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Molecular Beacons for Tissue Engineering Applications

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

By

Jennifer Alexander, M.Sc.

September 2012

National Centre for Biomedical Engineering Science, National University of Ireland, Galway

Head of Department: Professor Frank Barry
Research Supervisors: Dr. Yury Rochev and Prof. Abhay Pandit
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<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>3'-UTR</td>
<td>Three prime untranslated region</td>
</tr>
<tr>
<td>4S-PEG</td>
<td>Pentaerythritol poly(ethylene glycol) ether tetrasuccinimidyl glutarate</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>BHQ</td>
<td>Black hole quencher</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CdTe</td>
<td>Cadmium telluride</td>
</tr>
<tr>
<td>CO2</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CPP</td>
<td>Cell penetrating peptide</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>Cy</td>
<td>Cyanine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBM</td>
<td>Endothelial basal medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FAM</td>
<td>6-carboxyfluorescein</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>FISH</td>
<td>Fluorescent in situ hybridization</td>
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<td>Fluorescent resonance energy transfer</td>
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<td>Mesenchymal stem cell</td>
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<td>Poly(ε-caprolactone)</td>
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<td>pDNA</td>
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<td>Poly(glycolic acid)</td>
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<td>poly(D,L-lactic co-glycolic acid)</td>
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<td>PLLA</td>
<td>Poly(l-lactic acid)</td>
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<td>Quantum dots</td>
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<td>S/B</td>
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<td>SDS</td>
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<td>siRNA</td>
<td>Small interfering ribonucleic acids</td>
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<td>Transactivator of transcription</td>
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<td>Tris(2-carboxyethyl)phosphine</td>
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</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
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ABSTRACT
A complete spatiotemporal profile of the mechanisms involved in the synthesis, processing, and transport of RNA will improve our understanding of cell function and behaviour under various biological cues, which could potentially advance the field of biomaterials and tissue engineering towards designing more functional biomaterial scaffolds. Molecular beacons (MBs) have been extensively used in biomedical research and have the ability to provide spatiotemporal pattern of specific mRNA expression in live cells that can be visualized in real-time at the single cell level.

The aim of this research was to develop a detection system that can be used to monitor gene expression in living cells associated with 3D scaffolds.

The first stage of the research was dedicated to the design of molecular beacons targeting therapeutic genes of interest in cardiovascular tissue engineering. FAM-labelled DNA molecular beacons targeting human eNOS and IL-10 mRNAs were designed that were able to detect their nucleic acids targets with high specificity and sensitivity as well as access their intracellular mRNA targets in living cells emitting fluorescence signals that were stable for 2-3 h.

The next stage was to develop a molecular beacon detection platform for monitoring changes in transcription gene expression levels in living cells embedded into a 3D collagen scaffolding system. For the first time the use of TAT-peptide conjugated MB to monitor mRNA in a 3D in vitro system was reported. It was shown that TAT-peptide linked molecular beacons can monitor GAPDH mRNA expression in 3D type I collagen scaffold of 1 mm thickness and that delivery can be completed with fast kinetics (~1h). Also, spatial distribution of cells in 3D can be visualized by optically sectioning the scaffolds. Although MB technology can be used to detect mRNA abundance in cells in 3D, it may be necessary to perform quantification with another complementary technique such as RT-PCR, unless next generation beacons can be stand alone.

In the final stage of the research spatiotemporal efficacy of a dual-gene therapy model for cardiovascular tissue engineering based on a 3D collagen scaffold was assessed. It was found that IL-10 and eNOS MBs were able to detect changes in mRNA expression and demonstrated that the model supported both sustained and delayed release of functional genes, which was confirmed by RT-PCR results.
CHAPTER 1: MONITORING TRANSCRIPTIONAL GENE EXPRESSION IN 3D IN VITRO MODELS
1.1 Introduction

Tissue engineering (TE) aims to provide scaffolds to support, regenerate or replace lost function in tissues or organs by manipulation of biological cues [1-7]. Monitoring of gene expression is fundamental to tissue engineering as it can give insight into efficacy of the scaffold, the interaction of cells with the supporting scaffold environment, as well as the efficiency of gene therapy approaches [8-10].

Spatiotemporal monitoring of gene expression has facilitated the understanding of the changes that occur in human diseases, from the regulatory mechanisms involved, to the phenotypic relationship associated with gene expression. Both the messenger RNA (mRNA) and the functional protein products can be measured. Measurements at the mRNA levels are dominant as, generally, the techniques are more sensitive, simpler, and have a higher throughput than protein-based ones [11, 12]. Further, RNAs have many important functions in living cells from physically conveying and interpreting genetic information, providing structural support for molecular machines, catalyzing chemical reactions, to regulation of gene expression [13]. These functions are controlled through the expression levels and the spatiotemporal distribution of the specific RNA in the cell. Furthermore, mRNA expression levels are highly dynamic and indicate cellular responses to environmental stimuli which affect both the cell morphology and phenotype, and, for most genes, alterations in mRNA levels likewise coincide with protein levels [11, 14, 15]. Several techniques exist to measure gene expression at the molecular level and have been applied to 3D in vitro matrices. Techniques such as reverse transcription polymerase chain reaction (RT-PCR), DNA microarray, fluorescent in situ hybridization (FISH) and northern blot are used in the biomaterials and tissue engineering fields to quantify gene expressions of cells at the mRNA level in the 3D environments. The aim of this review is to examine the conventional methods used to monitor gene transcription levels in 3D in vitro models, evaluate the strengths and limitations of these methods and share insights into live cell imaging of gene transcription in the 3D matrix using molecular beacons. After summarizing the 3D in vitro model systems, techniques for quantification of mRNA will be discussed, followed by key parameters that effect gene transcription levels. Finally, critical insights into the
application of molecular beacons for spatiotemporal gene expression in 3D \textit{in vitro} models will be assessed.

1.1.1 The 3D \textit{in vitro} model

Generally, the 3D \textit{in vitro} model employs biomaterial scaffold, cells, and growth-stimulating factors \cite{7}. Scaffolds are typically constructed from synthetic polymers (e.g. poly(glycolic acid)(PGA), poly(l-lactic acid) (PLLA), poly(D,L-lactic co-glycolic acid) (PLGA) copolymers, poly( ε-caprolactone) (PCL) and ethylene glycol-based copolymers,\cite{2} natural polymers (e.g., collagen, gelatin and fibrin), inorganic materials (e.g. hydroxyapatite, tricalcium phosphate and titanium) and composites (e.g., PCL/PLGA/ collagen composite). The role of these scaffolding materials is to mimic, even partially, the extracellular matrix (ECM) \cite{16, 17}.

Cell may be associated with these scaffolds to form a 3D construct using two approaches: A cell-based approach in which cells are seeded \textit{in vitro} onto the scaffold which serves as a delivery vehicle for biomolecules \cite{18} or a tissue-like approach in which cells are seeded into the scaffold and allowed to make their own matrix \cite{7}.

Collagen is extensively utilized in TE approaches because of its biocompatibility, low immunogenicity and ability to initiate signalling pathways (via the cell surface receptors, integrins, discoidin domain receptors and glycoprotein VI). Of the collagens, collagen Type I is the the most widely used.

