA molecular weight of ~7000. If longer times are required, optimise and observe the growth kinetics using Gel Permeation Chromatography.

5. Precipitate the reactant, slowly and drop wise, in a five fold volume of hexane and allow to settle after 30 minutes. Remove hexane by decanting and evaporate using a vacuum.

E.2. Deactivation Enhanced Atom Transfer Radical Polymerisation of Linear pDMAEMA co-branched PEGDA/PEGMEA

1. Restore block 1 (linear pDMAEMA in 30 mls THF)

2. Add the following (in Table E.2) to a two-neck flask with a suitable sized magnetic stirring bar.

Table E.2: Chemicals and monomers required for linear linear pDMAEMA co-branched PEGDA/PEGMEA

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>PMDETA</th>
<th>DMAEMA</th>
<th>EbrIB</th>
<th>THF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Role</td>
<td>Initiator</td>
<td>Monomer</td>
<td>Ligand</td>
<td>Reaction Solvent</td>
</tr>
<tr>
<td>Molar Ratio</td>
<td>1</td>
<td>127</td>
<td>0.5</td>
<td>n/a</td>
</tr>
<tr>
<td>Volume Required</td>
<td>73 μl</td>
<td>15 ml</td>
<td>103.8 μl</td>
<td>15 mls</td>
</tr>
</tbody>
</table>

3. In this reaction, L-Ascorbic Acid (L-AA) is employed as a catalyst/reducing agent.
4. Resuspend required mass (moles) of L-AA in H₂O. If necessary make a concentrated solution in order to minimise the volume of H₂O added to the reaction.

5. Seal the two-neck flask and de-gas the solution using argons with an inlet needle and outlet needle in each neck of the flask. De-gas for at least 20 minutes.

6. In the meantime set the oil bath to a temperature of 50 °C.

7. Add suspended L-AA to the reaction with the use of a syringe to avoid opening the seal on the flask.

8. At this point take a sample for t = 0.

9. Begin reaction by placing in the oil bath at 50 °C.

10. Take samples at regular time points and investigate the growth of the PEGDA/PEGMEA branching using Gel Permeation Chromatography (GPC).

11. When required, halt the reaction by purging the system with oxygen. At this point, protect the resultant polymer from light at all times.

12. Precipitate the reactant slowly and dropwise in a five fold volume of 1:1 Hexane: Di-ethyl Ether.

13. Allow to stand for 30 minutes and vacuum as before.

14. Dissolve remainder polymer in acetone, and dialyse in ddH₂O with a membrane MWCO of 8,000. Protect from light and dialyse for three days, changing the water at as many intervals as possible. This removes any remaining monomer, or unreacted copper.

15. Freeze dry after dialysis is complete.

16. After freeze drying, aliquot the resultant polymer and store, protected from light at -20 °C.

E.3. Gel Permeation Chromatography of Synthesized Polymers

1. Under argon pressure, gently remove samples (approximately 1 mL maximum) before/during/after reaction process.

2. Dilute sample in dimethyl formaldehyde (DMF) ten-fold and then run sample through a silica gel column to remove any copper present in the sample.

3. Filter the polymer solution. Small solids and particles may clog the GPC resulting in expensive repairs. Every sample that is run on the GPC must be filtered. Screw needle (with cover) on glass syringe. Remove cover.

4. Draw up the polymer solution through the needle into the glass syringe. Draw up an additional 1-2 mL air so that the syringe contains both polymer solution and air.

5. Replace cover on needle. Remove the needle carefully. Discard needle in sharps container.

6. Hold the syringe upright so that the air is at the port of the syringe. Screw a PTFE filter on the end of the glass syringe.
7. Turn the needle upside down so that the polymer solution is at the port of the syringe. Empty syringe of contents through the filter into a clean 1 mL GPC vial. Push the “extra” air through the filter to clear all the polymer solution of the filter.

8. Cap the GPC vial immediately and dispose of the PTFE filter. Load glass vials in GPC carousel tray for analysis.

F. Characterization of miR-Complexing Agent Polyplexes

F.1. UV Spectroscopy

1. Start-up NanoDrop™.
2. Wipe the pedestal clean with a Kimwipe™.
3. The software will require a distilled water sample to initialize, then will ask for a blank sample – this is a sample with no DNA in it, or any other buffer solutions, etc. Only 1-2 μL required.
4. Fill in the Sample ID space, then start reading samples.
5. The output of the NanoDrop™ is a spectral representation of the UV absorbance of the sample. Normally, the peak absorbance is at 260 nm. If the samples are tightly polyplexed, this peak may be closer to 300 nm. Once polyplexed, the concentration measurement is no longer reliable.

F.2. Gel Electrophoresis for Characterisation of the Electrophoretic Mobility of Complexes

1. Add 0.7 g agarose in 100 mL TAE buffer (for large gel) or 0.35 agarose in 50 mL TAE buffer (small gel).
2. Add about 2 mL extra liquid to account for boiling and mix in an Erlenmeyer flask, and put into microwave.
3. Leave in microwave until boiling.
4. Remove from microwave with hot mitts or something to protect hands from heat.
5. Allow to cool until can be held comfortably in hand.
6. Add 10 μL Syber® Safe dye (Do not add until the solution is cool enough or the experiment will be ruined).
7. Pour immediately into plate, add comb, and remove any bubbles. Leave to cool and set
8. Pipette samples into wells.
9. Run gels at 80-100V and leave the current on ‘Auto’. Check that bubbles are forming along the wires at the bottom.
10. Check after 15 minutes – dye should have moved slightly out of the wells. Check that the direction is right and that all seems to be in order. If you leave it much after this, it is too late to rectify it.
11. Check every half hour/hour. Depending on the gel, dye, etc. it can take 3-4 hours to run. Make sure to stop it by the time that the dye reaches about ¾ of the way along the gel.

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

13. Dispose of gel appropriately.

G. Preparation, Propagation, Isolation and Purification of Plasmid

G.1. LB Agar Plates

1. Place 1 tablet of Luria broth (LB) agar into 50 ml of distilled water.
2. Place in autoclave.
3. When removed let cool until it can be handled comfortably.
4. For cultures that require kanamycin use 10 µg/ml, hence 10 mg/L.
5. Prepare Bunsen burner and plates.
6. Remove the lid of the LB agar.
7. Run the top across the Bunsen flame.
8. Pour into the plate in close vicinity to the flame.
9. Let plates set at room temperature.

G.2. Digestion of Reporter Plasmid

1. Prepare the following in a sterile eppendorf:
   - Sterile deionized water 16.3 µl
   - RE10X Buffer 2 µl
   - Acetylated BSA (10 µg/µl) 0.2 µl
   - Plasmid DNA (1 µg/µl) 1 µl
2. Mix by pipetting and then add
   - XhoI restriction enzyme (10 units/µl) 0.5 µl
   - XbaI restriction enzyme (10 units/µl) 0.5 µl
3. Mix gently by pipetting, close tube, centrifuge for a few seconds in a microcentrifuge and incubate for 3 hours at 37 °C.
4. Halt reaction by heat inactivation at 65 °C for 25 minutes.
5. Purify the product by using Qiaquick™ PCR purification kit (Appendix I.3)

G.3. Qiaquick™ PCR Purification Kit Protocol

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix.
2. Place a Qiaquick™-spin column in a 2 ml collection tube.
3. To bind DNA, apply the sample to the Qiaquick™ column and centrifuge for 30-60 seconds.
4. Discard flow-through. Place the Qiaquick™ column back into the same tube.
5. To wash, add 0.75 ml Buffer PE to the Qiaquick™ column and centrifuge for 30-60 seconds.
6. Discard flow-through and place the Qiaquick™ column back in the same tube. Centrifuge the column for an additional 1 minute.
7. Place the Qiaquick™ column in a clean 1.5 ml microcentrifuge tube.
8. To elute DNA, add 50 μl Buffer EB (10 mM Tris-Cal, pH 8.5) or water (pH 7.0-8.5) to the centre of the Qiaquick™ membrane and centrifuge for 1 minute. Alternatively for increased DNA concentration, add 30 μl elution buffers to the centre of the Qiaquick™ membrane, let the column stand for one minute, and then centrifuge.

G.4. Annealing of Oligonucleotides

1. Dilute both oligonucleotides (supplied by user) to 1μg/μl.
2. Combine 2 μl of each oligonucleotide with 46 μl of Oligo Annealing Buffer.
3. Heat at 90 °C for three minutes, then transfer to a 37 °C water bath for 15 minutes.
4. Use the annealed oligonucleotides immediately, or store at -20 °C.

G.5. Ligation of Oligonucleotides into Digested Vector

1. Dilute annealed oligonucleotides 1:10 in nuclease-free water to a final concentration of 4 ng/μl per oligonucleotide. Ligate 4 ng of annealed oligonucleotides and 50 ng of linearized vector using a standard ligation protocol.
2. The 2X Rapid Ligation Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes by making single-use aliquots of the buffer after the buffer is thawed for the first time. Store the aliquots at −20 °C.
3. Vortex the 2X Rapid Ligation Buffer before each use.
4. Mix the reactions by pipetting volumes according to Table G.1. Incubate the reactions at room temperature for 5 minutes. Alternatively, the reaction can be incubated for one hour at room temperature or overnight at 4 °C.

Table G.1: Reagent volumes for ligation of oligonucleotides into vector.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Negative Control</th>
<th>2X Rapid Ligation Buffer</th>
<th>pGeneClip™ Vector (50ng/μl)</th>
<th>annealed oligonucleotides A and B (4ng/μl each)</th>
<th>Nuclease-Free Water</th>
<th>T4 DNA Ligase (3 units/μl)</th>
<th>Total volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Rapid Ligation Buffer</td>
<td>5μl</td>
<td>5μl</td>
<td>1μl</td>
<td>1μl</td>
<td>3μl</td>
<td>1μl</td>
<td>10μl</td>
</tr>
<tr>
<td>pGeneClip™ Vector (50ng/μl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>annealed oligonucleotides A and B (4ng/μl each)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4 DNA Ligase (3 units/μl)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

5. The ligation reaction mixture can be used directly for the transformation of competent cells.
G.6. Transformation

1. Turn on water bath at 42 °C.
2. Take out plates for transformation to warm up.
3. Fill ice box.
4. Put 1 μg plasmid into ‘plasmid +’ tubes.
5. Label another tube control.
6. Take one tube of XL1Blue (bacteria) from box in -80 °C freezer and thaw on ice.
7. When thawed, add 50 μl bacteria to each tube.
8. Flick to mix.
9. Leave on ice for two minutes.
11. Place in ice for two minutes.
   Note: Can also repeat four times by 40 seconds on ice, 40 seconds at 42 °C.
12. Add 1 ml room temperature LB broth (no antibiotic).
13. Tape up-right on shaker for 30-45 minutes.
14. Prepare plates, Bunsen burner, spreader, 70% ethanol, etc. Add 100 μl of inoculums to ‘ampicilin +’ plates (or ‘kanamycin +’ plates depending on plasmid).
15. Take spreader out of 70% ethanol and burn off quickly in flame. Let cool (touch off edge of LB agar).
16. Spread cells over plate.
17. Let sit for ~20 minutes then put in 37 °C incubator.

G.7. Plasmid Propagation

1. Prepare LB (2.5 L each for Giga) for growing up cells.
2. Add RNase to P1 (1 vial/bottle).
3. Prepare 70 % ethanol by adding 40 ml 100% ethanol to endotoxin-free H₂O.
4. Check P2 for precipitation – can warm to 37 °C.
5. Put buffer P3 at 4 °C.
6. Pick a colony off an old plate and use it to inoculate a 1 ml eppendorf of LB.
7. Incubate for ~eight hours.
8. Streak a plate and allow to grow overnight at 37 °C.
Appendices

G.8. Gigaprep™ Protocol for Extraction of Plasmid DNA

P1, P2, P3, Buffer FWB2, Buffer ER, Buffer QN, and Buffer TE are all supplied in Gigaprep™ Kit.

1. Pick a single colony from a freshly streaked selective plate and inoculate a starter culture. Incubate for eight hours at 37 °C with vigorous shaking (~300 rpm). Use 50 ml tubes.
2. Pour 2.5-5 ml starter into 2.5 L ‘LB + antibiotic’. For ampicilin: 20 μg/ml. For kanamycin: 10-30 μg/ml.
3. Incubate at 37 °C for 12-16 hours. Needs to be in a shaker.
4. Switch to large vessels for a total volume of ~8 L. Vessel should be four times larger than volume. Therefore must split into 3 x 2.5 L flasks. Place in shaker overnight (37 °C).
5. Harvest by centrifugation at 6,000 g for 15 minutes at 4 °C.
6. Remove supernatant.
7. Attach a Qiafilter™ (Mega/Giga) to a glass bottle that can be vacuum-connected. (1L of larger).
8. Resuspend pellet in 125 ml P1.
9. Add 125 ml P2 (warmed and mixed homogeneously). Invert vigorously 4-6 times and incubate at room temp for five minutes.
10. Add 125 ml of pre-cooled P3 and invert four to six times. Mix well until fluffy material has formed and lysate is no longer viscous anymore.
11. Pour lysate into prepared cartridge and incubate at room temperature for ten minutes. Alternatively, centrifuge at 10,000 rpm for 30 minutes, transfer supernatant and centrifuge again at 10,000 rpm for 15 minutes.
12. Switch on vacuum and turn off when done.
13. Add 50 ml Buffer FWB2 to cartridge and gently stir precipitate with sterile spatula. Turn on vacuum again.
14. Add 300 ml Buffer ER to lysate, invert bottle ten times and put on ice for 30 minutes (This step is only for endo-free kits). Near the end of the 30 minutes, equilibrate Qiagen-tip™ 1000 by adding 75 ml Buffer QBT and empty via gravity flow.
15. Add filtered lysate and allow to enter resin via gravity flow.
16. Wash tip in total of 600 ml Buffer QN (can pre warm).
17. Precipitate DNA by adding 70 ml room temperature isopropanol to elute DNA.
18. Mix and centrifuge immediately at 15,000 g for 30 minutes at 4 °C (or 5,000 g for 60 minutes).
19. Wash pellet in 10 ml of endotoxin free room temperature 70% ethanol and centrifuge at same conditions.
20. Air dry pellet and re-dissolve in suitable volume of Buffer TE.
Appendices
G.9. Sequencing of Constructed Plasmid DNA