1.2 Quantification of mRNA

Messenger RNA ranges from 1-100 copies per cell/gene \cite{11, 19} and quantification is routinely performed using reverse transcription-polymerase chain reaction (RT-PCR). Microarrays quantify the expression profiles for thousands of genes simultaneously, while fluorescent \textit{in situ} hybridization (FISH) provides spatiotemporal localization patterns of the mRNA expression levels in the single cell, and northern blot probes information about the size and integrity of quantified mRNA. The basic process and duration of these techniques are summarized in Figure 1.
1.2.1 Reverse transcription polymerase chain reaction (RT-PCR)

PCR is a molecular technique invented by Kary B. Mullis in the 1980s. This breakthrough technique allows the exponential amplification of DNA. Further development of the PCR technology incorporated fluorescent dyes (e.g. SYBR Green, TaqMan probes) and led to the real-time PCR method in which the accumulated PCR products are detected, as the name implies, in real-time. Ultimately, RNA was enzymatically converted to complementary DNA (cDNA) for amplification using real-time PCR and this created the real-time reverse transcription polymerase chain reaction (RT-PCR) method.

RT-PCR is routinely used to quantify gene expression of a cell population from extraction of purified RNA obtained from cell lysates. Typically, 0.1-1µg of purified RNA is sufficient to produce cDNA required for the PCR, which is about 5 x 10^3-5 x 10^4 cells considering an average of 20 pg of RNA per mammalian cell. RT-PCR is a highly sensitive, specific and reproducible method that can be used to detect and compare RNA levels within a dynamic range of greater than eight orders of magnitude [20-25]. Furthermore, RT-PCR is particularly useful in samples presenting low RNA concentrations, low transcript copy number or small changes in mRNA expression levels [22, 23, 26-32]. However, purification, cDNA synthesis, and other processing techniques performed before PCR analysis can result in loss of RNA transcripts [33, 34]. Also, scaffold material may present some challenges during RNA isolation from 3D constructs. Noteworthy, polysaccharides (agarose, alginate, hyaluronate, chitosan, and dextran) and proteoglycans contaminate the lysate and reduce solubility of the isolate which may result in low-quality RNA [35, 36]. To address this, a high salt precipitation of the contaminants is indicated as per manufacturer’s protocol when using organic (guanidine isothiocyanate-phenol-based) extraction methods (e.g. TRIzol, TRI Reagent). Also, a combined method in which extracted RNA is followed by spin-column purification step use for plant RNA material may gave high-quality RNA yields [35]. Others have shown that a cationic surfactant, cetyltrimethylammonium bromide buffer may improve purity and yields compared to organic extractions [36]. In addition, constructs with high collagen content may require treatment with collagenase before RNA extraction [37].
Typically, RT-PCR is performed on RNA extracted from 3D constructs or from cells harvested from constructs. When extracted directly from 3D constructs, pre-treatment with EDTA–based solutions [38], pulverization [39, 40], or freezing in liquid nitrogen [41-43] may be required to disrupt cells. Alternatively, the cells may be first harvested using trypsin [44, 45]. RNA isolation may be performed using filter-based, spin-column kits [41, 45-62], organic extraction [9, 38, 39, 42, 63-76], or a combination of both methods [64, 77, 78].

RT-PCR has been used to establish the rationale for the design of the biomaterial scaffold by evaluating the cellular responses associated with the 3D construct. That is, to elucidate the cells ability to proliferate, differentiate and commit to specific tissues. Thus, cell/tissue specific genes are investigated. Tissue specific genes have been studied in a variety of cells in 3D constructs employing natural [38, 55, 79-81], synthetic [39, 82], inorganic [42] and composite [40, 59] biomaterials.

RT-PCR has also been employed to study transcript gene expression levels when developing physiologically relevant 3D in vitro model systems for tumorigenesis [66] and mammary tissue [44].

Currently, RT-PCR method has expression profiling capability for up to 384 genes using Taqman® low density arrays (TLDA). TLDA are customized microfluidic cards preloaded with gene specific primers and probes [83]. The sensitivity and reproducibility of this system is comparable to conventional RT-PCR, but with added advantages of allowing multiple genes to be studies on single samples, reducing sample and reagent volumes, and reducing costs [84]. Some studies have used TLDA to evaluate specific cell response to biomaterial scaffolds [85].

The RT-PCR product may be quantitatively analysed using gel electrophoresis followed by densitometric image analysis [51, 61, 86, 87] or electrophoretic analysis using Agilent 2100 [63]. However, this end-point method has lower specificity, reproducibility and sensitivity as well as a narrow dynamic range compared to RT-PCR [23, 88].

Some genes of interest investigated from 3D scaffolds using RT-PCR are presented in Tables 1-4.
Table 1.1 Genes related to extracellular-matrix

<table>
<thead>
<tr>
<th>Gene name</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggrecan</td>
<td>[38, 39, 53, 56, 63, 70, 72, 73, 76, 81]</td>
</tr>
<tr>
<td>Biglycan</td>
<td>[51]</td>
</tr>
<tr>
<td>Bone sialoprotein</td>
<td>[40, 42, 45, 50, 64, 68]</td>
</tr>
<tr>
<td>Cartilage oligomeric matrix protein</td>
<td>[39, 75]</td>
</tr>
<tr>
<td>Collagen Type I</td>
<td>[38-40, 42, 45, 46, 49-51, 53, 56, 60, 64, 65, 69, 72, 73, 75, 76, 81]</td>
</tr>
<tr>
<td>Collagen Type II</td>
<td>[38, 39, 53, 56, 63, 65, 70, 72, 75, 76, 81]</td>
</tr>
<tr>
<td>Collagen Type III</td>
<td>[75]</td>
</tr>
<tr>
<td>Collagen Type VI</td>
<td>[75]</td>
</tr>
<tr>
<td>Collagen Type IX</td>
<td>[63, 81]</td>
</tr>
<tr>
<td>Collagen Type X</td>
<td>[76, 81]</td>
</tr>
<tr>
<td>Collagen Type XI</td>
<td>[63]</td>
</tr>
<tr>
<td>Decorin</td>
<td>[51]</td>
</tr>
<tr>
<td>Matrix metalloproteinase 1</td>
<td>[76]</td>
</tr>
<tr>
<td>Matrix metalloproteinase 2</td>
<td>[78]</td>
</tr>
<tr>
<td>Matrix metalloproteinase 3</td>
<td>[39]</td>
</tr>
<tr>
<td>Matrix metalloproteinase 13</td>
<td>[39, 76]</td>
</tr>
<tr>
<td>Osteonectin</td>
<td>[42, 49, 69]</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>[42, 45, 49, 50, 68]</td>
</tr>
<tr>
<td>Proteoglycan 4</td>
<td>[39]</td>
</tr>
<tr>
<td>Tenascin C</td>
<td>[51]</td>
</tr>
<tr>
<td>Tissue inhibitor of metalloproteinase-1 or 3</td>
<td>[39]</td>
</tr>
<tr>
<td>Versican</td>
<td>[72]</td>
</tr>
</tbody>
</table>
**Table 1.2 Growth factors and receptor genes**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone morphogenetic protein 2</td>
<td>[42, 46, 68]</td>
</tr>
<tr>
<td>Bone morphogenetic protein 4</td>
<td>[68]</td>
</tr>
<tr>
<td>Calgizzarin</td>
<td>[54]</td>
</tr>
<tr>
<td>Elongation factor 2</td>
<td>[54]</td>
</tr>
<tr>
<td>Growth and differentiation factor-5</td>
<td>[75]</td>
</tr>
<tr>
<td>Inhibitor of differentiation-1</td>
<td>[75]</td>
</tr>
<tr>
<td>Kinase insert domain receptor (KDR), VEGFR2</td>
<td>[55, 80]</td>
</tr>
<tr>
<td>Mast/stem cells growth factor receptor, c-kit</td>
<td>[55]</td>
</tr>
<tr>
<td>Midkine</td>
<td>[48]</td>
</tr>
<tr>
<td>Neurogenic homolog 4</td>
<td>[48]</td>
</tr>
<tr>
<td>Survivin</td>
<td>[48]</td>
</tr>
<tr>
<td>Transforming growth factor, beta 1,</td>
<td>[42, 76]</td>
</tr>
<tr>
<td>Transforming growth factor, beta 2</td>
<td>[48]</td>
</tr>
<tr>
<td>Transforming growth factor, beta 3</td>
<td>[76]</td>
</tr>
<tr>
<td>Vascular endothelial growth factors (VEGFs)</td>
<td>[41, 46, 48, 66]</td>
</tr>
</tbody>
</table>
### Table 1.3 Genes involved in regulatory functions