Table G.9: Fasta report of plasmid sequencing
Template
Primer
Status LeftClip RightClip Length
--------------------------------------------------------------------------------pMIR29B
For 7228
PASSED
13 - 1023 1010
pMIR29B
Rev 282
PASSED
14 - 996 982
pMIR29B
T7 Term prim FAILED
- 385
Ligated sequences are highlighted in BOLD
Fasta sequences (5`→3`):
> pMIR29B_For-7228 -- 13..1023 of sequence
ACGCCAGAGGGCGGCAGATCGCCGTGTAATTCTAGTTGTTTAAACGAGCTCGCTAGCCTCGAGTA
GCGGCCGCTAGTAACACTGATTTCAAATGGTGCTATCTAGAGTCGACCTGCAGGCATGCAAGCT
GATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTAGTTGGCTGCTGCCACCGCTGAGCAATAACTA
GCATAACCCCTTGGGGCGGCCGCTTCGAGCAGACATAACATTGATGAGTTTGGACAAACCACAACT
AGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTTAACCATTAT
AAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAGGTTCAGGGGGAGAT
GTGGGAGGTTTTTTAAGCAAGTAAAACCTCTACAAATGTGGTAAAATCGAATTTTAACAAAATATT
AACGCTTACAATTTCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATAC
GCGGATCTGCGCAGCACCATGGCCTGAAATAACCTCTGAAAGAGGAACTTGGTTAGGTACCTTCTG
AGGCGGAAAGAACCAGCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGCTCCCCAGC
AGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGAAAGTCCCCAGGCTC
CCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGAACCATAGTCCCGCCCCTAACT
CCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTT
TTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCAGAAGTAGTGAGGAGGCTTTTT
TGGAGGCCTAGGCTTTTGCAAAAAGCTTGATTCTTCTGACACAACAGTCTCGAACCAAAGGCTGGA
GCCACCATGG
GGTACCACCGAGGTCGGAAACCAAGCTCTGACAACACAGTCTTCTTAGTTCGAAAAACGTTTTCGG
ATCCGGAGGTTTTTTCGGAGGAGTGATGAAGACTTATCGAGTCTCCGGCTCCGCCGGAGCCGGAGA
CGTATTTATTTTTTTTAATCAGTCGGTACCCCGCCTCTTACCCGCCTTGACCCGCCTCAATCCCCGCC
CTACCCGCCTCAATCCCCGCCCTGATACCAAGACTGATTAACTCTACGTACGAAACGTATGAAGAC
GGACGACCCCTCGGACCCCTGAAAGTGTGGACCAACGACTGATTAACTCTACGTACGAAACGTAT
GAAGACGGACGACCCCTCGACCCCTGAAAGGTGTGGGATTGACTGTGTGTAAGGTGTCGACCAAG
AAAGGCGGAGTCTTCCATGGATTGGTTCAAGGAGAAAGTCTCCAATAAAGTCCGGTACCACGACG
CGTCTAGGCGCATACGCCACACTTTATGGCGTGTCTACGCATTCCTCTTTTATGGCGTAGTCCTTTA
ACATTCGCAATTATAAAACAATTTTAAGCTAAAATGGTGTAAACATCTCCAAAATGAACGAATTTT
TTGGAGGGTGTAGAGGGGGACTTGGACTTTGTATTTTACTTACGTTAACAACAACAATTGAACAAA
TAACGTCGAATATTACCAATTTTATTTCGTTATCGTAGTGTTTAAAGTGTTTATTTCGTAAAAAAAG
TGACGTAAGATCAACACCAAACAGGTTTGAGTAGTTACAATACAGACGAGCTTCGCCGGCGGGGTTCC
CCAATACGATCAATAACGAGTCGCCACCGTCGTCGGTTGATCGAAGGAAAGCCCGAAACAATCGTCG
GCCTAGTCGAACGTACGGACGTCCAGCTGAGATCTATCGTGGTAAACTTTAGTCACAATGATCG
CCGGCGATGAGCTCCGATCGCTCGAGCAAATTTGTTGATCTTAATGTGCCGCTAGACGGCGGGAG
ACCGCA
> pMIR29B_Rev-282 -- 14..996 of sequence
CGTAGGCGGTCCCGATGCCGGACTGCTTTGTTGGTCGCGGTAAGACTAGTGGGGGCTTCCCCTGCT
GTTCGGACCGCGTCATCCGTTCCACCACGGGAAGAAGCTCCGATTCCACCACCTGAACCTGTGGCC
ATTCTGTGACCCACACTTGGTCGCGCCGCTCGACACGCAGGCACCGGGGTACTAGTACTCGCCGAT
GCAATTGTTGGGGCTCCGATGTTTGCGAGAGTAGCTGTTCCTGCCGACCGACGTGTCGCCGCTGTA
GCGGATGACCCTGCTCCTGCTCGTGAAGAAGTAGCACCTGGCCGACTTCTCGGACTAGTTTATGTT
CCCGATGGTCCATCGGGGTCGGCTTGACCTCTCGTAGGACGACGTTGTGGGGTTGAGAAGCTGCGG
CCCCAGCGGCCGGACGGGCTGCTGCTACGGCCGCTCGACGGGCGGCGTCAGCAGCACGACCTTGT
GCCATTTTGGTACTGGCTCTTCCTCTAGCACCTGATACACCGGTCGGTCCAATGTTGGCGGTTCTTC
GACGCGCCACCACAACACAAGCACCTGCTCCACGGATTTCCTGACTGCCGTTCAACCTGCGGGCGT
TCTAGGCGCTCTAAGAGTAATTCCGGTTCTTCCCGCCGTTCTAGCGGCACATTAAGATCAACAATTT
GCTCGAGCGATCGGAGCTCATCGCCGGCGATCATTGTGACTAAAGTTTACCACGATAGATCTC
AGCTGGACGTCCGTACGTTCGACTAGGCCGACGATTGTTTCGGGCTTTCCTTCGACTCAACCGACG
ACGGTGGCGACTCGTTATTGATCGTATTGGGGAACCCCGCCGGCGAAGCTCGTCTGTACTATTCTA
TGTAACTACTCAAACCTGTTTGGTGTTGATCTTACGTCACTTTTTTTACGAAATAAACACTTTAAAC
ACTACGATAACGAAATAAACATTGGTAATATTCGACGTTATTTGTTCATTGTTGTT

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H. In Vitro Transfection

H.1. Gaussia Transfection of Cultured Cells

1. A standard transfection assay of 10,000 cells uses 1 μg of G. Luciferase encoding plasmid DNA at optimum w/w ratios of DNA: transfection agent.
2. Twenty-four hours prior to transfection, seed 10,000 cells in the well of a 24 well plate. The use of cell culture inserts are convenient if confocal microscopy studies are to be performed.
3. On the day of transfection, prepare plasmid complexes under a cell culture laminar hood.
4. For a single transfection, add 1 μg of plasmid DNA to suitable amount of transfection reagent in a sterile eppendorf.
5. Adjust volume to 50 μl with serum free media.
6. Gently vortex sample for five seconds and let stand at room temperature for a further ten minutes.
7. Remove media from cells and add the complexed DNA.
8. Adjust volume to standard culture volume with standard cell media and place in cell culture media.
9. At selected time periods (usually every two days), remove media from the cells and store at -20 °C for analysis.
10. Replace media with standard cell culture media and return to cell culture incubator.
11. At each time point, repeat steps 9 and 10.

H.2. Gaussia Luciferase Transfection Analysis

1. Thaw the samples that were collected according to Appendix O. Also thaw the luciferase buffer supplied with the kit. For analysis of a single sample, the following protocol should be followed:
2. Prepare luciferase dye (1 X) from stock solution supplied in kit (100 X). Use the supplied buffer (1 X) for diluting. Note: This dye is sensitive to light so protect with tin foil.
3. Prepare a fresh opaque (white is best but be consistent with all measurements) 96 well plate.
4. Place 100 μl of PBS into a single well.
5. Add 10 μl of cell culture media which is removed from transfected cells.
6. Prepare plate reader for sample loading (ideally a plate reader with injection needles should be used but if not available use a multi channel pipette and test one column per test run). Plate reader settings should be set to luminescence (1.0 seconds).
7. Add luciferase dye which is prepared according to step 3.
8. Close plate reader lid and read sample.
9. A luciferase reading will appear on screen which is an indication of the level of transfection.

**H.3. Dual Luciferase Transfection Analysis**

1. Remove multiwell plates containing mammalian cells from the incubator. Make certain that the plates are compatible with the type of luminometer being used.

2. Measuring firefly luciferase activity: Add a volume of Dual-Glo® Luciferase Reagent equal to the culture medium volume to each well and mix. For 96-well plates, typically 75 μl of reagent is added to cells grown in 75 μl of medium. For 384-well plates, typically 20 μl of reagent is added to cells grown in 2 μl of medium.

3. Wait at least ten minutes, then measure the firefly luminescence (consult the luminometer manual for proper use of the instrument). Optimal results will be generated if the luminescence is measured within two hours of addition of Dual-Glo® Luciferase Reagent.

4. Measuring Renilla luciferase activity: Add a volume of Dual-Glo® Stop & Glo® Reagent equal to the original culture medium volume to each well and mix. As noted in Step 2, this volume is typically 75 μl for 96-well plates and 20 μl for 384-well plates. **Note:** Dual-Glo® Stop & Glo® Reagent should be added to plate wells within 4 hours of addition of Dual-Glo® Luciferase Reagent.

5. Wait at least 10 minutes, and then measure luminescence. Renilla luminescence should be measured in the same plate as the firefly luminescence was measured (Step 3). Optimal results will be generated if the luminescence is measured within 2 hours of addition of Dual-Glo® Stop & Glo® Reagent.

6. Calculate the ratio of luminescence from the experimental reporter to luminescence from the control reporter. Normalize this ratio to the ratio of a control well or series of control wells that are treated consistently on all plates. This normalization provides optimal and consistent results from the Dual-Glo® Luciferase Assay System.

7. Ratios can then be calculated from the Normalized Ratios. This is expressed on a scale of 0-1 where 0 is complete inhibition of a reporter protein (Renilla) and 1 is no inhibition whatsoever. **Positive control:** Renilla/Luciferase Transfection only  

**Negative control:** Cells only

\[
Ratio = \frac{LUMINESCE_{Firefly}}{LUMINESCE_{Renilla}}
\]

Relative Inhibition

\[
RI = \frac{(Ratio_{sample} - Ratio_{negative control})}{(Ratio_{positive control} - Ratio_{negative control})}
\]
I. **Cell Culture**

1. **Aseptic Technique**

   1. Ensure inside of hood is as clean as possible (spray with Virkon then 70% Ethanol). Spray everything entering the hood with 70% Ethanol.
   2. Assume every exposed surface is contaminated – if anything touches the hood or your hands or lab coat, it most likely will have contamination.
   3. The insides of sterile containers are the only clean areas (i.e., sterile media, sterile plates, flasks, tubes, etc.).
   4. Keep sterile containers sealed.
   5. Contamination often starts near the lids of containers – never touch lids or necks of flasks or bottles with the tip of a pipette.
   6. If something is contaminated, do not open it in the hood.

2. **Feeding Flasks**

   1. Use aseptic technique (as always).
   2. Clean hood, spray all equipment, take flask from incubator, spray with ethanol and wipe clean with tissue.
   3. Remove media from flask. **Note:** when pipetting, never let the liquid get up to the cotton at the top of the pipette as this will break pipette boy will be broken.
   4. Put media in waste container, or use aspirator to remove.
   5. Pipette in new media (three sizes of flask commonly used: T25, T75, and T175)
      - T25: 3-5 mL media, T75: 8-10 mL media, T175: 25-30 mL media
   6. Return flask to incubator.

3. **Cell Splitting**

   1. Remove media from flask.
   2. Wash flask with Hanks balanced salt solution or Dulbecco’s PBS by pipetting in 5-10 mL, tilting the flask to get the liquid covering the full surface, then removing it.
   3. Add enough 0.25% Trypsin-EDTA to cover the bottom of the flask when it’s lying in the proper configuration (T25: 1 mL, T75: 3 mL, T175: 7-10 mL).
   4. Put in incubator for five minutes (enzyme is active at 37 °C).
   5. Take flask out and look at under microscope. Cells should be rounded and moving around if the flask is examined. If the cells are still adhered to the bottom, try tapping the side of the flask gently. If they don’t come off, leave it for another minute or two.
   6. Once all (or almost all) cells are free, spray the flask and return it to the hood.
7. Add an equal volume of 10% serum media to the flask (ie. if you added 5 mL T/E, add 5 mL media) (deactivates the trypsin).
8. Remove all of the liquid and transfer this to a sterile centrifuge tube (15 or 50 mL).
9. Spin for five minutes at 400 g.
10. Remove the supernatant and resuspend the pellet (the cells) in new media.
11. Count and seed into new flasks.

I.4. Thawing Cells

1. Wearing protective gloves and face shield, remove tube containing cells from liquid nitrogen cylinder.
2. Warm tube in palm of hands or in waterbath.
3. When tube contents have thawed transfer to a 15 ml tube.
4. Add 10 ml or appropriate pre-warmed media with gentle aspiration.
5. Centrifuge the tube at 1500 rpm for five minutes.
6. Remove the supernatant and discard.
7. Add 10 ml or appropriate pre-warmed media with gentle aspiration to homogeneously distribute cells.
8. Transfer the contents of the tube to a new tissue culture flask.
9. Label the flask with name, date and cell type.
10. Place the flask in an incubator set at 37 °C and 5% CO₂.
11. Refresh media every two to three days.