<table>
<thead>
<tr>
<th>Gene name</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha fetal protein</td>
<td>[47] [87]</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>[48]</td>
</tr>
<tr>
<td>Core binding factor alpha 1(Runx2)</td>
<td>[40, 42, 45, 49, 64]</td>
</tr>
<tr>
<td>Glyceraldehyde-3 phosphate dehydrogenase</td>
<td>[77]</td>
</tr>
<tr>
<td>GATA binding protein 2</td>
<td>[47]</td>
</tr>
<tr>
<td>GATA binding protein 4</td>
<td>[55, 59, 80]</td>
</tr>
<tr>
<td>Heat shock proteins 47; 70,72,90</td>
<td>[80];[55]</td>
</tr>
<tr>
<td>Hypoxia-inducible factor 1 alpha</td>
<td>[46, 66]</td>
</tr>
<tr>
<td>Myocyte-specific enhancer factor 2C (Mef-2c)</td>
<td>[80]</td>
</tr>
<tr>
<td>Myosin heavy chain 10 non-muscle</td>
<td>[60]</td>
</tr>
<tr>
<td>Neuronal differentiation 1</td>
<td>[47]</td>
</tr>
<tr>
<td>NK2 homeobox 5 (Nkx2.5)</td>
<td>[55, 59, 80]</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>[42, 54, 65]</td>
</tr>
<tr>
<td>Osterix</td>
<td>[42]</td>
</tr>
<tr>
<td>Pancreatic and duodenal homeobox 1</td>
<td>[87]</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptors gamma2</td>
<td>[69]</td>
</tr>
<tr>
<td>Pou5f1</td>
<td>[47]</td>
</tr>
<tr>
<td>Sox 9</td>
<td>[70, 73, 81]</td>
</tr>
</tbody>
</table>
## Chapter 1: Monitoring transcriptional gene expression in 3D in vitro models

### Table 1.4. Specific markers

<table>
<thead>
<tr>
<th>Cells/tissue</th>
<th>Gene name</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipocytes</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leptin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adipsin A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adiponectin, Glucose transporter type 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Resistin</td>
<td>[74]</td>
</tr>
<tr>
<td>Brain</td>
<td>Neurofilament heavy chain</td>
<td>[87]</td>
</tr>
<tr>
<td>Cardiac muscle tissue</td>
<td>Troponin I</td>
<td>[55, 78]</td>
</tr>
<tr>
<td></td>
<td>Troponin T</td>
<td>[59, 80]</td>
</tr>
<tr>
<td></td>
<td>Connexin 43</td>
<td>55, 78</td>
</tr>
<tr>
<td></td>
<td>Actin, alpha, cardiac muscle 1</td>
<td>[59]</td>
</tr>
<tr>
<td></td>
<td>Atrial natriuretic peptide</td>
<td>[57]</td>
</tr>
<tr>
<td>Cardiac myocytes</td>
<td>Ankyrin-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cardiac ankyrin repeat protein, Cytochrome c oxidase subunit VIII heart/muscle</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adrenergic receptor beta-1</td>
<td>[9]</td>
</tr>
<tr>
<td></td>
<td>Adrenergic receptor beta-1(β1-AR), Sarcoplasmic reticulum calcium-ATPase</td>
<td></td>
</tr>
<tr>
<td>Endothelial lineage</td>
<td>CD146, melanoma cell adhesion molecule</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td>Von Willebrand factor</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td>Vascular endothelial-cadherin</td>
<td>[80]</td>
</tr>
<tr>
<td>Mammary cells</td>
<td>Casein-α and casein-β</td>
<td>[44]</td>
</tr>
<tr>
<td>Osteoblasts</td>
<td>Alkaline phosphatase</td>
<td>[40, 42, 49, 54, 64, 68]</td>
</tr>
<tr>
<td>Skin</td>
<td>Keratin</td>
<td>[87]</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td>SM22 alpha (transgelin)</td>
<td>[60]</td>
</tr>
<tr>
<td></td>
<td>α-smooth muscle actin</td>
<td>[58]</td>
</tr>
<tr>
<td></td>
<td>Myocardin,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Smoothelin</td>
<td>[58]</td>
</tr>
<tr>
<td></td>
<td>Calponin I</td>
<td>[60]</td>
</tr>
<tr>
<td></td>
<td>Smooth muscle myosin heavy chain</td>
<td>[60]</td>
</tr>
<tr>
<td></td>
<td>α-myosin heavy chain</td>
<td>[62]</td>
</tr>
</tbody>
</table>
1.2.2 DNA microarrays

There are currently two DNA microarray platforms available, cDNA-based and oligonucleotide-based arrays. Complementary DNA microarrays first developed by Brown laboratory at Stanford University in the 1995 use amplified cDNA clones (500-1000 nucleotides) spotted on a glass surface, while oligonucleotide arrays use short DNA oligonucleotide (30-40 nucleotides) synthesized on a solid substrate [89-91]. Microarray is a high throughput technique that allows the expression profiles of thousands of different genes to be detected simultaneously from hybridization of labelled cDNA or cRNA with immobilized DNA (cDNA clones or oligonucleotides) fragments [91, 92]. Some oligonucleotides arrays (Affymetrix, CA, USA), typically require 0.5-5µg RNA and can provide specific detection limits in the range of 1:10^3 to 1:2 x 10^6 with a dynamic range up to 4 orders of magnitude [93]. Due to the enormous amount of data generated by microarrays, accuracy of selected gene expression levels deduced from these assays is typically validated using RT-PCR [94]. Microarray is well suited for the tissue engineering field and has been used to monitor global gene expression in 3D constructs for applications related to skin [95, 96], cartilage [75], the cardiovascular system [9, 60] and traumatic brain injury [48]. Typically, constructs were fabricated from natural, synthetic or composite biomaterials.

1.2.3 Fluorescent in situ hybridization (FISH)

FISH is a molecular biology technique, introduced some 30 years ago, that allows the spatiotemporal expression pattern of transcripts at the single cell level using fixed cells [97, 98]. Several FISH-based techniques (reviewed in [98]) such as RNA-FISH and cellular quantitative in situ hybridization assay (QISH) [99] has since been developed. For RNA-FISH complementary mRNA target sequences in fixed and permeabilized cell preparations on slides are hybridized using labelled DNA/RNA probe, visualized in situ by fluorescent microscopy, and relative expression levels quantified based on signal intensity. This technique has been used to visualize the transcription level of up 11 gene transcripts in a single cell by fluorophore combinations [100].