I.5. Cell Seeding

1. When cells have become ~80% confluent in tissue culture flask remove from incubator.
2. Remove cell media and discard.
3. Add 5 ml of pre-warmed Hanks’ balanced salt solution.
4. Gently agitate the cell culture flask.
5. Remove Hanks balanced salt solution and discard.
6. Repeat steps 3-5.
7. Add 3 ml of pre-warmed trypsin-EDTA.
   Note: 3 ml is sufficient for T-75 flasks. Use 5 ml for T-175 flasks.
8. Place in incubator for five minutes.
9. Upon removal from incubator view the cells under light microscope. If cells have not detached, gently tap the base of the flask to dislodge the remaining cells.
10. Return to laminar flow hood and add 7 ml of cell media.
12. Remove contents of flask and deposit in a 15 ml tube.
13. Centrifuge the tube at 1500 rpm for five minutes.
14. Remove supernatant and discard taking care not to disturb the cell pellet at the base of the tube.
15. Add media (5-10 ml) to resuspend the pellet. Aspirate the media to homogenise the cells.
16. Remove a sample for use with the haemocytometer. 50 μl is sufficient. Place the sample in a 1.5 ml eppendorf.
17. Add 50 μl Trypan blue.
18. Assemble hemocytometer and inject a sample into either side with the 200 μl pipette.
19. Place the haemocytometer under the light microscope and count the number of cells in the centre square on opposing sides of the hemocytometer.
20. Calculate the average cell number on opposing sides and multiply by 2 to account for Trypan blue dilution.
21. Multiply by 10,000 to calculate the number of cells/ml in 15 ml tube. Depending on the volume, the total cell number can be calculated.
22. Dilute the cells as per number required per well; i.e. if 10,000 cells are required per well then dilute cells to 10,000 cells/ml.
23. Place sterile scaffolds and glass coverslips (controls) at the base of the well.
24. Add 1 ml of cell media containing appropriate number of cells.
25. Label the well plate with name, date and cell type.
26. Place well plate in incubator.

I.6. Freezing Cells

1. When freezing down cells, aspirate the media off cell pellet after it has been centrifuged.
2. Resuspend the pellet in freezing media (freezing media: 45 ml FBS, 5 ml DMSO. Make up freezing media and store at 4 °C). Cell number determines amount of freezing media. Generally ~1 ml per vial, 500,000 cells to 5 million cells per vial.
3. Put cells in Mr. Frosty container and put into -80 °C freezer immediately. Cells can be then put into liquid nitrogen after 24 hours.

I.7. Cell Counting

1. Take 50 μl of the cell suspension and add to 50 μl Trypan blue.
2. Add 10 μl of this cell/trypan blue suspension to each side of a haemocytometer (Trypan blue is excluded by live cells – blue cells are dead, clear cells are alive, see Figure I.1 for schematic).
3. Count cells on both sides and get the average
4. To calculate the total cell number: average cell no./box x 10^4 X dilution factor (in this case 2) x original volume cells were suspended in (in this case 2 ml).
Appendices

Figure I.1: Example haemocytometer grid
I.8. *Isolation of Rat Neonatal Cardiac Fibroblasts*

1. Every medium, HEPES buffer and PBS should be pre-warmed to 37 °C. Treating cardiomyocytes with cold solutions will impair their functionality. Also prepare an ice-cold aliquot of PBS.

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

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

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

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

6. Let cells settle down and wash once with pre-warmed HEPES buffer containing 5 % horse serum. Eventually, spin cells down for one minute at 340 g (if cardiomyocytes are required)

7. Resuspend cells in adhesion medium (20 ml per 10 hearts) and plate suspension on uncoated 10 cm dishes. Incubate for 1 - 1.5 hours at 37 °C/5 % CO₂. Repeat this step. During this time, fibroblasts will stick and spread on the plate whereas cardiomyocytes remain suspended.

8. Collect and count the cells to determine cell density. Expect a yield of 0.8 - 1 x 10^6 cardiomyocytes per heart. Store cells in warm medium or buffer.

I.9. *alamarBlue® Assay*

1. Remove media from cells in culture.

2. Add 1000 µl/200 µl of Hanks’ balanced salt solution into the required number of wells in a sterile 24/96 well plate.

3. Prepare alamarBlue® working solution in Hanks’ balanced salt solution (ratio 1:9 respectively). Add 500 µl/200 µl depending on conditions.

4. Incubate for two hours at 37 °C.

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

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

7. Subtract the absorbance values of media only from the absorbance values of the absorbance values of alamarBlue® in media. This gives the absorbance of alamarBlue® in media absorbance
of media only. Denote these values \( AO_{LW} \); absorbance of oxidized form at lower wavelength, and \( AO_{HW} \); absorbance of oxidized form at higher wavelength.

Calculate correlation factor:

\[
R_0 = \frac{AO_{LW}}{AO_{HW}}
\]

8. To calculate the percent of reduced alamarBlue®

\[
AR_{LW} = \left(A_{LW} - \left(A_{HW} \times R_0\right)\right) \times 100
\]

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

\[
\text{% Reduction: } = \left(\frac{A_{LW} - \left(A_{HW} \times R_0\right)_{\text{sample}}}{A_{LW} - \left(A_{HW} \times R_0\right)_{\text{control}}\text{positive}}\right) \times 100
\]

J. Generation of Antibody Fragments from Hybridoma Culture

J.1. Extraction of Monoclonal Antibody from Hybridoma Supernatant

1. After the required period, remove cells from hybridoma supernatant by centrifugation at 1500 rpm, for five minutes. Sterile filter supernatant through 0.2 μm pore filter to remove any remaining cell debris.

2. Add solution to sample filter cup (to a maximum of 70 mL); seal with supplied cap. Place sample filter cup into filtrate collection cup.

3. Place Centricon Plus-70™ assembly in centrifuge bucket.

**Caution:** Before proceeding, check centrifuge clearance by manually moving bucket to its full-swing position. Counterbalance centrifuge with a second bucket containing a second Centricon Plus-70 unit and an equal volume of sample or water.

4. Spin at up to 3,500 × g until desired concentration is achieved. A typical spin time is 15–20 minutes, depending on solute type and concentration.

**Note:** If the centrifuge vibrates excessively, stop and rebalance it.

Remove Centricon Plus-70™ unit from centrifuge and separate the sample filter cup from the filtrate collection unit.

Turn the concentrate/retentate cup upside down and place on top of the sample filter cup.

5. Carefully invert unit, place in centrifuge, and counterbalance with a similar device. Spin at no more than 1,000 × g for up to two minutes.

6. Remove the retentate cup containing the concentrated sample from the sample filter cup. Keep the filter cup inverted during this process.
7. Remove the sample with a pipette or cap the retentate cup and store sample for later use.

**J.2. Antibody Extraction from Protein Fraction**

1. Equilibrate column and buffers to room temperature. Set table top centrifuge to 1,000 g.
2. Prepare sample for purification by diluting in Binding Buffer to a minimum of 5 ml, or a maximum volume of 10 ml.
3. Loosen top cap on spin column and snap off bottom closure. Place column in a 50 ml collection tube, centrifuge for one minute and discard the flow-through.
4. Equilibrate column by adding 10 ml of Binding Buffer. Centrifuge for one minute and discard the flow-through. Repeat this step once.
5. Cap bottom of column with the included rubber cap. Apply sample to column and tightly cap top. Incubate at room temperature with end-over-end mixing for ten minutes.
6. Loosen top cap and remove bottom cap. Place column in a new 50 ml collection tube and centrifuge for one minute.

**Note:** This first collection tube contains the non-bound sample components and may be analyzed to assess binding efficiency and capacity.

7. Place column in a new 50 ml collection tube. Wash column by adding 10 ml Binding Buffer. Centrifuge for one minute and collect wash fraction. Repeat wash two additional times for a total of three washes.
8. Add 500 μl of Neutralization Buffer to three 50 ml collection tubes and place the spin column into one of the tubes.
9. Add 5 ml of Elution Buffer to the column and centrifuge for one minute. Transfer the spin column to another tube that contains Neutralization Buffer, saving the collected solution as the first elution fraction. Repeat this step two times to obtain three fractions.
10. Determine which fraction(s) contain the purified antibody by measuring the relative absorbance of each fraction at 280 nm. If required for downstream applications, exchange the buffer using Zeba™ Desalt Spin Columns, or by using Slide-A-Lyzer® Dialysis Cassettes (see related Thermo Scientific Products section).
11. To regenerate the column for storage or re-use, add 10 ml of Elution Buffer and centrifuge for one minute. Repeat once. Wash column with 10 ml of Storage Solution. Add 10 ml of Storage Solution and store column at 4 °C. Typically, the immobilized protein column may be used up to ten times without significant loss in binding capacity, although the actual number of effective usages may vary.

**J.3. Pepsin Digestion and Reduction of Monoclonal Antibodies Preparation of F(ab’)_2**
1. Gently swirl the immobilized pepsin vial to obtain an even suspension. Seat the spin column frit with an inverted 200 μl pipette tip.

2. Using a wide-bore or cut pipette tip, place 0.25 ml of the 50% slurry (i.e. 0.125 ml of settled resin) into a 0.8 ml spin column. Twist off the bottom tab of the column and place into 2.0 ml microcentrifuge tube. Centrifuge column at 5,000 x g for one minute and discard buffer.

3. Wash resin with 0.5 ml digestion buffer. Centrifuge column at 2,000 x g for one minute and discard buffer. Cap bottom of spin column with included rubber cap.

4. Twist off the bottom closure of a Zeba™ Desalt Spin Column and loosen cap. Place column in a 15 ml collection tube.

5. Centrifuge column at 1,000 x g for two minutes to remove storage solution. Place a mark on the side of the column where the compacted resin is slanted upward. Place column in centrifuge with the mark facing outward in all subsequent centrifugation steps. Note: resin will appear compacted after centrifugation.

6. Add 1 ml of digestion buffer to column. Centrifuge at 1000 g for two minutes to remove buffer. Repeat this step three additional times, discarding buffer from the collection tube.

7. Place column in a new collection tube, remove cap and slowly apply 0.5 ml sample to the centre of the compacted resin bed.

8. Replace cap and centrifuge at 1, 000 g for two minutes to collect the sample. Discard the column after use.

9. If IgG sample is 0.5-8 mg/ml (i.e. 250 μg to 4 mg, no further preparation is necessary. If sample volume is less than 0.5 ml, add digestion buffer to a final volume of 0.5 ml.

**J.3.1 Fragment Generation**

1. Add 0.5 ml of the prepared IgG sample to the spin column containing the equilibrated immobilized pepsin. Place top cap and bottom plug on the spin column.

2. Incubate digestion reaction for the appropriate time, with an end-over mixer or a tabletop rocker at 37 °C. Maintain constant mixing of resin during incubation.

3. Remove bottom cap and place column into a 2.0 ml microcentrifuge tube. Centrifuge column at 5,000 g for one minute to separate digest from the immobilized pepsin.

4. Wash resin with 0.5 ml of PBS. Place column into a tube and centrifuge at 5000 g for one minute. Repeat this step once.
Figure J.1: (a) SDS page electrophoresis of protein fractions of washes (W) and eluent fractions (F) during antibody purification process. (b) Western blot using goat anti-mouse biotinylated antibody clearly showing antibody present in fractions 1, 2, and 3. (F1, F2 and F3). See Appendices N and O for detailed protocols.
5. Add both wash fractions to the digested antibody. Total sample volume should be 1.5 ml. Discard the immobilized pepsin. **Note:** for best results, evaluate the digest and wash fraction via SDS-PAGE to assess digestion completion. Protein purification is only required to remove undigested IgG and degraded Fc do not bind to Protein A. The resulting F(ab)\(_2\) is non-reducing. SDS derived from human and mouse IgG will migrate with an apparent molecular weight of ~110 kDa.

**J.3.2 F(ab')\(_2\) Purification**

1. Equilibrate the Nab™ Protein A column, PBS and IgG elution buffer to room temperature. Set centrifuge to 1000 g.
2. Loosen top cap on the Protein A column and snap off bottom closure. Place column in a 15 ml collection tube and centrifuge for one minute to remove storage solution. Discard the flow-through.
3. Equilibrate column by adding 2 ml of PBS. Centrifuge for one minute and discard the flow-through. Repeat this step once.
4. Cap bottom of column with the included rubber cap. Apply sample to column and cap the top tightly. Resuspend the resin and sample by inversion. Incubate at room temperature with end-over-end mixing for ten minutes.
5. Loosen top cap and remove bottom cap. Place column in a new 15 ml collection tube and centrifuge for one minute. Save the flow-through as this fraction contains F(ab')\(_2\) and Fc fragments.
6. For optimal recovery, wash column with 1 ml of PBS. Centrifuge for one minute and collect flow-through. Repeat and combine wash fractions with the F(ab')\(_2\) fraction from step 5.
7. Measure protein concentration using the BCA assay or by measuring the absorbance at 280 nm. Use an estimated extinction coefficient of 1.4. Assuming complete IgG digestion, F(ab)\(_2\) yields may vary from 50 to 70 % depending on the amount of starting antibody and the protein assays used.
8. If desired, perform dialysis (50K MWCO) to remove the Fc fragments.

**J.3.3 Regeneration of Column**

1. Apply 1 ml of IgG elution buffer to the Protein A Column and centrifuge for one minute. Repeat this step two times to obtain three fractions which will contain undigested IgG. To save the undigested IgG, add 100 μl of a neutralization buffer to each of the elution fractions.
2. Add 3 ml of IgG elution buffer to the column and centrifuge for one minute. Discard flow-through and repeat.
3. Add 3 ml of PBS to the column and centrifuge for one minute.
4. For storage, add 3 ml of 0.02% sodium azide in PBS to column. Replace top and bottom caps. Store column upright at 4 °C. Columns can be regenerated at least ten times without significant loss of binding capacity.

**J.4. Reduction of Di-sulfide Bonds (Production of Fab’s from F(ab’)₂)**

1. Equilibrate immobilized reductant column and prepared equilibration buffers to room temperature before use. Perform all steps of procedure at room temperature.

2. Remove top cap from the immobilized reductant column and pour off storage solution which contains 0.02% sodium azide.

**Note:** When uncapping a column, always remove the top cap first to avoid drawing air into the resin bed. If the top porous disc in the column is dislodged, push it down to the resin bed surface (but avoid compressing the resin bed itself).

3. Twist off bottom column tab and stand column upright.

4. Equilibrate/wash the immobilized reductant by adding 5 ml of buffer #1 to the column and allowing it to drain through.

5. Prepare DTT activation solution: Dissolve 15 mg of DTT in 10 ml buffer #1 (results in 10 mM DTT).

6. Activate the column by applying 10 ml of DTT activation solution to the column and allowing it to drain through.

7. To remove the activating DTT, wash the column with 10 ml of buffer #1. (If buffer #2 is chosen and used for the sample, wash column with only 5 ml of buffer #1, followed by 5 ml of buffer #2).

8. Apply 1 ml of peptide or protein solution to the column. Allow the sample to completely enter the resin bed. (Flow will stop automatically when the liquid drains down to the top porous disc).

9. Cap top and bottom of column (white bottom tips supplied) and incubate for 60 minutes for protein samples; no incubation is needed for peptide samples.

10. Recover reduced peptide or protein sample from the column by applying 9 ml of the appropriate buffer and collecting separate 1 ml fractions as they emerge from the column tip. To ensure that the volume of each fraction is exactly 1 ml, apply only 1 ml of buffer at a time and collect the entire volume that emerges until column flow stops; then change collection tubes and apply the next 1 ml of buffer. This method utilizes the stop-flow feature of the top porous disc.

11. Identify the fractions that contain the peptide or protein (now reduced) by determining which ones have peak absorbance at 280 nm (usually the first two fractions). Be aware that some peptides do not absorb significantly at 280 nm and cannot be assayed by this method.
Alternatively, identify fractions that contain the reduced peptide or protein by specifically measuring for sulphydryl groups using the Ellman’s Reagent.

12. Reuse the immobilized reductant column by simply repeating the procedure from the beginning; the DTT activation step will regenerate the column. Four cycles of regeneration (5 total uses) are possible with each immobilized reductant column. For prolonged storage of used columns, first wash them with phosphate buffer or water containing 0.02% sodium azide, and then replace top and bottom caps and store the columns upright at 4 °C.

K. **SDS -PAGE Analysis of Extracted Proteins**

**K.1. BioRad Protein Assay to Quantify Protein**

1. Prepare dye reagent by diluting one part Dye Reagent Concentrate with four parts DDI water. Filter through a Whatman #1 filter (or equivalent) to remove particulates. This diluted reagent may be used for about two weeks when kept at room temperature.

2. Prepare three to five dilutions of a protein standard, which is representative of the protein solution to be tested. The linear range of this microtiter plate assay is 0.05 mg/ml to approximately 0.5 mg/ml. Protein solutions are normally assayed in duplicate or triplicate.

3. Pipet 10 μl of each standard and sample solution into separate microtiter plate wells.

4. Add 200 μl of diluted dye reagent to each well. Mix the sample and reagent thoroughly using a microplate mixer. Alternatively, use a multi-channel pipet to dispense the reagent. Depress the plunger repeatedly to mix the sample and reagent in the well. Replace with clean tips and add reagent to the next set of wells.

5. Incubate at room temperature for at least five minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than one hour.

6. Measure absorbance at 595 nm.

**K.2. Sample Preparation**


2. Samples: 1 μg/μl.

3. Markers: 1 μl of Precision Plus Protein Dual Colour Standards™ (Bio-Rad, 161-0374) in 20 μl of 5X sample buffer.

4. Empty well: 10 μl of 1X sample buffer (diluted from 5 X sample buffer with water).

5. For mini-gel; 10-well prepare a volume of 15 μl per well with at least 1μg of sample/control per well.

6. Vortex samples and centrifuge briefly.
7. Store at 4 °C. Prior to use, heat samples at 95 °C for five minutes, vortex and briefly centrifuge. Load carefully in each well.

**K.3. Preparation of Gels**

1. See Table K.1 and K.2 for list of materials and quantities to make gels.
2. Thaw aliquot of ammonium persulphate (APS).
3. Clean glass plates with 70% ethanol and wipe dry with microscopic tissues.
4. Set the gel making apparatus ensuring that the glass plates fit snugly to the platform.
5. Check for leaks by pouring water prior to making the gels.
6. Add gel ingredients to make a 5% resolving gel. This can be done in 15/50 ml conical tube.
7. Add APS and TEMED last, right before gels are to be poured as this initiates polymerisation.
8. Carefully pour prepared mixture into space between the glass plates until it reaches about 1 cm from the bottom of the wells etched out by the comb. Keep excess to monitor polymerisation.
9. Overlay gel with 10% ethanol to seal gels.
10. Leave for approximately 30 minutes. A line at the ethanol-gel interface will appear which indicates polymerisation.
11. Prepare stacking gel according to table. Again, add APS/TEMED immediately before use.
13. Add APS and TEMED to stacking gel and carefully add to separation gel. Immediately insert the comb, taking care to avoid trapping any air bubbles. Keep excess to monitor polymerisation.
14. Allow to set for 10-15 minutes and, in the meantime, denature samples and standards as described above.
15. After gels have been set, slowly remove combs.
17. Fill the upper/inner chambers with 1 X Running buffer.
18. Wash well with running buffer with a hypodermal needle syringe.
19. Load all samples, standards and markers.
20. Put upper chamber on the main chamber and close the lid. Run the gels
21. For a mini gel: run at 50 V until the front reaches the end of the stacking gel (±35 minutes), then adjust to 120 V until the front reaches the end of the separating gel (±1 hour).
22. Separate the glass plates and release the gel slowly into ddH₂O.
23. Proceed with coomassie or silver staining, or western blotting of separated protein.
Table K.1: 5% Separation gel (1 mm thickness) for collagen mini gel (Protean II® Bio-Rad)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide/Bis (37.5:1)</td>
<td>830 μl</td>
</tr>
<tr>
<td>1.875 M Tris-HCl pH 8.8</td>
<td>1000 μl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>50 μl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>3070 μl</td>
</tr>
<tr>
<td>Ammonium Persulphate (APS, 100 mg/ml)</td>
<td>42 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 μl</td>
</tr>
<tr>
<td>Total Volume</td>
<td>5000 μl</td>
</tr>
</tbody>
</table>

Table K.2: 3% Stacking gel (1 mm thickness) for collagen mini gel (Protean II® Bio-Rad)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide/Bis (37.5:1)</td>
<td>200 μl</td>
</tr>
<tr>
<td>1.25 M Tris-HCl pH 6.8</td>
<td>200 μl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>33 μl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>1550 μl</td>
</tr>
<tr>
<td>Ammonium Persulphate (APS, 100 mg/ml)</td>
<td>17 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>2 μl</td>
</tr>
<tr>
<td>Total Volume</td>
<td>2000 μl</td>
</tr>
</tbody>
</table>

L. **Western Blot Analysis of Transferred Proteins**

*Note:* Prepare the transfer buffer, blotting pads, and blotting membranes before performing the transfer. Preparation of the transfer buffer and materials for transfer may be made while electrophoresis of the gel is in progress.

Wear gloves at all times during the entire blotting procedure to prevent contamination of gels and membranes, and to avoid exposure to irritants commonly used in electrophoresis and blotting procedures.

Do not touch the membrane or gel with bare hands. This may contaminate the gel or membrane and interfere with further analysis.

Prepare the appropriate buffer for gel type from the recipe according to Table L.1.
Table L.1: Transfer buffer recipe for western blot procedure.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novex® Tris-Glycine Transfer Buffer (25 x)</td>
<td>40 mL</td>
</tr>
<tr>
<td>Methanol</td>
<td>200 mL</td>
</tr>
<tr>
<td>Deionized Water</td>
<td>760 mL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>1,000 mL</strong></td>
</tr>
</tbody>
</table>

Use ~ 700 mL of transfer buffer to soak the blotting pads until saturated. Remove air bubbles by squeezing the blotting pads while they are submerged in buffer. Removing air bubbles is essential as they can block the transfer of biomolecules.

PVDF membrane: Pre-wet the PVDF membrane for 30 seconds in methanol, ethanol, or isopropanol. Briefly rinse in deionized water and then place the membrane in a shallow dish containing 50–100 mL transfer buffer for several minutes.

Nitrocellulose/Nylon membrane: Place the membrane directly in a tray containing the transfer buffer for several minutes.

Filter paper: Soak briefly in transfer buffer immediately before using.

Gel: Use the gel immediately following the SDS PAGE described in Appendix K. Do not soak the gel in transfer buffer.

Remove the gel from the cassette for transfer after completion of electrophoresis as described below. One may continue electrophoresis of the gel at a low voltage of 5 V. The gel can be left in the unit for a few hours until it is ready to transfer the gel.

1. After electrophoresis, separate each of the three bonded sides of the gel cassette by inserting the gel knife into the gap between the cassette’s two plates. The notched (“well”) side of the cassette should face up.
2. Push up and down on the knife handle to separate the plates. Repeat on each side of the cassette until the plates are completely separated.
   **Caution:** Use caution while inserting the gel knife between the two plates to avoid excessive pressure towards the gel.
3. The gel may adhere to either side of plates upon opening the cassette. Carefully remove and discard the plate without the gel. The gel remains on the other plate.
4. Remove wells on the gel with the gel knife.
5. Place a piece of pre-soaked filter paper on top of the gel, and lay just above the “foot” at the bottom of the gel (leaving the “foot” of the gel uncovered). Keep the filter paper saturated with transfer buffer and remove all trapped air bubbles by gently rolling over the surface using a glass pipette.

6. Turn the plate over so the gel and filter paper are facing downwards over a gloved hand or clean flat surface covered with a piece of Parafilm®.

7. Remove gel from the plate using the following methods:

8. If the gel rests on the longer (slotted) plate, use the gel knife to push the foot out of the slot in the plate and the gel will fall off easily.

9. If the gel rests on the shorter (notched) plate, use the gel knife to carefully loosen the bottom of the gel and allow the gel to peel away from the plate.

10. When the gel is on a flat surface, cut the “foot” off the gel with the gel knife.
    
    **Note:** Once you have removed the gel from the unit and the cassette, perform the transfer immediately.

11. Wet the surface of the gel with the transfer buffer and place pre-soaked transfer membrane on the gel. Remove air bubbles by rolling a glass pipette over the membrane surface.

12. Place the pre-soaked filter paper on top of the transfer membrane. Remove any trapped air bubbles.

13. Place two soaked blotting pads into the cathode (-) core of the blot module. The cathode core is the deeper of the two cores and the corresponding electrode plate is a darker shade of gray. Carefully pick up the gel membrane assembly with a gloved hand and place on the pad in the same sequence, such that the gel is closest to the cathode plate.

14. Add enough pre-soaked blotting pads to rise 0.5 cm over the rim of the cathode core. Place the anode (+) core on top of the pads. The gel/membrane sandwich should be held securely between the two halves of the blot module ensuring complete contact of all components.
    
    **Note:** To ensure a snug fit, use an additional pad since pads lose their resiliency after many uses. Replace pads when they begin to lose resiliency and are discolored.

15. Position the gel membrane sandwich and blotting pads in the cathode core of the XCell II™ Blot Module to fit horizontally across the bottom of the unit. There should be a gap of ~ 1 cm at the top of the electrodes when the pads and assembly are in place.

    Hold the blot module together firmly and slide it into the guide rails on the lower buffer chamber. The blot module fits into the unit in only one way, such that the (+) sign is seen in the upper left hand corner of the blot module. The inverted gold post on the right hand side of the blot module fits into the hole next to the upright gold post on the right side of the lower buffer chamber.

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16. Depending on the mini-cell that that is being used, follow the appropriate instructions for positioning the wedge:

**Note:** When properly placed, the rear wedge will not be flush with the top of the lower buffer chamber. There will be a gap between the rear wedge and lower chamber.

17. Fill the blot module with transfer buffer until the gel/membrane sandwich is covered in transfer buffer.

18. Do not fill all the way to the top as this will generate extra conductivity and heat.

19. Fill the outer buffer chamber with ~ 650 mL deionized water by pouring in the gap between the front of the blot module and front of the lower buffer chamber. The water level should reach approximately 2 cm from the top of the lower buffer chamber. This serves to dissipate heat produced during the run.

20. Place the lid on top of the unit.

21. With the power turned off, plug the red and black leads into the power supply.

22. For overnight blotting, perform transfer in the cold room with low power to prevent overheating. Transfer at constant voltage of 10–15 V overnight. Depending on the transfer efficiency, adjust the transfer conditions accordingly.

23. Remove blot from the transfer apparatus.

*L.1. Ponceau Stain 1% (v/v) acetic acid)*

1. Add in the order listed:
   
   10 ml Milli-Q Water
   
   0.3 ml glacial acetic acid (use glassware to measure)
   
   0.033 g Ponceau S
   
   Fill to 30 ml with MiliQ water and store at room temperature.

*L.2. Immunostaining of Transfer Membrane from Western Blot*

1. Block nonspecific sites with blocking reagent for 20-60 minutes at room temperature (RT) with Tris Buffered Saline with 0.1% Tween-20 (TBST) containing 5 % (w/v) of bovine serum albumin (BSA). For best results, block for 1 hour at RT. Blots also may be blocked overnight at 2-8 °C.

2. Remove the blocking reagent and add the appropriate primary antibody dilution. Dilute antibody to the appropriate dilution factor with TBST containing 5 % (w/v) BSA. Incubate blot for 1 hour with shaking.

3. If desired, blots may be incubated with primary antibody overnight at 2-8 °C.

4. Wash membrane by suspending it in TBST and agitating for ≥5 minutes. Replace TBST at least four to six times.
5. Increasing the TBST volume and/or the number of washes may help reduce background.
   **Note:** Briefly rinsing membrane in TBST before secondary incubation will increase wash efficiency.

6. Incubate blot with the appropriate HRP-conjugate dilution (dilute in TBST containing 5 % (w/v) BSA for one hour at RT with shaking. Repeat Step 5 to remove unbound HRP-conjugate.
   **Note:** Membrane must be thoroughly washed after incubation with the HRP-conjugate.

7. Prepare working solution by mixing equal parts of the stable peroxide solution and the luminol/enhancer solution.

8. Use 0.1 ml working solution per cm² of membrane. The working solution is stable for 24 hours at room temperature.
   **Note:** Exposure to the sun or any other intense light can harm the working solution. For best results keep the working solution in an amber bottle and avoid prolonged exposure to any intense light. Short-term exposure to typical laboratory lighting will not harm the working solution.

9. Incubate blot with working solution for five minutes.
   **Note:** Perform previous steps to stain for GAPDH, β-actin or another appropriate loading control. This is essential to the evaluation of the previous proteins detected.

10. Remove blot from working solution and place it in a plastic membrane protector. (A plastic sheet protector works very well, although cling film may also be used.) Use an absorbent tissue to remove excess liquid and to carefully press out any bubbles from between the blot and the membrane protector.

**M. Protein Blot Array**

**M.1. Protein Extraction**

1. Retrieve protein from storage and keep on icebox.

2. Prepare lysis buffer. Avoid using SDS or other strongly denaturing detergents. In general, non-ionic detergents, such as Triton X-100 or NP-40 are best, although zwitterionic detergents, such as CHAPS, or mild ionic detergents, such as sodium deoxycholate will work. Use no more than 2% v/v total detergent Avoid the use of sodium azide Avoid reducing agents, such as dithiothreitol (DTT) or mercaptoethanols. In this instance, commercially available Lysis Buffer from Sigma Aldrich was used.