QISH is an RNA-FISH assay in which cells are fixed in a 96-well plate and mRNA target sequences are hybridized using photo-biotin labelled cDNA probes [99].
Consequently, the biotin is detected by streptavidin and biotinylated alkaline phosphatase, followed by measurement of the optical density using a plate reader.

FISH is a complex and laborious technique with low sensitivity compared to RT-PCR [101]. Added to this, this technique provides very limited temporal resolution of RNA [13].

RNA-FISH has been used to detect the expression levels of genes related to the ECM in cells grown in collagen [102, 103] and alginate [38] scaffolds. Bone-related genes of cells harvested from hydroxyapatite (HA) ceramics [104], calcium phosphates ceramics [105], cements [106] and titanium [107] may be analysed by QISH.

1.2.4 Northern blot
This molecular biology technique was developed by James Alwine and colleagues in the 1970s. Basically, purified RNA lysates are gel electrophoresed, transferred to nitrocellulose membranes then hybridized with labelled DNA or RNA probes for the identification and quantification of mRNA [91, 108]. Northern blot also provides information about the size and integrity of mRNA in a sample [28]. Although this method is simple, it typically requires large quantities of RNA (~10 µg) and may result in mRNA degradation [109]. Also, sensitivity is much lower compared with RT-PCR.

RNA extracted directly from 3D constructs containing cells embedded in collagen [110, 111], PLGA [112], PGA [110], gelatin/PCL construct [113] and PLLA/hydroxyapatite [114] scaffolds, as well as cells harvested from hydroxyapatite, glass-ceramics, and titanium constructs [115] have been analysed using northern blots.

1.3 Comparison of mRNA quantification techniques
The techniques used to analyse mRNA expression in 3D in vitro models are summarized in Figure 1.1
Figure 1.1 Schematic of mRNA quantification techniques with typical times needed for assay completion.
1.4 Key parameters for tissue engineering

1.4.1 Sampling regime

Cells undergo transcriptional control during various stages of development from proliferation, differentiation to maturation. For example, observations of the temporal expression levels of bone-related genes in osteoblast cultures over a 35d period showed differential expression during the proliferation, maturation and mineralization stages [116]. For instance, Laurie et al has shown that changes in gene expression levels occurred for laminin receptor, laminin, and collagen IV in mouse kidney cells collected between 16 day gestation and 3 weeks after birth [117]. Thus, the use of time points is an important consideration for gene expression analysis when evaluating the performance of 3D scaffolds, and should coincide with the synthesis, release and activation at different stages of development.

An overview of the literature showed that gene expression analyses of 3D constructs for cardiac specific genes are generally performed at day 7. On the other hand, analysis for bone related genes are typically performed at 2-3 time points chosen from 7, 14 or 21 d.

1.4.2 Spatial distribution

The microenvironment in which the cell interacts with the ECM, soluble growth stimulating factors and surrounding cells provides structural integrity and controls gene expression [118]. Thus, the spatial distribution and organization of cells within the 3D constructs determines the level of cellular interaction and thus influence cellular function and formation of new tissue [119]. It follows that initial seeding densities considerations should ensure sufficient quantities of cells are uniformly seeded onto or embedded in the scaffold. High density cell cultures increase cell-cell interactions and communication while promoting secretion of extracellular matrix and growth factors, which may enhance [120] or inhibit [121] proliferation. However, the absence of cell-cell interactions in experimental systems tend to show the lowest proliferation rates [121].

The physical location of cells within 3D constructs may influence the spatiotemporal gene expression levels [41, 76] as can cell morphology [122] and surface patterns [123].
1.4.3 Cells

The four major tissue types, namely muscle (skeletal, cardiac, smooth), nerve (nerves, brain), epithelial (e.g. skin) and connective (e.g. cartilage, bone) tissues consist of specialized cells expressing specific genes [124], [125] in addition to the housekeeping genes that are present in all cells. Thus, transcription gene expression levels in cells may be tissue source specific. Hence, consideration of an appropriate cell source is important in the development of 3D in vitro models for specific TE applications. Equally important are the proliferation capability, differentiation, and tissue specific commitment of the cells in the 3D constructs [80].

1.5 Project rationale

1.5.1 Background

Based on the discussion above, it is clear that a wealth of information can be gained from gene expression analysis of cells associated with 3D constructs. However, when using lysates to quantify relative gene expression, the information is limited to temporal expression of the average cell with no spatial information. Although the in situ hybridization technique gives insight into the spatiotemporal gene expression of cells in constructs, this method requires fixation which may alter the mRNA location. A complete spatiotemporal profile of the mechanisms involved in the synthesis, processing, and transport of RNA will improve our understanding of cell function and behaviour under various biological cues, which could potentially advance the field of biomaterials and tissue engineering towards designing more functional biomaterial scaffolds [13].

Molecular beacons [126] have been extensively used in biomedical research and have the ability to provide spatiotemporal pattern of specific mRNA expression in live cells that can be visualized in real-time at the single cell level. These oligonucleotide probes are designed to have a stem-loop conformation and recognize targets with a measurable fluorescence signal (illustrated in Figure 1.2) with high signal-to-background ratios [13]. Furthermore, in the stem-loop conformation, the molecular beacon (MB) is unhybridized and typically elicits low fluorescence signal which enables detection of
probe–targets hybrids to be performed without the separation of excess probes. This detection without separation capability of MB is ideal for 3D scaffolds.

Figure 1.2 Schematic of molecular beacon. In the absence of complementary nucleic acid target, the beacon has a hairpin shaped structure and fluorescence is quenched; however, on hybridization with target the fluorescence is restored.

1.6 Objectives
The overall goal of this project was to develop a detection system that can be used to monitor gene expression in living cells associated with 3D scaffolds.

Objectives: Phase-I (Chapter 2)
Aim: Design, characterize and evaluate MBs targeting therapeutic genes.
- Design eNOS and IL10 molecular beacons.
- Characterize MBs by evaluating sensitivity and specificity for target nucleic acid, signal/background ratios, and thermal denaturation profiles.
- Evaluate whether sites on target mRNA are accessible to the MBs.

Objectives: Phase II (Chapter 3)
Aim: Develop a molecular beacon detection platform for monitoring changes in transcription gene expression levels in living cells embedded into a 3D collagen scaffolding system.
- Develop and characterize Type I collagen scaffold.
• Select an efficient method for MB delivery.
• Transfect HFFF2 cells using GAPDH siRNA.
• Quantify siRNA knockdown of GAPDH mRNA in 3D scaffold.
• Evaluate GAPDH mRNA expression levels in transfected cells in the 3D scaffold using MB technology and RT-PCR.

Objectives: Phase-III (Chapter 4)
Aim: Assess the spatiotemporal efficacy of a dual-gene therapy model based on a 3D collagen scaffold loaded with pIL-10 polyplexes and peNOS polyplexes encapsulated into microspheres.

• Characterize the scaffold for sustained and delayed release profiles of loaded polyplexes over a 14d period.
• Evaluate temporal expression levels of IL-10 and eNOS mRNA in cells transfected via 3D scaffolding gene carriers using RT-PCR.
• Evaluate spatiotemporal expression of IL-10 and eNOS mRNA in cells transfected via 3D scaffolding gene carriers using MBs

1.7 References
Chapter 1: Monitoring transcriptional gene expression in 3D in vitro models


Chapter 1: Monitoring transcriptional gene expression in 3D in vitro models


Chapter 1: Monitoring transcriptional gene expression in 3D in vitro models


Chapter 1: Monitoring transcriptional gene expression in 3D in vitro models


Chapter 1: Monitoring transcriptional gene expression in 3D in vitro models


Chapter 1: Monitoring transcriptional gene expression in 3D in vitro models


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2.1 Introduction
The design of the molecular beacon plays a crucial role in its ability to detect targets with high specificity and sensitivity. Optimal probes in biomedical research should satisfy several requirements: (1) have a high specificity for target, (2) elicit signals that be easily measured on target detection, (3) provide high signal-to-background ratio, (4) be stable, so differentiation can be made between true and false positive events (5) and achieve highly efficient delivery to living cells that (6) occur with fast probe-hybridization kinetics [1, 2]. Molecular beacons have the potential to satisfy all these requirements. Added to this, un-hybridized probes do not need to be removed from the system which is ideal for intracellular measurement of mRNA expression levels. Along with high specificity and sensitivity for their nucleic acid targets, these probes have shown the ability to detect single nucleotide polymorphisms (SNPs) [3].

2.2 Molecular beacons
Molecular beacons are antisense oligonucleotide hairpin probes with four essential components: loop, stem, fluorophore, and quencher (Figure 2.1A). The single stranded loop end is generally 15-35 nucleotides [4] and carries the complementary sequence to the target nucleic acid (mRNA, DNA) which allows the beacon to bind to the target. The short 4-7 nucleotides double stranded (from complementary pairing) stem keeps the fluorophore and quencher, each covalently attached to one of its ends, in close proximity (1-10 nm) to each other and quenching of the fluorescence of the MB is achieved mainly on the principle of contact (static) quenching, although fluorescence resonance energy transfer (FRET)-quenching is possible, illustrated in (Figure 2.2). In the presence of the target, the complementary sequences of the loop and target are hybridized and the MB spontaneously changes conformation and fluorescence is restored (Figure 2.1A).

The selection of a fluorophore-quencher pair is not restricted; however the choice could provide optimal signal-noise background ratios and multiplexing function of the MB.

There are two types of MB, conventional and shared-stem. In a conventional MB (Figure 2.1A), the stem is chosen independent of the target sequence, while in
shared-stem MB the full sequence of one arm of the stem is chosen depending on the target sequence (Figure 2.1B).
Figure 2.1 Illustrations of molecular beacons.
A. Conventional molecular beacon. B. Shared stem molecular beacon.
In (A), the beacon stem is designed independent of the target sequence
In (B), the beacon stem is designed dependent of the target sequence
Figure 2.2 Principle of static (contact) and FRET quenching.

*Static quenching:* strong coupling (via hydrogen bonds, induced dipole interactions and hydrophobic interactions) of the reporter (R) and quencher (Q) forms ground-state complex (RQ) with unique absorption spectrum. RQ absorb energy, become excited and quickly returns to ground state without the emission of energy (= no fluorescence light emitted).

*FRET quenching:* Weak dipole-dipole coupling of R (donor) and Q (acceptor) occurs at distances between 1-10 nm. Radiationless energy is transferred from R in the excited state to Q, returning R to ground state. The mechanism requires the emission spectrum of R to overlap the absorption spectrum of Q.

Typically, the efficiency of quenching in static conditions are independent of spectral overlap or R and Q, but not so in FRET conditions [5].

(Adopted from [6]).
2.3 Molecular beacon design and function

The three major challenges in molecular beacon design are specificity, melting temperature, and the secondary structure of the target mRNA.

2.3.1 Specificity

To ensure specificity for target mRNA, the loop sequence of the molecular beacon is designed to be complementary to the target and the sequence is interrogated using BLAST (basic local alignment search tool) or similar software to determine uniqueness. Firstly though, the secondary structure (folding) of the mRNA is predicted using software such as Mfold [7] to identify single stranded regions, and the regions of interest selected based on probe/RNA interactions determined using, for example, the OligoWalk software [8]. Mfold uses a model based on minimum free energy of base pairing to compute optimal or suboptimal mRNA structures. Some have used the ss-count and p-num values computed in Mfold to select regions for the beacon loop sequence [9]. From the group of predicted secondary structures given, the ss-count value indicates the number of times the particular base (nucleotide) is single-stranded, while the p-num value, indicates whether the base pair predicted is well or poorly (high p-num value) determined. Thus, high ss-count values of more than 50% together with low/high p-num value are considered favourable for beacon loop design. To further select loop sequences that bind strongly to the mRNA target, the thermodynamic parameters generated using OligoWalk may be employed. OligoWalk predicts the MB loop/RNA interactions based on equilibrium affinity at 37°C and allows manipulation of probe length and concentration with limited choice of backbone chemistry to RNA or DNA. The thermodynamic values that are of interest during the selection of the target region are duplex and overall ΔG. Simply, the more negative thermodynamics (-ΔG) indicate the stronger bond. Thus, regions in which the -ΔG of the duplex (binding of the beacon loop and the mRNA) is more negative than the overall -ΔG by 10 or less may be selected as target regions [9].

2.3.2 Melting temperature

The melting temperature is controlled by the length and sequence of the stem of the beacon and influences specificity and S/B noise (sensitivity). Typically, the stem is
designed with 75-100 % G-C content. However, adjustment of the lengths and G-C content of the stem can be used to manipulate the melting temperature of the beacon for the specific application. For instance, in live cell environments melting temperatures greater than 37°C are required for MB to discriminate its target; MB with a lower melting temperature will be prone to opening in the absence of target presenting lower S/B noise and decrease specificity.

The fluorophore−quencher pair may attract or repulse each other. Attractive forces between fluorophore and quencher helps to stabilize the stem. Thus, the selected fluorophore−quencher combinations may influence the melting temperature as well as the quenching efficiency of the MB [5].

### 2.3.3 Secondary structure of mRNA

The folded secondary structure of mRNA needs to be taken into consideration in the MB design and efforts made to avoid targeting double-stranded regions which may be inaccessible. Still, accessibility of the mRNA region is not guaranteed as models used to predict mRNA structure may not be accurate. Thus accessibility of target to the beacon can only be determined by evaluating the MB in the intracellular environment. Hence, multiple beacons are typically designed to target different regions of the mRNA [10].

The structure design of molecular beacon is crucial to its performance. The probe and stem lengths influence both melting temperatures and hybridization kinetics of the MB in the presence of targets [3, 11], which is illustrated in Figure 2.3. Also, the fraction of beacons present at varying temperatures in each of the three conformational states: hybridized to target, unhybridized and random coil states, illustrated in Figure 2.4 is influenced by the lengths and sequences of the both the loop and stem. Furthermore, sensitivity of the molecular beacons is also influenced by the stem length. A stem length that is too short (very unstable) or too long (very stable) may result in low S/B noise due to opening in the absence of target or inadequate opening in the presence of target.
Figure 2.3 Probe and stem lengths relationship with (A) melting temperature and (B) hybridization kinetics (rate constant) of molecular beacons in the presence of targets. (Adopted from Ref [11]).
Figure 2.4 Thermodynamic phase transitions of molecular beacons in the presence of targets.

Phase 1: Hybridized state with open beacons producing highest fluorescence signal at low temperatures.

Phase 2: Unhybridized state with closed beacons producing weakest fluorescence signal at melting temperature of duplex.

Phase 3: Random coil state with unhybridized beacons randomly opening producing low background fluorescence signal above melting temperature.

(Adopted from ref [3]).
Chapter 2: Design, characterization and evaluation of molecular beacons

Two critical considerations are essential for the design of molecular beacons for intracellular detection of target mRNA. Firstly, the designed beacons must find the target mRNA, a process of sound MB design already discussed above. Secondly, the beacon must be efficiently delivered into the cells by an effective method.