3. Place approximately 1 gram of tissue into eppendorf suitable for TissueLyserLT bead mill (Qiagen) with 1 ml lysis buffer and one stainless steel bead. The TissueLyserLT functions by vertically oscillating the rupture chamber at a high speed to move the stainless steel bead up and down rapidly within the eppendorf to rupture and homogenise the associated tissue.
4. Place eppendorfs into the pre-cooled bead mill chamber and begin rupture for one minute. Check samples to see if complete homogenisation has taken place. Otherwise repeat rupture until complete homogenisation occurs, taking care to keep the ependorfs cool.

**M.2. Protein Blot**

1. The cell culture supernates should be dialyzed with a Dialysis tube (Item A) before the biotin labeling procedure. Recommended loading is 2.5~3.0 ml of cell culture supernates into a dialyzer and dialyzing with at least 2,000 ml 1X PBS buffer (pH = 8) at 4 °C. Change the 1 x PBS buffer and dialyze again. Allow at least three hours for each dialysis step, stirring gently. The sample volume may be altered after dialysis.

2. Briefly spin down Internal Control tube (Item C) before use. Add 100 ml 1X PBS, pH=8.0 into the Internal Control tube, pipette up and down to dissolve the powder. Transfer 2 ml dialyzed sample into a new tube. Add 40 ml prepared Internal Control into the tube. Mix well. Immediately before use, briefly spin down the Labeling Reagent tube (Item B). Add 100 ml 1X PBS into the tube, pipette up and down or vortex to dissolve the powder to prepare 1X Labeling Reagent solution. Add an appropriate amount of prepared Labeling Reagent into above tube with sample in step 2, mix well immediately. Incubate the reaction solution at room temperature for 30 min with gentle shaking. Gently tap the tube to mix the reaction solution every 5 min. 7.2 ml of 1X Labeling Reagent for labeling 1 mg total protein in supernates.

3. Add 5 ml Stop Solution into the above reaction solution and then use the spin column to remove free biotin. Twist off the spin column’s bottom closure and loosen the cap. Place the column into a 50 ml collection tube. Centrifuge column at 1,000 g for three minutes to remove storage solution. Add 5 ml 1X PBS into column, centrifuge at 1,000 g for three minutes to 1X PBS. Repeat additional two times to wash the column. Place the column in a new collection tube, slowly load the sample to the center of the compact resin bed. Centrifuge the column at 1,000 g for three minutes to collect sample. Stored at –80 °C until testing. Discard column after use.

4. Place each membrane into the provided tray ("-" mark is on the antibody printed side). **Note:** The printed side should be facing upward. Add 2.5 ml Blocking Buffer and incubate at room temperature for one hour to block membranes. Decant Blocking Buffer from each container. Add 2.5 ml of sample into each array membrane, and cover with the lid. Incubate at room temperature with gentle shaking for 2 hours. Dilute sample using Blocking Buffer.

5. Decant the samples from each container, and wash three times with 3 ml of 1X Wash Buffer I at room temperature with shaking and five minutes per wash. Dilute 20X Wash Buffer I with deionized or distilled water. Decant the 1X Wash Buffer I from each container. Wash three times with 3 ml of 1X Wash Buffer II at room temperature with gentle shaking. Add 2.5 ml of
500 fold diluted HRP-conjugated streptavidin (e.g. add 10 ml of HRP-conjugated streptavidin to 5 ml of Blocking Buffer) to each membrane.

**Note:** Mix tube containing 500X HRP-Conjugated Streptavidin well before use since precipitation may form during storage. Incubate at room temperature with gentle shaking for two hours.

6. Add 2.5 ml of Detection Buffer C and 2.5 ml of Detection Buffer D into a tube (for detecting 2 membranes); Mix both solutions; Drain off excess wash buffer. Place membrane protein side up (“-” mark is on the protein side top left corner) on a clean plastic plate or its cover (provided in the kit). Pipette 2.2 ml of the mixed Detection Buffer on to each membrane and incubate at room temperature with shaking for 2 minutes. Ensure that the detection mixture is evenly covering the membrane without any air bubbles. Gently place the membrane with forceps, protein side up, on a piece of plastic sheet (“-” mark is on the protein side top left corner). Cover the array with another piece of plastic sheet. Gently smooth out any air bubbles. Avoid using pressure on the membrane. Work as quickly as possible. The signal can be detected directly from the membrane using a chemiluminescence imaging system or by exposing the array to x-ray film (we recommend using Kodak X-Omat™ AR film) with subsequent development. Expose the membranes for 40 Seconds. Then re-expose the film according to the intensity of signals. If the signals are too strong (background too high), reduce exposure time (eg, 5–30 seconds). If the signals are too weak, increase exposure time (eg, 5–20 min or overnight). Or re-incubate membranes overnight with 1X HRP-conjugated streptavidin, and repeat detection on the second day. Save membranes at –20 °C to –80 °C for future reference.

**M.3. Image Analysis (Using LUTS Protein Membrane Array)**

1. Open images in ImageJ. Begin LUTS Protein Membrane Array.
2. Determine and select the array settings for detection on the images obtained from protein array chemiluminescence.
3. Perform intensity profiles on all array images.
4. Masterise all images using the options on the LUTS Protein Membrane Array (see Figure M.1).
5. Normalise all blots using positive controls and blanks.
6. Export all data images and numerical values of intensity for further analysis.
Figure M.1: Protein membrane array intensity profiles of (a) initial intensities and (b) normalised intensities.
N. Michael–type Addition of Fab’s to Hyperbranched Polymer

N.1. Ellman’s Assay for Quantitation of Reduced Fragments Material Preparation

Reaction buffer: 0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA
Cysteine hydrochloride monohydrate: M.W. = 175.6
Ellman’s reagent solution: Dissolve 4 mg Elman’s Reagent in 1 ml of reaction buffer.

1. Prepare a set of cysteine standards by dissolving cysteine hydrochloride monohydrate at the following concentrations in reaction buffer according to Table N.1.

Table N.1: Guidelines for preparation of cysteine standards for Ellman’s assay.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Volume of Reaction Buffer</th>
<th>25 ml Amount of Cysteine (M.W. = 175.6)</th>
<th>5 ml of Standard A Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100 ml</td>
<td>26.34 mg</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>B</td>
<td>5 ml</td>
<td>25 ml of Standard A</td>
<td>1.25 mM</td>
</tr>
<tr>
<td>C</td>
<td>10 ml</td>
<td>20 ml of Standard A</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>D</td>
<td>15 ml</td>
<td>15 ml of Standard A</td>
<td>0.75 mM</td>
</tr>
<tr>
<td>E</td>
<td>20 ml</td>
<td>10 ml of Standard A</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>G</td>
<td>30 ml</td>
<td>0 ml</td>
<td>0.0 mM (Blank)</td>
</tr>
</tbody>
</table>

2. Prepare a set of test tubes, each containing 50 μl of Elman’s reagent solution and 2.5 ml of reaction buffer.

3. Add 250 μl of each standard or unknown to the separate test tubes prepared in step 2.

   **Note:** For the unknown(s), make dilutions so that the 250 μl sample applied to the assay reaction has a sulfhydryl concentration in the working range of the standard curve (0.1-1.0 mM is ideal).

4. Mix and incubate at room temperature for 15 minutes.

5. Measure absorbance at 412 nm.

6. Plot the values obtained for the standards to generate a standard curve. Determine the experimental sample concentrations from this curve.

   **Note:** The most accurate results are obtained from the linear portion of the standard curve; i.e., the portion yielding an r² value equal to 1.0. One or more of the high standards may exceed the linear range.
N.2. Michael-type Addition Conjugation of Antibody Fragments to pDMAEMA co-branched PEGDA/PEGMEA

1. Note molar concentration of thiols calculated using Ellman's Assay in Appendix O.1.
2. From $^1$H NMR data calculate the moles of free vinyl present per mole of pDMAEMA co-branched PEGDA/PEGMEA.
3. Degas 0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA using argon for one hour prior to experiment.
4. Using 0.1 M sodium phosphate, pH 8.0 containing 1 mM EDTA as the reaction buffer (10 x), add antibody fragment containing solution and polymer containing solution together. If possible, resuspend polymer in 0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA to maintain equilibrium of reaction buffer.
5. React at room temperature under gentle rocking for at least 30 minutes. Keep samples for analysis using SDS-PAGE and for use as complexing reagent in further experiments.

O. Rat Excisional Model

1. Anaesthetize animal using isoflurane and oxygen. In the induction phase 5% of isoflurane will be used until surgical levels of anaesthesia are reached.
2. The percentage of isoflurane is then reduced to maintain these levels for the duration of the procedure (to about 2%).
3. Shave the hair off the dorsum areas of the rats.
4. Swab the surgical area with 4% chlorohexidine or povidone iodine to control bacterial contamination of the surgical field.
5. Record wound size in the animal using tracing paper.
6. Cover the wounds with a standard bio-occlusive transparent dressing (e.g. Opsite® Smith & Nephew).
7. Place additional wound dressing/jackets on the animals with the intention of preventing wound disturbance.
8. Identify animals using tail marking with a permanent marker.
9. Closely monitor the animals until full recovery from anaesthesia is evident.
10. House the animals individually housed to avoid interference with the dressing. House the animals in transparent cages and provide standard environmental enrichment.
11. Sacrifice the animals at the required time periods using an overdose of sodium pentobarbital solution or CO$_2$ asphyxiation.
12. Trace the healed areas on sterile tracing papers and photograph prior to excision and future histomorphometry.
13. House animals appropriately and monitor regularly using the following distress scoring sheet.
# Distress Scoring Sheet

Animals reaching scores of 12 or more will be euthanised.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symptom</th>
<th>Score</th>
<th>Date/Time</th>
<th>Date/Time</th>
<th>Date/Time</th>
<th>Date/Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>Normal</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lack of grooming</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Piloerection, ocular and nasal discharge</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Piloerection, hunched up</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Above and eyes half closed</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural behaviour</td>
<td>Normal</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Minor changes</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Less mobile, but alert</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Restless or still, not alert</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydration</td>
<td>Normal</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abnormal pinch test</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory signs</td>
<td>No dressing disturbance</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mild oozing through dressing</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overt discharge from wounds</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dressings severely damaged</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dressing removed and wounds severely damaged</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Provoked behaviour</td>
<td>Normal</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Minor depression or exaggerated response</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moderate change in expected behaviour</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Very weak and precomatose</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>0-19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure O.1: Excisional wound model with four 1 cm² wounds created in the back of female Lewis rats at (a) initially after surgery and (b) day 28 after surgery. The wounds have completely closed at the end time-point.
Figure O.2: Measurements of gross wound area using tracing paper (normalized to tracings performed at day 0). Data presented is the mean ± standard deviation (n = 6) analysed by one-way ANOVA and Tukey’s post-hoc test, p < 0.05. No statistically significant difference is detected between any of the groups investigated.
14. Import images into ImageJ and trace the wound area. Calculate normalized wound contraction area index using the following formula:

\[
\text{Normalized Wound Contraction Index} = \frac{A_{w_0} - A_{w_t}}{A_{w_0}}
\]

where \(A_{w_0}\) is wound area at time of surgery (day 0) and \(A_{w_t}\) is the wound area at the end time point (28 days). Data is presented in Figure O.2.

**P. Generation of In Vitro Human Skin Equivalents**

**P.1. Isolation of Keratinocytes**

1. Collect data from the biopsy (procedure, date, patient age, gender, region extraction, dimensions, weight, location of procedure).
2. Place biopsy in 94 mm Petri dish.
3. Remove gross adipose tissue with scalpel.
4. Cut the biopsy into small 0.5 cm\(^2\) portions.
5. Rinse twice with PBS plus and a third time with PBS minus.
6. Place tissue into small petri dish.
7. Add 20 mls of dispase (or more precisely 1.25 ml/cm\(^2\)).
8. Incubate for 18 hours maximum at 4 °C (under static condition).
9. After incubation period, carefully remove epidermis which has dissociated from the underlying dermis.
10. Remove dispase solution and rinse epidermal tissue with PBS plus (retain dermis portion for fibroblast isolation).
11. Remove PBS plus, rinse with PBS minus.
12. Place epidermis in a tube, and add 5 mls of trypsin.
13. Rinse petri dish with a further 5 mls of trypsin and add to the tube.
14. Incubate for five minutes in a 37°C waterbath and vortex briefly every two minutes.
15. Stop tyrpsin activity with 1 ml of FCS.
16. Disrupt the solution by pipetting continuously for five minutes until all the tissue has been disrupted.
17. Strain solution with a 100 \(\mu\)m cell strainer.
18. Rinse the strainer three times with a 5 ml PBS rinse.
19. Centrifuge the keratinocyte suspension that has gone through the strainer for five minutes at 1000 rpm.
20. Resuspend the pellet in KGM-2 Ready media.
21. Seed 600,000 cells/ T75 flask.

**P.2. Isolation of Fibroblasts**

1. Place dermal pieces previously acquired in a fresh petri dish.
2. Wash twice with PBS minus and add to a centrifuge tube.
3. Add 40 ml of collagenase to the tissue (more precisely 0.625 ml/cm²).
4. Incubate for four hours at 37 °C.
5. Separate the remaining undigested pieces using a cell strainer and rinsing three times using PBS plus.
6. Centrifuge the cell suspension for five minutes at 1500 rpm.
7. Wash once with serum containing DMEM.
8. Centrifuge again for five minutes, this time at 1000 rpm.