2.4 Intracellular delivery of probes

The relatively lipophilic nature of the plasma membrane presents a barrier for polyanionic antisense oligonucleotides (ODN) and prompted the need to develop robust methods for efficient intracellular delivery. The main methods can be divided into two (2) categories: endocytic and non endocytic. Endocytic delivery is usually accomplish via cationic/polycationic molecules such as liposomes and dendrimers, while non-endocytic, by microinjection, electroporation, cell penetrating peptides (CPPs), and Streptolysin O (SLO) permeabilization. Transport mechanism, cellular uptake, distribution, dynamics, lifetime, and availability of the oligonucleotides to targets may vary according to the methods used.

Endocytic delivery methods is mediated via the endocytic pathway in which membrane specific surface receptors on cells bind to the ODN and uptake the molecules during incubation of around 2 h [12]. Endocytosis of labelled ODN, generally, exhibit a punctuate fluorescence pattern. Cellular uptake may be enhanced by stable complex formation between anionic ODN and cationic liposomes or dendrimers. However, only a small amount, about 0.01-10 percent [13], of the internalized ODN may be available to their cytoplasmic targets in the cytosol due to entrapment of the by ODN in endosomes and eventual degradation by hydrolytic enzymes (nucleases) predominantly present in lysosomes. Clearly, labelled ODN that can escape the endosomes or are nuclease resistant could avoid this difficulty. Complexes form between DNA and “fractured” cationic polyamidoamine dendrimers (e.g. Superfect™) have been shown to escape the endosomes [14]. It is postulated that the fractured dendrimers extend fully to bind DNA then collapse into compact structure at neutral pH. On internalization into acidic endosomes fractured dendrimers become protonated, release the DNA and swell causing endosomes to rupture so that DNA can escape.
Microinjection is a widely used technique for intracellular delivery of ODN. The ODN is injected directly into the cytoplasm or nucleus of individual cell. Thus, this method does not allow for high amount of cells to be analysed at the same time. Electroporation uses short (µs to ms) electrical pulse to produce transient pores in the membranes of cells. Small molecules are believed to enter by through the pores via diffusion, while macromolecules (e.g. DNA) enter by electrophoretic transport [15]. Electroporation perform in micro-litre volumes can achieve uniform cytosolic distribution of probe in live cells with transfection efficiency of 93% and average cell viability of 86 % [16].

Streptolysin O (SLO) is an oxygen-labile bacterial toxin that is activated by thiols (e.g. dithiothreitol) and by tris(2-carboxyethyl) phosphine, TCEP). The toxin binds to cholesterol containing membranes of cells to form pores about 30 nm in diameter. The mechanism relies on the self association of SLO with the cholesterol membrane to form amphiphilic oligomers ring/rod-shaped structures. These structures penetrate into the apolar domains of the membrane to create protein-lined transmembrane channels or large apertures in the bilayer [17]. Typically, 0.2U/ml SLO concentration and 5-15 min incubation time at 37°C is used to deliver MB into cells, but optimization may be necessary due to the varying cholesterol content of different cells. SLO is inactivated by serum media with typical resealing of the cells occurring at 37°C for 1h. In this study, SLO mediated delivery of molecular beacons was selected to evaluate transcriptional gene expression in two-dimensional (2-D) cultures.

Cell penetrating peptides (CPPs) also termed protein transduction domains are mainly derived from viral proteins and are capable of crossing the plasma membrane and taking cargoes along. The mechanism by CPPs translocation is still unclear as there is evidence to support both a non-endocytic pathway occurring in a temperature-independent manner and an endocytic pathway mediated via membrane receptors [18, 19]. It is suggested, however that the mechanism may be dependent on the CPP type, cell type, and size of the cargo. CPPs have been used to deliver peptides [20, 21], nucleic acids [22], proteins [20, 23], nanoparticles [24], and liposomes [25] into live cells. The net positive charge of the CPPs is associated with the amino acids lysine and/or arginine present. Of the various CPPs, HIV-1-Tat (48-60) peptides are the best characterized. These peptides are derived from the 86 amino
acid HIV-1 virus transactivator of transcription (Tat) protein domain. Tat-peptide conjugated to molecular beacons have been delivered to primary cells [2] in 2D and were internalized within 30 min with 100% efficiency. In this study Tat-peptide linked molecular beacons was selected to evaluate transcriptional gene expression in three-dimensional (3-D) cultures (Chapter 3).

2.5 Backbone chemistry modifications
The backbone chemistry of antisense oligonucleotide is a crucial parameter to consider for delivery to the intracellular environment. The simplest beacon is synthesized using a phosphodiester DNA (PO) backbone which is made up of phosphate and sugars groups linked by ester bonds. PO beacons are limited to short term (few hours) analysis in living cells. Firstly, beacons with DNA backbone chemistry may be prone to degradation by endonucleases, in endosomes/lysosomes, before reaching intended targets. Degradation separates fluorophores from quencher and results in fluorescence signal which may be falsely interpreted as hybridization. Secondly, ribonuclease H (RNase-H) an endoribonuclease that is found in both the nucleus and the cytoplasm of all cells may cleave the RNA target of the RNA–DNA duplex formed by the beacon resulting in decrease fluorescence as the beacon loses its target to assume the unhybridized state. Thirdly, binding proteins that interact with beacons may cause instability and opening of beacons which results in false positive fluorescence signal.

To provide beacons that are nuclease resistant and stable for many hours in living cells, modification of the DNA backbone chemistry have been used to synthesize molecular beacons. Substitution a sulphur atom for one of the non-bridging oxygen in the phosphate backbone of PO results in phosphorothioate DNA (PS). This modification increases the resistance to endonucleases, but results in lower affinity (decreases the melting temperature) for RNA compared to PO [26]. Also, PS show increased susceptibility to RNase H digestion [26] and may be prone to nonspecific protein binding [27].

Addition of a methyl group at the 2’ position of the PO sugar backbone results in a 2’- which besides being nuclease resistant has the stability of DNA molecules (as oppose to RNA molecules which are less stable). 2’-O-methyl analogs possess greatly increased melting temperature with higher affinity for their RNA targets
compared to PO [28] Also, 2’-O-methyl RNA analogues have been shown to hybridized to their target with faster kinetics [28] and are not susceptible to RNase H digestion.

Introduction of a methylene bridge linking the 2’-oxygen and 4’-carbon in the sugar backbone of PO locks the sugar ring into an RNA mimicking conformation that is termed lock nucleic acid (LNA) [29, 30] analogue. Lock nucleic acids are resistant to nucleases and single-stranded binding proteins and show high affinity for RNA and DNA [30].

Substitution of the PO phosphate backbone for N-(2-aminoethyl)glycine backbone produces peptide nucleic acid (PNA) analogues with neutral charges and obey the characteristic Watson-Crick base pairing rule [31]. The neutral charge of PNAs results in high affinity for DNA/RNA, compared to PO, at low to medium ionic strength [32].