**P.3. Generation of Organotypical Skin Model**

1. To generate a volume for a 500 ml Nunc cell culture insert, the collagen and the Neutralisation Solution need to be added at a ratio of 2:1. At time of experiment, place both solutions separately on ice.
2. Trypsinise fibroblasts, centrifuge, count and adjust cell suspension concentration to 1 x 10⁶ cells/ml. 50,000 cells are used per 500 ml of collagen.
3. Remove supernatant and resuspend the cells in a required volume of cooled Gel neutralisation solution, carefully without making bubbles.
4. Transfer the suspension into the collagen solution, resuspend gently but quickly.
5. Using a multi-step pipette, pipette the collagen-cell mixture using a 500 ml pipette rapidly into each cell culture insert.
6. Allow to stand for 10-20 minutes at 37 °C, to allow gelation to occur.
7. Submerge the gels with DMEM containing 10 % FCS and place in incubator.
8. One day after creating the gels remove the media and coat gels with fibronectin. Allow to coat for 30 minutes in an incubator.
9. During this time, trypsinise keratinocytes, centrifuge and resuspend in KGM-2 (5% FCS) at 1 x 10⁶ cells/ml.
10. Seed cells on the gels at 1 x 10⁵ cells/gel (100 μl).
11. After 45 minutes incubation in an incubator, submerge gels with KGM-2 (5% FCS).
12. Over the days, decrease the concentration the KGM-2 media contains (5 – 2 %) and resulting at 0 % FCS by day 6/7. Perform each media change every two days.
13. Aspirate the medium above the gel completely, while not touching the gel with the pipette tip.
Figure P.1: Isolated human keratinocytes in culture
14. Add airlift medium (KGM-2 kit ready mixed with 3.1 ml of 300 mM CaCl$_2$ solution) in the surrounding outer space (of the wells), filling the media to the meniscus of the gel medium.

15. Pay attention to the media consumption of the cells, and carry out media change every two to three days until day 12. After 12 days, these are the skin models for testing.

Q. Fixation Methods

Q.1. Formalin Fixation for Tissue

**Note:** Formalin crosslinks everything, so all endogenous RNases are destroyed. Important for further RNA extraction of FFPE-samples. Not suitable for fixing cells.

1. Wash the tissue sample in RNase-free water or PBS.
2. Fix the sample in an adequate volume of 10% Formalin in PBS for exactly 12 hours. Wash in RNase-free water.
3. Put the sample then in 70% ethanol (prepared with RNase-free water and ethanol with molecular biology grade) and store the sample at 4 °C until starting the embedding procedure.
4. Start embedding machine (starts directly in 70% ethanol), so that no additional water steps are performed.

Q.2. Fixation with 4% Paraformaldehyde in PBS: for Cells

**Note:** Paraformaldehyde crosslinking is not so strong and therefore not all endogenous RNases may be destroyed. Should be used to fix cells, with formalin they will be excessively fixed.

1. Wash the sample with PBS.
2. Fix the sample in an adequate volume of 4% paraformaldehyde in PBS.
3. Tissues can also be left in this solution for up to 48 hours.

R. Staining Methods

R.1. Haematoxylin and Eosin Staining

1. Place microtomed slides in an oven at 60 °C overnight.
2. Hydrate:
   - Place in Xylene for 10 minutes
   - Place in new Xylene for 5 minutes
   - 100% ethanol for 3 minutes
   - 95% ethanol for 3 minutes
   - 70% ethanol for 3 minutes
   - Rinse in ddH$_2$O and leave for 5 minutes
3. Stain in Hematoxylin for eight minutes.
4. Wash in running tap water for eight minutes.
5. Differentiate in 1% acid alcohol for 30 seconds.
   \textbf{Note:} prepare the 1% acid alcohol fresh and be strict with the 30 seconds time).
6. Wash slides in running H\textsubscript{2}O for five minutes.
7. Blue in 0.2 % ammonia H\textsubscript{2}O or saturated lithium carbonate for 30 seconds to one minute.
8. Wash in running tap water for five minutes.
9. Rinse in 95\% EtOH – dip ten times.
10. Counterstain in Eosin/Phloxine solution for 30 seconds – 1 minute.
11. Dehydrate:
    
    95\% ethanol for 5 minutes
    100\% ethanol for 5 minutes twice
    Xylene for 5 minutes twice.

\textbf{R.2. Russell Movat Pentachrome Stain}

\textbf{10\% alcoholic Haematoxylin:}

20 g Haematoxylin
+ 200 ml absolute ethanol
\textbf{Note:} long stirring is needed to dissolve it

\textbf{Universal iodine solution (Weigert's Iodine solution):}

4 g potassium iodide
+ 2 g iodine
200 ml ddH\textsubscript{2}O

\textbf{1\% Alcian blue solution:}

2 g Alcian blue
+ 200 ml 3\% glacial acetic acid

\textbf{Crocein scarlet-acid fuchsin:}

\textit{Croceins scarlet (Stock solution)}

0.2 g Crocein scarlet
+ 200 ml 0.5\% glacial acetic acid

\textit{Acid fuchsin (Stock Solution)}

0.2 g Acid fuchsin
+ 200 ml 0.5\% glacial acetic acid

\textit{Crocein scarlet – acid fuchsin (Working Solution)}

Mix 8 parts Crocein Scarlet “stock” with 2 parts Acid Fuchsin “stock”
Appendices

**Note:** for substances, which are hard to dissolve use a pinch of thymol.

1. Deparaffinize and hydrate (ten minutes each)
   - Xylene x 2
   - 100% ethanol x 2
   - 70% ethanol x 2
   - 50% ethanol x 2
   - ddH₂O x 2
     
     Mix components in the order shown below just prior to use to make Verhoeff’s elastic stain:
     
     50 ml 10% alcoholic haematoxylin
     50 ml absolute ethanol
     50 ml 10% ferric chloride
     50 ml universal iodine solution

3. Place slides in Verhoeff’s elastic stain for 15 minutes.
4. Rinse slides in lukewarm running tap water for five minutes, followed by distilled water.
5. Differentiate sections in 2% ferric chloride until elastic fibers are sharply defined (check using microscope) ~ one to two minutes.
6. Rinse slides in distilled water and place in 5% sodium thiosulfate for 1 minute.
7. Rinse slides in running tap water for five minutes.
8. Place slides in 3% glacial acetic acid for three minutes.
9. Place slides directly in 1% alcian blue solution for 30 minutes.
10. Rinse slides thoroughly in warm running Tap water for one minute, then rinse in distilled water.
11. Place slide in crocein scarlet-acid fuchsin for two minutes.
12. Rinse slide through three changes of distilled water.
13. Dip slides five times in 1% glacial acetic acid.
14. Place slides in two changes of 5% phosphotungstic acid for two minutes each. Check sections under microscope; stop differentiation when connective tissue is clear and before the elastic fibers are de-stained.
15. Dip slides five times in 1% glacial acetic acid.
16. Dehydrate slide through three changes of fresh absolute ethanol.
17. Place slides in alcoholic saffron solution for 30 minutes.
18. Dehydrate slides through three changes of fresh absolute ethanol.
19. Clear slides through three changes of fresh xylene or xylene Substitute.
20. Coverslip using a permanent mounting media (Entellan®).
21. View under microscope and observe the following staining:
Elastic fibers, nuclei: \textcolor{black}{black}
Collagen: \textcolor{yellow}{yellow}
Mucins: \textcolor{blue to green}{blue to green}
Muscle: \textcolor{red}{red}
Fibrinoid: \textcolor{intense red}{intense red}

R.3. \textit{Polarized Light Microscopy}
1. Deparaffinize and hydrate (ten min each)
   - Xylene for two minutes-in the hood (toxic fumes)
   - 100\% ethanol for 2 minutes
   - 70\% ethanol for 2 minutes
   - 50\% ethanol for 2 minutes
   - ddH$_2$O for 2 minutes
2. Incubate in 0.1\% Picrosirius for one hour at room temperature.
3. Rinse with deionised water.
4. Place in 0.1N HCl for two minutes.
5. Dehydrate by reversing the hydration steps.
6. Place in xylene prior mounting with DPX Mounting medium and leave to set.

A BX-Pol polarizing attachment can be applied to a Fluorescent Microscope (Olympus BX51). The attachment consists of an Analyzer (U-ANT mounted in U-TAD, U-TP530 is a tin piece of 530nm of \textgreek{gamma}) and polarizer (U-POT). The analyzer is placed in the slot of the dummy slider of U-D6RE revolving nosepiece and polarizer in the filter receptacle on the microscope frame. The images obtained are polarigraphs of the specimen rich in collagen. Under polarized light, collagen fibres glow with bright colours against black background. In general collagen type I forms thick fibres with tightly packed fibrils, on the contrary type III forms thin fibres with slackly disposed and fine fibrils. Therefore the colour displayed varies from yellow to red of collagen type I to green of collagen type III.
Figure R.1 Polarigraphs of wound edges, where collagen distribution and organization is shown. Thicker, collagen type I-like fibers, polarise to display as red and yellow fibres whereas thinner, collagen type III-like fibers, polarise to display as green fibres.
Procedure
1. Rotate the revolving nosepiece to swing in appropriate objective lens (x 40 objective).
2. While looking through the eyepieces, rotate the polarizer to the position where the field of view is the darkest.
3. Place a specimen on the stage and start observation.
4. Adjust the field iris diaphragm until circumscribes the field-of-view.
5. Capture images.

R.4. Immunohistochemical Staining of Paraffin Embedded Sections
1. Deparaffinize and hydrate (10 min each)
   - Xylene for 2 minutes in the hood (toxic fumes)
   - 100% ethanol for 2 minutes
   - 70% ethanol for 2 minutes
   - 50% ethanol for 2 minutes
   - ddH₂O for 2 minutes Pour ddH₂O in pressure cooker (less than ½).
2. Immerse slides in staining dish filled up completely with the buffer (if both buffers need to be used, do the Tris-EDTA first, cool down, wash with PBS once, and proceed with the Citrate).

Antigen Retrieval Buffers
- Tris-EDTA buffer (10 mM Tris-Base, 1 mM EDTA, .05% Tween20 pH 9.0)
  - Tris Base 1.21 g
  - EDTA 0.37 g
  - ddH₂O 1000 ml
  - Tween-20 0.5 ml
- Citrate Buffer (10mM Citric acid, .05% Tween-20, pH 6.0)
  - Citric Acid (anhydrous) 1.92 g
  - ddH₂O 1000 ml
  - Tween-20 0.5 ml
3. Cook for ~eight minutes in microwave until yellow knob pops up, then 30 more seconds.
4. Let sit for five to ten minutes outside of the cooker.
6. Put on ice for 10-15 min until cooled down.
7. Wash w/PBS 3x (1 x 5drops and aspirate).
8. Incubate with 1% TritonX-100 solution in PBS-30 min (skip the Triton step if the antibodies are against the membrane antigens, as Triton lyses the membranes).
9. Wash with PBS.
10. PapPen™ the circles around tissue (hold the slides horizontal and test the PapPen™ before using on the slide).
11. Incubate for 30 min in goat block at room T (use the serum of the species in which the secondary antibody was raised in, i.e. normally goat. However, when using goat primary antibodies, use the donkey serum for blocking).

**Goat Block (keep on ice before use)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% BSA (stabilizer)</td>
<td>0.5 g</td>
</tr>
<tr>
<td>0.1% Triton x100</td>
<td>50 µl</td>
</tr>
<tr>
<td>0.1% cold-water fish skin gelatine</td>
<td>50 µl</td>
</tr>
<tr>
<td>0.05% Tween-20</td>
<td>25 µl</td>
</tr>
<tr>
<td>Add PBS up to 50 ml</td>
<td></td>
</tr>
</tbody>
</table>

12. Take 25 ml out and add 2% goat serum (0.5 ml). Use the remaining 25 ml as primary antibody dilution buffer for step 8. Flip filter both solutions.
13. Wash with PBS.
14. Wash in 0.05% Tween-PBS for five minutes (x3) (for 150 ml PBS add 75µl Tween-20).
15. Incubate overnight at 4 ºC with primary antibodies solution. Can use both antibodies on same section if they are from different animals.
16. Dilute the primary antibodies in dilution buffer (prepared in step 11) to make a total of 300 µl for each section.
17. Use large culture dishes with wet tissue paper to place the slides in, cover, and place in fridge over night.
18. Wash in PBS or in 0.05% Tween-PBS on shaker five minutes (x3) or by suction three times. Perform all following steps in the dark.
19. Dilute secondary antibodies 1:250 (example: AlexaFluor® 488 (green) anti-rabbit and AlexaFluor® 594 (red) anti-mouse) in dilution buffer to make 300 µl for each section (one solution for all).
20. Centrifuge the antibody solutions.
21. Incubate the tissue for 30 min with secondary antibodies at room temperature.
22. Wash with PBS for five minutes (x3) and take out the mounting medium to thaw.
23. Incubate in DAPI solution for 10-15 min at room temperature in dark.
24. DAPI: 1ml stock + 4ml PBS, flip filter.
25. Wash with PBS for five minutes (x3).
26. Mount the sections using Prolong Gold Anti Fade solution (Molecular Probes; stored at −20 ºC, thaw for ~15 min upside down before use), put the slides flat at 4 ºC overnight and seal the glass cover slip edges next day with nail polish (wait 24 hours till imaging).

R.5. TUNEL Staining

1. Deparaffinise, rehydrate and wash cryosections in PBS for 30 minutes solution.
2. Incubate tissue sections for 15-30 minutes at 21-37 ºC with Proteinase K solution. (The Proteinase K solution contains 2 ml 50X Proteinase K in 98 ml PBS buffer)
3. Rinse slides with PBS two times for five minutes each time.
4. Incubate with Blocking solution for ten minutes at 15-25ºC. The Blocking solution contains 3% H₂O₂ in methanol.
5. Rinse slides with PBS two times for five minutes each time.
6. Positive control: Before beginning the labeling procedure, incubate the fixed and permeabilized cells or sections with 100 ml DNase I Solution for 10-30 minutes at 21-37°C to induce DNA strand degradation. (DNase I Solution contains 10000 U/ml-50000 U/ml DNase I (grade I) depending on the sample to be stained in DNase I buffer (the concentration of DNase I is 10000 U/ml– 20000 U/ml for cell sample, 20000U/ml – 30000U/ml for cryopreserved section, and 30000U – 50000U/ml for paraffin-embeded sections). One example of DNase I buffer is 10 mM CaCl₂, 6 mM MgCl₂, and 10 mM NaCl in 40 mM Tris-HCl, pH 7.9)
7. Rinse slides with PBS two times for five minutes each time. Then keep the area around the samples dry.
8. Add 50 ml TUNEL Reaction Mixture on samples. Add a coverslip and incubate for 60 minutes at 37°C under wet conditions, protected from light. (The TUNEL Reaction Mixture contains 45 ml Equilibration Buffer, 1 ml Biotin-11-dUTP, and 4 ml TdT, freshly prepared) Note: Add 50 mL TUNEL Reaction Mixture into the negative control. To ensure a homogeneous dispersal of TUNEL Reaction Mixture across the cell monolayer and to avoid loss to evaporation, samples should have to be covered with parafilm or coverslip during incubation.
9. Rinse slide with PBS three times for five minutes each time. Then keep the area around the samples dry.
10. Add 50 ml Streptavidin-HRP solution on samples. Incubate the slide under wet conditions, protected from light for 30 minutes at 37°C.
11. (The Streptavidin-HRP solution contains 0.5 ml Streptavidin-HRP in 99.5 ml PBS. Note: To ensure a homogeneous dispersal of Streptavidin-HRP solution across the cell monolayer and to avoid loss to evaporation, the samples should be covered with parafilm or a coverslip during incubation.
12. Rinse slide with PBS three times for five minutes each time.
13. Add 50 ml DAB - working solution and incubate slide for 30 second-5 minutes at 15-25°C. (The DAB - working solution: 2.5 ml DAB-A (brown cap) Solution in 50 ml H2O, then add 2.5 ml DAB-B (gray cap) Solution and 2.5 ml DAB-C (yellow cap) Solution, freshly prepared)

14. Rinse slide three times with PBS and counterstain using Mayer’s Haematoxylin for eight minutes. Wash in running water to remove unbound Haematoxylin. Mount under glass coverslip (such as with PBS/glycerol) and analyze with light microscope (Alternative: Samples can be counterstained prior to analysis by light microscope).