Features of the main classes of modified oligonucleotides are summarized in Table 2.1.
Table 2.1 Features of modified molecular beacons

<table>
<thead>
<tr>
<th>Type</th>
<th>Modification</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phosphorothioate</strong></td>
<td><strong>Phosphate backbone</strong></td>
<td>Nuclease resistant</td>
</tr>
<tr>
<td></td>
<td>Sulphur atom replaces a</td>
<td>Lower affinity for RNA compared to PO</td>
</tr>
<tr>
<td></td>
<td>non-bridging O atom</td>
<td>Susceptible to RNase H</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vulnerable to single-stranded binding proteins</td>
</tr>
<tr>
<td><strong>2’-O-methyl RNA</strong></td>
<td><strong>Sugar backbone</strong></td>
<td>Nuclease resistant</td>
</tr>
<tr>
<td></td>
<td>Addition of methyl group</td>
<td>High affinity for RNA and DNA</td>
</tr>
<tr>
<td></td>
<td>to 2’OH position</td>
<td>Do not support to RNase H</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vulnerable to single-stranded binding proteins</td>
</tr>
<tr>
<td><strong>Lock nucleic acid</strong></td>
<td><strong>Sugar backbone</strong></td>
<td>Nuclease resistant</td>
</tr>
<tr>
<td></td>
<td>2-O and 4’C are linked</td>
<td>High affinity for RNA and DNA</td>
</tr>
<tr>
<td></td>
<td>by methylene linker</td>
<td>Do not support to RNase H</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resistance to single-stranded binding proteins is LNA design dependent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Better structural stability</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low fluorescence background in living cells</td>
</tr>
<tr>
<td><strong>Peptide nucleic acid</strong></td>
<td><strong>Phosphate backbone</strong></td>
<td>Nuclease resistant</td>
</tr>
<tr>
<td></td>
<td>Phosphodiester backbone</td>
<td>High affinity for RNA and DNA</td>
</tr>
<tr>
<td></td>
<td>replaced with N-(2-aminoethyl)glycine units</td>
<td>Low solubility in aqueous environment</td>
</tr>
</tbody>
</table>

(Modified from [33]).
The principal objective of this phase of the study was to develop molecular beacons targeting the therapeutic genes interleukin-10 (IL-10) and endothelial nitric oxide synthase (eNOS). To this end, several molecular beacons were designed. The sensitivity and specificity for the intended targets was determined and the accessibility of intracellular mRNA region for the molecular beacons was evaluated.

2.6 Materials and methods

2.6.1 Materials
Molecular beacons with DNA backbone chemistry were used in this study. The beacons and synthetic DNA are shown in Table 2.2. The beacons were synthesized by Eurofins MWG, Ebersberg, Germany, unless otherwise mentioned.

The human eNOS plasmid used in this study has an eNOS gene sequence encoded into a pcDNA3 vector containing the CMV promoter and was a kind donation from Dr. Karl McCullagh (Regenerative Medicine Institute, National University of Ireland-Galway, Ireland). The plasmid encoding human IL10, pORF-hIL10, was purchased from InvivoGEN (CA, USA).
Table 2.2 The design of probes and target oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Sequence (5'-3')</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unmodified MB</strong></td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>29</td>
</tr>
<tr>
<td>IL-10 #1</td>
<td>26</td>
</tr>
<tr>
<td>IL-10 #2</td>
<td>25</td>
</tr>
<tr>
<td>Random</td>
<td>27</td>
</tr>
<tr>
<td>GAPDH</td>
<td>29</td>
</tr>
<tr>
<td>Random</td>
<td>27</td>
</tr>
<tr>
<td><strong>Dual FRET MB</strong></td>
<td></td>
</tr>
<tr>
<td>Poly-dT (donor)</td>
<td>50</td>
</tr>
<tr>
<td>eNOS (acceptor)</td>
<td>35</td>
</tr>
<tr>
<td><strong>Modified MB</strong></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>29</td>
</tr>
<tr>
<td>Random</td>
<td>27</td>
</tr>
<tr>
<td><strong>Synthetic DNA</strong></td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>29</td>
</tr>
<tr>
<td>IL-10 #1</td>
<td>26</td>
</tr>
<tr>
<td>IL-10 #2</td>
<td>25</td>
</tr>
<tr>
<td>GAPDH</td>
<td>29</td>
</tr>
</tbody>
</table>

Molecular beacon stem is indicated in **bold caps** and the **underlined** bases were shared with the probe on hybridization.
2.6.2 Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and cultured in endothelial basal medium (EBM-2) supplemented with the EGM-2 bullet kit (Lonza). Human mesenchymal stem cells (HMSCs) were obtained from REMEDI, NUIG and were maintained in MEM α, medium (Gibco) supplemented with 10% research grade FBS (ThermoScientific Hyclone), 1% penicillin/streptomycin (10,000 units/ml) and 1 ng/ml fibroblast growth factor-2 (BD Biosciences, Bedford, MA).

2.6.3 Design of eNOS and IL-10 molecular beacons

Molecular beacons labelled at the 5’-end with FAM fluorophore and the 3’-end with BHQ 1 were designed to target a human eNOS or IL-10 mRNA following the protocol outlined in [9]. Briefly, the secondary structure of the target mRNAs were predicted using Mfold software [7] and single stranded regions were selected based on both ss-counts and low/high p-num values. Then, the Oligowalk software [8] was used to select the optimal sequence that binds strongly to target mRNAs and the sequence were evaluated using BLAST to ensure specificity for the target. The procedure is illustrated in Figure 2.5. A random control beacon that does not have any target in mammalian cells was included in the study. Dual FRET beacons targeting eNOS were previously described in [34] and were designed to target the 3’-UTR region human eNOS and the Poly-(A) tail. The eNOS targeting beacon (acceptor beacon) was labelled at the 5’-end with Cy5 and at the 3’-end with BHQ-2 while the poly-T beacon (donor beacon, Eurogentec, Belgium) was labelled at the 5’-end with BHQ-2 and at the 3’-end with Cy3.

2.6.4 Hybridization assays

To assess the sensitivity and specificity of MBs, complementary DNA oligonucleotides for eNOS and IL-10 were synthesized. Concentrations of 200 nM MBs in PBS were incubated at 37°C for 1h in the presence of complementary synthetic DNA ranging from concentrations of 0-500 nM, in a final volume of 100µl. Fluorescence intensity was measured at excitation/emission of 485/535 (for FAM) using the Wallac Victor2 1420 plate reader (Perkin Elmer). The fluorescent signal from molecular beacon only well was subtracted from all wells. A curve was
generated from these values to determine the lowest concentration of DNA detectable by the MBs.

2.6.5 Signal/background ratio
The signal background (S/B) ratio was used to demonstrate that MBs undergo a measurable fluorogenic response in the presence of target. The fluorescence intensity ($F_{\text{buffer}}$) of 100 µl PBS in 96-well black plate was measured followed by the fluorescence intensity ($F_{\text{closed}}$) when 10 µl of 5µM target DNA was added and the stable fluorescence intensity ($F_{\text{open}}$) when 10 µl of 1µM MB was finally added to the system. Fluorescence intensity was measured at excitation/emission of 485/535 nm (for FAM) using the Wallac Victor2 1420 plate reader (Perkin Ekmer). The background signal ($F_{\text{buffer}}$) was subtracted from ($F_{\text{closed}}$) and $F_{\text{open}}$ signals before calculation of the S/B ratio and quenching efficiency.

2.6.6 Thermal denaturation profile
The melting temperatures of FAM eNOS and IL-10 molecular beacons with their complementary DNA were determined. The fluorescence of the beacon solutions in the absence or presence of synthetic target DNA was measured as a function of temperature and based on a protocol previously described [35]. Briefly, 25 µl solutions containing 200 nM molecular beacons dissolved in 4 mM MgCl$_2$, 10 mM Tris.HCl (pH 8.0) with no target, or 400 nM synthetic DNA targets were reacted. Fluorescence of each solution was monitored in parallel in 1 °C step each 60s as the temperature decreased from 80 to 25°C using the StepOne plus instrument (Applied Biosystems).