S. RNA Extraction

S.1. Extraction from Cells and Scaffolds

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

16. Transfer column to a new 1.5 ml eppendorf. Add 30 μl RNase-free water onto the column, incubate at room temperature for one minute and centrifuge for one minute at 8,000 g for 15 seconds.

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

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

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

T. Reverse Transcription of RNA to cDNA

T.1. Reverse Transcription of RNA to cDNA for Regular PCR

Preparation before Reverse Transcription

Clean the work surface and spray RNAse away. Wipe dry all the pipettes and gloves with RNAse away. Use sterile nuclease free tubes which are pre-chilled on ice. Use 1μg of RNA template and 0.5 μg of oligo dT primers and random primers. Denature the target RNA and primers by incubation at 70 °C for 5 min. Quick-chill on ice for 5 min.

Recipe for Reverse Transcription Reaction Mix

1. Begin with highest volume. Add the reverse transcriptase enzyme at the last. Prepare the reaction mix on ice and keep on ice until incubation. Follow Table T.1 for volumes.

2. After combining all components, vortex gently to mix. After mixing, place the tube with reaction mix into the reverse transcription machine and run the program as detailed in Table T.2.

3. After reaction is complete proceed with polymerase chain reaction (PCR) or store cDNA at -20 °C for future use.
Table T.1: Reaction components and quantities required for a single reverse transcriptase reaction

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

Table T.2: PCR machine steps for reverse transcriptase of RNA to cDNA

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

Table T.3: Genomic DNA elimination mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>25 ng – 5 μg</td>
</tr>
<tr>
<td>Buffer GE</td>
<td>2 μl</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>Variable</td>
</tr>
<tr>
<td>Total Volume</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

Table T.4: Reverse-transcription mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 X Buffer BC3</td>
<td>16 μl</td>
</tr>
<tr>
<td>Control P2</td>
<td>4 μl</td>
</tr>
<tr>
<td>RE3 Reverse Transcriptase Mix</td>
<td>8 μl</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>12 μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>40 μl</td>
</tr>
</tbody>
</table>
T.2. **Reverse Transcription of RNA to cDNA for RT² Profiler PCR Array**

1. Thaw reagents of the kit and briefly centrifuge for 10 – 15 seconds to bring the contents to the bottom of the tubes.
2. Prepare the genomic DNA elimination mix for each RNA sample according to Table T.3. Mix gently by pipetting up and down and then briefly centrifuge.
3. Incubate the genomic DNA elimination mix for five minutes at 42 °C, then place immediately on ice for at least one minute.
4. Prepare the reverse-transcription mix according to Table T.4.
5. Add 10 μl reverse transcription mix to each tube containing 10 μl genomic DNA elimination mix. Mix gently by pipetting up and down.
6. Incubate at 42 °C for exactly 15 minutes. Then immediately stop the reaction by incubating at 95 °C for five minutes.
7. Add 91 μl RNase-free water to each reaction. Mix by pipetting up and down several times.
8. Place the reactions on ice and proceed with the real-time PCR protocol, or alternatively store the reactions at -20 °C.

U. **Real-time PCR**

**U.1. Regular PCR**

1. Dilute cDNA template so as to obtain a final concentration of 20ng per well.
2. Make sure that the cDNA concentration must not exceed 100ng/reaction and 10% of the final volume.
3. Add the components listed in Table U.1 to prepare master mix.
4. For a triplicate reaction, add cDNA template in triplicate and also add a negative control by replacing cDNA with nuclease free water.
5. Mix well the master mix by pipetting and add to each well to obtain final volume of 25ml.
6. Add the plastic cover provided by supplier on the PCR plate.
7. Centrifuge 1 minute at 1400 rpm. Place the plate in the machine.
8. Open the step one software and enter the details to map the plate on the software.
9. Choose Sybr Green filter for each well.
10. Choose the endogenous control from the plate and enter in the software.
11. Set up the steps in accordance to the gene and melting temperature (T_m) of the primers. A general program is detailed in Table U.2.
Table U.1: Polymerase chain reaction components

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

Table U.2: Typical real time PCR program.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Ramp Rate</th>
<th>No. Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial step</td>
<td>50 °C</td>
<td>2 minutes</td>
<td>Maximal/fast mode</td>
<td>1</td>
</tr>
<tr>
<td>PCR initial activation step</td>
<td>95 °C</td>
<td>5 minutes</td>
<td>Maximal/fast mode</td>
<td>1</td>
</tr>
<tr>
<td><strong>Two step cycling</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>10 seconds</td>
<td>Maximal/fast mode</td>
<td>35</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60 °C</td>
<td>30 seconds</td>
<td>Maximal/fast mode</td>
<td></td>
</tr>
<tr>
<td><strong>Final Step</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>15 seconds</td>
<td>Maximal/fast mode</td>
<td>1</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60 °C</td>
<td>20 seconds</td>
<td>Maximal/fast mode</td>
<td></td>
</tr>
<tr>
<td>Final denaturation</td>
<td>95 °C</td>
<td>15 seconds</td>
<td>Maximal/fast mode</td>
<td></td>
</tr>
</tbody>
</table>

U.2. **Protocol for PCR using RT² Profiler PCR Array**

1. Briefly centrifuge the RT² SYBR Green Mastermix to bring the contents to the bottom of the tube.
2. Prepare the PCR components mix in a 5 ml tube or a loading reservoir depending on the RT² Profiler PCR Array format as described in Table U.4.
3. Dispense the PCR components mix into the RT² Profiler PCR Array depending on the RT² Profiler format.
4. Carefully remove the RT² Profiler PCR Array from its sealed bag.
5. Add PCR components mix to each well of using an 8-channel pipettor and the 384EZLOAD covers provided.
6. Place 384Load cover 1 (white) on the plate. Add 10 μl PCR components mix for sample 1 to the open wells. Remove and discard cover. Repeat with cover 2 (yellow), 3 (black) and 4 (red) on the plate.
7. Carefully, tightly seal the RT² profiler PCR Array with optical adhesive film.
8. Centrifuge for one minute at 1000 g at room temperature to remove any bubbles. Visually inspect the plate from underneath to ensure no bubbles are present in the wells.

9. Place the RT² Profiler PCR Array on ice while setting up the PCR cycling program.

10. Proceed in real-time cycler as normal.

Table U.3: PCR components mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 x RT² SYBR Green Mastermix</td>
<td>650 µl</td>
</tr>
<tr>
<td>cDNA synthesis reaction</td>
<td>102 µl</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>548 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>1300 µl</td>
</tr>
</tbody>
</table>

Table U.4: Cycling conditions for the Roche LightCycler 480®

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Duration</th>
<th>Temperature</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 minutes</td>
<td>95 °C</td>
<td>HotStart DNA Taq Polymerase is activated by this heating step</td>
</tr>
<tr>
<td>45</td>
<td>15 s</td>
<td>95 °C</td>
<td>Perform fluorescence data collection</td>
</tr>
<tr>
<td></td>
<td>1 minute</td>
<td>60 °C</td>
<td></td>
</tr>
</tbody>
</table>
Table U.5: List of genes investigated in rat wound healing PCR array

<table>
<thead>
<tr>
<th>Extracellular Matrix &amp; Cell Adhesion:</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECM Components: Col14a1, Col1a1, Col1a2, Col3a1, Col4a1, Col4a3, Col5a1, Col5a2, Col5a3, Vtn.</td>
</tr>
<tr>
<td>Remodeling: CTGS, CTSK, Ctsl1, F13a1, F3 (Tissue Factor), Fga (Fibrinogen), MMP-1a,</td>
</tr>
<tr>
<td>Enzymes: MMP-2, MMP-7, MMP-9, Plat (tPA), Plau (uPA), Plaur (uPAR), Plg, Serpine1 (PAI-1), TIMP-1.</td>
</tr>
<tr>
<td>Cellular: Cdh1 (E-cadherin), Itga1, Itga2, Itga3, Itga4, Itga5, Itga6, Itgav, Itgb1, Itgb3,</td>
</tr>
<tr>
<td>Cytoskeleton: Acta2 (a-SMA), Actc1, Rac1, Rhoa, Tagln.</td>
</tr>
</tbody>
</table>

Inflammatory Cytokines & Chemokines:
CCL-12, CCL-7 (MCP-3), CD-40LG (TNFSF5), CXCL-1, CXCL-11 (I-TAC/IP-9), CXCL-3, CXCL-5, IFNG, IL-10, IL-1B, IL-2, IL-4, IL-6.

Growth Factors
ANGPT1, CSF2 (GM-CSF), CSF3 (GCSF), CTGF, EGF, FGF10, FGF2, FGF7, HBEGF (DTR), HGF, IGF-1, MIF, PDGFA, TGFA, TGF-B1, TNF, VEGFA.

Signal Transduction:
TGFß: TGF-B1, TGF-BR3, STAT-3
WNT: CTNNB-1, WISP-1, WNT-5A.
Phosphorylation: MAPK-1 (ERK2), MAPK-3 (ERK1), PTEN.
Receptors: EGFR, IL-6ST (GP130).
Other: PTGS-2.

Housekeeping
B2M, HPRT-1, RPL13A, GAPDH, ACTB
V. **Fluorescent Labelling of siRNA with Cy™3**

1. Resuspend experimental RNA (maximum weight of 80 μg) to 20 μM in Nuclease-free Water.
2. Add 100 μl of Reconstitution Solution to the dry Cy™3 Labelling Regent. Perform all manipulations in the dark as the Cy™3 dye is light sensitive.
3. In a sterile, nuclease-free tube, assemble the reagents in the order shown in Table V.1, making sure to add the labelling Reagent last. Mix well by vortexing.
4. Incubate the reaction mix at a constant temperature of 37 °C for one hour in the dark.
5. Remove from incubation and to the tube add 5 μl of 5 M NaCl and 125 μL of ice cold 100 % ethanol.
6. Mix well and place the mixture at -20 °C (or colder) for 30-60 minutes. The labelling RNA precipitates during this incubation.
7. Pellet the labelled RNA by centrifugation at ≥8,000 g for 15-20 minutes. Carefully remove the supernatant; avoid disrupting the pellet. A red pellet siRNA pellet should be visible.
8. Gently add 175 μL of 70 % ethanol making sure not to disrupt the pellet, and centrifuge at ≥8,000 g for five minutes (use the highest speed compatible with your tubes). Carefully remove supernatant with a pipette. To remove the last traces of solution, respin the tube briefly and discard the supernatant.

### Table V.1: Reaction setup for labelling 5 μg 21-mer duplex siRNA.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free Water</td>
<td>18.3</td>
</tr>
<tr>
<td>10 X Labelling Buffer</td>
<td>5.0</td>
</tr>
<tr>
<td>21-mer duplex siRNA at 20 μM (~5μg/μl)</td>
<td>19.2</td>
</tr>
<tr>
<td>Cy™3 Labelling Reagent</td>
<td>7.5</td>
</tr>
<tr>
<td>Total Volume</td>
<td>50</td>
</tr>
</tbody>
</table>

9. Dry the RNA for five to ten minutes at room temperature. Do not dry the pellet for longer than five to ten minutes or it will be difficult to solubilise. Resuspend the RNA pellet in Nuclease-free Water or in the buffer of your choice. If desired, resuspend the labelled RNA in the same volume it was in before the labelling reaction. Remove an aliquot (1 μg) for analysis via electrophoresis (Figure V.1).
Figure V.1: Agarose gel electrophoresis of (a) unlabelled and (b) Cy™3 labelled siRNA. The migration of (b) is slower due to an increased molecular weight due to labelling with Cy™3. However, labelling may not be homogenous due to the observation of a very thick, weaker band at (b).
Appendices

W. Induction of Myocardial Infarction

1. Complete recovery of the anaesthesia after surgery is beneficial for circulation. Anesthetize the mice by an intraperitoneal injection of a solution of midazolame (5 mg/kg body weight), medetomidine (0.5 mg/kg body weight) and fentanyl (0.05 mg/kg body weight). Anesthesia is maintained by inhalation of isoflurane (0.7 – 1.5 Vol. % in O₂). For protecting the cornea, apply eye ointment.

2. Shave the thoracic wall of the narcotized mouse on the left side. Treat this area as well as the chin with depilatory cream. Clean and disinfect these areas with povidone-iodine.

3. Afterwards fix the mouse in a supine position with extremities taped on the operating table. For subsequent tracheotomy hyperextension of the neck position a cannula sheath under the neck. The right side of the mouse must be marginally uplifted.

4. Maintain the body temperature at 37 ± 1 °C using a controllable heating pad and a thermometer with a rectal probe.

5. Make a midline cervical skin incision by lifting the skin with straight forceps and cutting with the microscissor. Separate the salivary glands atraumatically. After gentle separation of the muscles (M. sternohyoideus, M. sternothyroideus) overlying the trachea fix aside with sutures (Prolene®, 6-0) and clamps. Now the trachea is visible. Make a cut between the fourth and fifth tracheal cartilage and attach a suture (Prolene®, 7-0) on the posterior tracheal cartilage. Fix a bull-dog clamp at the suture. Thus the suture can be used to facilitate the intubation. Subsequently, connect the tracheotomy cannula to a rodent ventilator via a tube. Artificial ventilation (120 strokes/min, 180 μl volume, 0.8 L/min oxygen flow) is recommended throughout the procedure.

6. To perform a thoracotomy at the left side the mouse, relocate the mouse on its right side. Incise the skin and dissect the subcutaneous tissue free. Transect the ventral serrated muscle of thorax and the intercostal muscles. Incise the thorax in the third intercostals space. Widen the opening with a rip retractor.

7. Displace the lung with the help of Ethiket® (Ethicon) to visualize the left auricle at whose tip the left anterior descending artery takes course toward the apex of the heart. Before occlusion, carefully open the pericardium. There are two anatomical patterns how the left anterior descending artery (LAD) takes course: first, there is a major singular LAD. Second, there exists a major bifurcation of the artery, which is close to the left auricle. Branches of the mouse LAD penetrate the myocardium close to their origin. Use Silk 6-0 to occlude the LAD just proximal to the site where the artery splits into two smaller branches or alternatively at the level of the tip of the auricle.

8. To later allow the reestablishment of blood flow, occlude the LAD by placing a 3 mm long piece of polyethylene tubing (Portex®, Smiths Medical, 0.28 mm ID, 0.61 mm OD) on the
Figure W.1: Tracheotomy and intubation. (A) A midline cervical skin incision is made and the salivary glands as well as the muscles overlying the trachea are gently separated. (B, C) The muscles are fixed aside with sutures and clamps. Thereby the trachea is visible. (D, E) A cut is made between the fourth and fifth tracheal cartilage. (E, F) Intubation is facilitated by attaching a suture on the posterior tracheal cartilage on which a small bulldog clamp is fixed.
Figure W.2: Thoracotomy. (A) The mouse is relocated on its right side and the skin is incised. (B, C) The subcutaneous tissue is dissected free. (D) The ventral serrated muscle of thorax is transected. (E) The intercostal muscles are incised in the third intercostal space. (F) The opening is widened with a rip retractor.
Figure W.3: Occlusion of the left descending artery (LAD). (A) By widening the intercostal space the left side of the heart and the lung become apparent. (B) After opening the pericardium the left auricle is visible (triangle) at whose tip the LAD (arrow) takes course towards the apex. (C, D) The LAD is occluded at the level of the tip of the left auricle. (E) A loose snare is formed. (F) Furthermore, a piece of polyethylene tubing is placed on the artery to later allow the reestablishment of blood flow.
artery and fix it in place with a ligature. Contract the knot by forming a loose snare. Discoloration of the left ventricle wall indicates interruption of the coronary flow and verifies the successful myocardial infarction.

9. Retain occlusion of the LAD must for a well defined period to achieve presentable infarct sizes.

10. Maintain ischemia 30 minutes. After this period, terminate the ischemia by removing the polyethylene tubing and opening the knot. Leave the suture in the myocardial tissue for the Evans Blue staining, which is performed before removing the heart. Confirm reperfusion visual inspection. Remove the rip retractor and deposit the silk 6-0 suture thread outside the thorax. Close the intercostal space and the pleura with interrupted suture by using Vicryl® 6-0.

11. For extubation, relocate the mouse in the supine position. As soon as the mouse breaths autonomously take the tracheotomy cannula off from the trachea. Close the latter with a single Vicryl® 8-0 knot. Remove the holding sutures and close the skin is closed with Prolene® 6-0 in interrupted sutures. Support the circulation of the mouse with Ringer’s solution and 5 % glucose solution by a subcutaneous injection. To antagonize the anaesthesia, a mixture consisting of atipamezole (2.5 mg/kg body weight) and flumazenil (0.5 mg/kg body weight) is administered subcutaneously. Allow the mouse to recover from the surgery under a heating lamp. Repeat the injection of buprenorphine in an interval of eight hours for three days. At the end of the reperfusion period, anesthetize the mouse again. Carry out the intubation as already described. Raise the isoflurane supply to 2 % volume.

12. Cut open the abdomen at the place where the xiphoid process of sternum can be found. Following this cut the diaphragm. Open up the thorax by cutting the rips on the right side of the sternum, shifting it to the left and fixating it with a clamp. Locate the silk suture and retie the left anterior descending artery ligature. Inject 0.5 ml of 5 % Evans Blue dye into the left ventricular cavity. The circulating dye is uniformly distributed throughout with the exception of the heart region supplied by the occlude coronary artery. This domain illustrates the area at risk, which is affected by the ischemia. Sacrifice the mouse by an intracoronary injection of 0.3 ml potassium chloride solution (1 M). Quickly remove the heart and clean with a sodium chloride solution. Embedding in Tissue Tec® it and freeze at -20 °C. Section the frozen heart transversely into five slides of approximately 1 – 2 mm breadth. Clean each section with sodium chloride solution and incubate in 1 % TTC for ten minutes.

13. After TTC-staining, viable myocardium stains red and the infarct region appears pale white. Further fix the slides in 4 % formaldehyde for two hours at room temperature. Thereafter, photograph the tissue sections on both sides using a digital camera mounted atop the stereo microscope. The infarct sizes can be determined by quantitative morphometric planimetry using an image analysis software program.
X. One-day microRNA ISH Protocol (using Mercury LNA microRNA ISH Optimization Kit (Formalin Fixed Paraffin Embedded Sections))

**Antibody Blocking Solution:** PBS, 0.1% Tween, 2 % Sheep serum, 1 % BSA

**Antibody Diluent:** PBS, 0.05 % Tween, 1% Sheep serum, 1% BSA,

**Levamisole (for blocking endogenous AP activity):** Prepare a 100 mM stock

**Proteinase-K Buffer:**

900 ml RNase-free water  
5 ml 1M Tris-HCL (pH 7.4)  
2 ml 0.5 M EDTA  
0.2 mL 5M NaCl  
Adjust volume to 1000 ml and autoclave

**PBS-T (0.1%), pH 7.4:** Add 1 mL of Tween-20 to 1 L of PBS and autoclave.

**KTBT (AP stop solution):** To 900 ml RNase-free water add:

7.9g Tris- HCl (50 mM)  
8.7 g NaCl (150mM)  
0.75 g KCl (10mM)  
Adjust volume to 1000 ml. Do not adjust pH and autoclave.

**Proteinase-K Reagent**

Prepare immediately before use. For a Proteinase-K concentration of 15 μg/ml: Add 7.5 μl Proteinase K stock to 10 ml Proteinase K buffer.

**Hybridization Mix (for microRNA ISH buffer and LNA™ Detection probes)**

Dilute the 2x microRNA ISH buffer 1:1 with RNase-free water, e.g. mix 1 ml 2 x microRNA ISH buffer with 1 ml RNase-free water to give 2 ml 1 x buffer.

For each probe to be used in the experiment, place the appropriate amount of LNA™ probe in a 2 ml non-stick RNase-free tube.

Denature the probes at 90 °C for four minutes.

Place the tubes in a table-top microcentrifuge and spin down shortly.

Immediately add the 2 mL 1 x microRNA ISH buffer to each of the tubes with the different LNA™ probes.
**Antibody Blocking Solution**

To make 10 mL blocking and 10 mL diluting solution start with 15 mL PBS-T and add 300 μl sheep serum. Label the tube as ‘blocking solution’.

Remove 5 ml from the tube in step 1, place in a new tube and label diluting solution. To the tube labelled blocking solution, add 330 μl 30 % BSA to give a final concentration of 1 %. The ‘blocking solution’ is now ready to use.

To the tube labelled diluting solution, add 5 mL PBS (to give 0.05 % Tween and 1 % sheep serum final concentration) and 330 μl 30 % BSA to give 1 % final concentration. The diluent solution is now ready for use.

**Anti-Digoxigenin (DIG) reagent:** Dilute the sheep- anti-DIP-AP antibody 1:800 in antibody diluting solution.

**Alkaline Phosphatase (AP) Substrate:** Immediately prior to use, dissolve a nitro-blue tetrazolium and 5-bromo-4-chloro-3’-indolyphosphate (NBT-BCIP) tablet in Milli-Q™ water according to the manufacturer’s instructions. Add levamisol to a final concentration of 0.2 mM.

1. Prior to deparaffinization place slides in 60 °C oven overnight to remove paraffin.
2. Deparaffinize slides in xylene and ethanol solutions at room temperature (RT) by placing slides with sections in slide rack.
3. Deparaffinization:
   - Xylene: 10 minutes
   - Xylene: 10 minutes
   - EtOH (99.9%): 5 minutes
   - EtOH (99.9%): 5 minutes
   - EtOH (95%): 5 minutes
   - EtOH (95%): 5 minutes
   - EtOH (70%): 5 minutes
   - EtOH (70%): 5 minutes
   - PBS: 5 minutes
4. Incubate slides with Proteinase-K at 37 °C for ten minutes.
5. Wash with PBS
6. Dehydrate slides:
   - PBS: 5 minutes
   - EtOH (70%): 2 minutes
   - EtOH (70%): 2 minutes
   - EtOH (95%): 2 minutes
EtOH (95%) 2 minutes
EtOH (99.9%) 2 minutes
EtOH (99.9%) 2 minutes

7. Air-dry the slides on clean paper towels afterwards for 15 minutes.

8. Apply slides on a flat surface and apply 25 μl of the hybridization mix. For initial protocol optimization, probe concentrations could be a) 1nM LNA™ U6 snRNA probe b) 40 nM double –DIG LNA™ microRNA probe. The probe concentration will need to be optimized for optimal microRNA ISH signal. Avoid touching the tissue sections with the pipette tip. Then apply a sterile cover glass onto each section, carefully avoiding air bubbles, and seal along all four edges with Fixogum™ (rubber cement). Place the slides in the hybridizer and start a program hybridizing for one hour. Hybridization temperature must be optimized for individual probes.

9. Remove Fixogum™ using tweezers. Avoid sliding the coverslip, which may damage the tissue. Then, carefully detach cover glass and place the slides into a slide rack placed within a glass jar containing 5 x sodium saline citrate (SSC) at RT.

10. Wash slides in glass jars according to the following list. To ensure sufficient stringency perform the washes in glass jars placed in a water bath set to the hybridization temperature:
   - 5 x SSC 5 minutes at Hybridization Temperature
   - 1 x SSC 5 minutes at Hybridization Temperature
   - 1 x SSC 5 minutes at Hybridization Temperature
   - 0.2 x SSC 5 minutes at Hybridization Temperature
   - 0.2 x SSC 5 minutes at Hybridization Temperature
   - 0.2 x SSC 5 minutes at Room Temperature

11. Transfer the slides to glass jars with PBS. Apply a hydrophobic barrier around the tissue sections using a PapPen® following the manufacturer’s instructions. Tissue sections are not allowed to dry out during this and the subsequent immunohistochemistry steps.

12. Place the slides in a humidifying chamber and incubate with blocking solution for 15 minutes at room temperature. NB. Perform all steps after this in the humidifying chamber.

13. Remove the blocking solution and apply anti-DIG reagent (sheep anti-DIG-AP at 1:800 in antibody diluting) and incubate for 60 minutes at room temperature.

14. Wash the slides 3 x 3 minutes with PBS-T.

15. Apply freshly prepared AP substrate to the sections and incubate the slides for 2 hours at 30 °C in the humidifying chamber. Protect from light during development.

16. Incubate the slides in KTBT buffer (2 x 5 minutes) to stop the reaction.

17. Wash with water (2 x 1 minute).

18. Apply 200 μl Nuclear Fast Red™ (nuclear counter stain) for one minute for nuclear counter staining.
19. Remove slides from the humidifying chamber to a slide rack placed within a glass jar containing tap water. Carefully rinse the slides with running tap water for approximately ten minutes.

20. Dehydrate the slides in ethanol according to the following:
   - EtOH (70%) 2 minutes
   - EtOH (70%) 2 minutes
   - EtOH (95%) 2 minutes
   - EtOH (95%) 2 minutes
   - EtOH (99.9%) 2 minutes
   - EtOH (99.9%) 2 minutes

21. Mount the slides directly with one to two drops of mounting media. Avoid air-drying sections at this step.

22. Allow to precipitate to settle overnight and analyze by light microscopy the subsequent day.

Y. Journal Publications and Conference Proceedings

Y.1. Journal Publications


Y.2. Conference Podium Presentations


5. Kelly J.L, Doody J, Zeugolis D, **Monaghan M**, Kelly G, Devocelle M, Kilcoyne M, Joshi L, Pandit A. Collagen fibers modified with polysialic mimetic peptide are a suitable material for use as a synthetic peripheral nerve graft. *Podium Presentation at the Irish Association of Plastic Surgery Meeting, Dublin, Ireland, 26th November 2010*


10. Mathew A, **Monaghan M**, Wang W, Pandit A. A linear-dendritic hybrid poly (2- (dimethylamino) ethyl methacrylate) as a highly effective gene delivery vector, *Podium Presentation at the European Society of Biomaterials Conference, Tampere, Finland, 11th-15th September 2010*

11. Réthoré G, **Monaghan M**, Dash B C, Pandit A. Chitosan-polyglutamic acid biodegradable nanospheres show a blood compatibility response that is dictated by size and surface charge, *Podium presentation at the 2nd World Congress of the Tissue Engineering and Regenerative Medicine International Society, Seoul, Republic of Korea, 2009*

**Y.3. Conference Poster Presentations**


2. Browne S, **Monaghan M**, Pandit A. A spatiotemporal biomaterial gene delivery system to modulate inflammation and Angiogenesis, *Poster Presentation at the Keystone Symposium on Cardiac Remodeling, Signaling, Matrix and Heart Function, Utah, USA. 7th – 12th April 2013*


7. **Monaghan M**, Pandit A. A 3D co-culture vascular model to quantify remodeling after injury with copolymers of poly (DL-Lactide-co-glycolic-acid) and poly (Lactic-co-glycolic- acid), *Poster presentation at the European Society for Biomaterials Conference, Lusanne, Switzerland, 8th-11th September 2009*


Y.4. Awards

1. Deutscher Akademischer Austausch Dienst, Short Term Travel Award to conduct research in University Hospital Tübingen, 2012
2. European Molecular Biology Organisation (EMBO) Short Term Travel Award to conduct research in Fraunhofer IGB, Stuttgart. 2011
3. Roche Best Poster Award, National Centre for Biomedical Engineering Science (NCBES) Research day, 2008
4. NUI Galway College of Engineering and Informatics Research Fellow Award, 2008s