2.7 Evaluation of molecular beacon target sites in live cells in 2D

2.7.1 Transfection of plasmids eNOS and IL-10
The day before transfection, 10,000 HUVECs and 25,000 HMSCs were seeded into 6-channel µ-slide (µ-slide VI$^{0.4}$, Ibidi, Germany) and 24-well glass bottom plate (MaTtex), respectively. Plasmids eNOS or IL-10 were combined with Superfect® (3mg/ml, Qiagen) to form polyplexes at ratio between Superfect® (SF) and plasmid DNA (µL:µg) of 2:1, according to manufacturer’s protocol. The ratio was found to
be less cytotoxic to cells. HUVECs in microfluidic chambers were transfected with 30 µl of transfection media containing 0.5 µg peNOS and 1µl Superfect in serum-free medium (HBSS). HMSCs were transfected with transfection medium containing 1µg of pIL-10 and 2 µl Superfect, according to manufacturer’s protocol. After 2-3h incubation, cells were rinsed three times with complete medium and cultured in 120 and 500 µl complete medium, respectively for 48h before MB delivery.

2.7.2 Transduction of AdeNOS
The day before transfection, 10,000 HUVECs were seeded into 6-channel µ-slide. Cells were transduced using 30 µl of adenovirus encoded with human eNOS (adeNOS) or no gene (adnull, serves as control) at a multiplicity of infection (MOI) of 100 in HBSS. The cells were incubated with the transduction media for 1h with occasional rocking every 15 minutes before 120 µl complete media was added for 48 h before MB delivery.

2.7.3 Molecular beacon delivery via activated Streptolysin O
HUVECs were incubated at 37°C/5% CO2 for 10 min with 30 µl of 0.2U /ml activated SLO containing 300 nM of FAM-labelled eNOS or control MB. For transduction experiments, HUVECs were incubated with 400 nM of dual FRET MB (200 nM Cy5- labelled eNOS and 200 nM Cy3-labelled Poly-T). HMSCs were incubated with 200 µl of 0.2U/ml activated SLO containing 400 nM of FAM-labelled IL-10 or control MB. The permeabilization solution was removed and cells were washed two times with complete medium. Fresh complete medium 150 or 500 µl, respectively, was added and cells were allowed to reseal at 37°C/5% CO2 for 1 h before imaging. Imaging was completed within 1-2h.

2.7.4 Imaging
Imaging was performed using Axiovert LSM-510 confocal microscope equipped with Plan-Apochromat 63 x/1.4 oil DIC or C-Apochromat 63 x/1.2 water objectives (Zeiss). Beacons were visualized with argon laser at excitation of 488 nm with signal detection at 530 nm for FAM. The 2D images were 512 x 512 pixels and acquisition was completed in 6.29 s (at scan speed 8 and an average of 4 frames) using a 1.6 Airy Disk. For Dual FRET beacons, imaging was performed using an Andor
Chapter 2: Design, characterization and evaluation of molecular beacons

Revolution Spinning Disk equipped with a 60X/1.2W objective (Andor) and excitation of 561 and emission between 665-680 nm.

2.8 Results and discussions

Three eNOS MBs and four IL-10 MBs were designed following the design process illustrated in Figure 2.5. The beacons were delivered to living cells and one eNOS MB and 2 IL-10 beacons gave the best signal and were thus chosen for further analysis.

The eNOS MB was designed with a 17-nucleotide loop and a 6-nucleotide stem. However, the first nucleotide (closest to the loop) on each side of the stem was shared to obtain a 19-nucleotide probe length on hybridization. IL-10 MB # 1 was designed with a 16 nucleotides probe length and 5-nucleotide stem sequence and IL-10 MB # 2 with a 15 nucleotides probe length and 5-nucleotide stem sequence (Table 2.2). The fluorophore FAM and quencher BHQ-1 was the chosen fluorophore-quencher pair as this combination has shown high quenching efficiency [5]. Also, FAM has a high quantum yield and BHQs do not emit fluorescence and so can increase signal background noise ratios.

2.8.1 Specificity and sensitivity of molecular beacons

Molecular beacons targeting a human eNOS mRNA and two regions of the human IL-10 mRNA were characterized for specificity and sensitivity, signal/background ratios and thermodynamic behaviour. All three beacons were found to be specific and sensitive for their targets in homogeneous assays (Figure 2.6). Specifically, incubation of 200 nM MB concentrations with varying concentrations of target DNA ranging from 0-500 nM showed beacon signal was concentration dependent and followed second order kinetics. Also, beacon fluorescence signal following hybridization was increased with the probe length (fluorophore-quencher distance); increased signals were observed from duplexes formed with 29 nucleotide eNOS MB, less with 26 nucleotide IL-10 MB#1 and the least with 25 nucleotide IL-10 MB#2.
2.8.2 Signal background noise ratio
The detection using molecular beacons is based on a fluoregenic response thus high signal background ratios are required to distinguish target signal from background noise. Further, the S/B ratio is an indicator of purity and quenching efficiency of the MB as free fluorophore in the beacon solution as well as inefficiently quenched beacons may give rise to high background signals. The three designed beacons evaluated had high S/B ratios and high quenching efficiencies (Figure 2.7) showing that FAM–BHQ-1 pair is a good combination.

2.8.3 Melting temperature of beacons
Thermal denaturation profiles (Figure 2.8) showed that eNOS and IL-10 molecular beacons can discriminate between targets over a range of temperatures and indicated correct phase transition characteristics of the molecular beacons. At low temperatures, the beacons were in their hairpin state and did not emit fluorescence. However, as the temperature was increased, the beacons eventually lost the hairpin shaped conformation separating the fluorophore and quencher to form random coils and a fluorescence signal was emitted. In the presence of DNA targets and at low temperature, the beacons spontaneously hybridized to targets and emitted high fluorescence signals which dropped substantially as the melting temperature of the MB-DNA duplex is surpassed and the beacon is returned to its unhybridized state separated from targets. Further increase in temperature caused the beacons to form random coils with the emission of fluorescence signal lower than hybridized state.
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Figure 2.5 Schematic showing the MB design process used in this study.
Figure 2.6 Specificity and sensitivity of eNOS and IL-10 molecular beacons. Fluorescence intensities of molecular beacons (200 nM) after 1h incubation at 37 °C with different concentrations of complementary DNA in PBS buffer, pH 7.4. The concentration of the DNA ranged from 0 to 500 nM. The signal from MB only concentration was used as background measurement and subtracted from the test signal.
Figure 2.7 Signal background ratio of IL-10 and eNOS molecular beacons. MBs (200nM) were incubated with 400nM target DNA in PBS. Fluorescence was recorded for PBS before addition of the MB and for the MB before addition of the DNA targets. A stable fluorescence signal from the duplex (MB-DNA) was recorded at 60 min. MB were excited at 485 nm and emission signal collected at 535 nm for FAM.
Figure 2.8 Thermal denaturation profiles for (A) eNOS MB, (B) IL-10 MB#1 and (C) IL-10 MB #2. Molecular beacons (200 nM) were incubated in the absence of target or the presence of 500 nM complementary DNA. The fluorescence signal was measured during 1 minute holds at every 1°C decrease in temperature from 80 to 25°C. (A). Probe length is 19 nucleotides (nt) and stem length is 6 nt, (B) Probe length is 16 nt and stem length is 5 nt, and (C) Probe length is 15 nt and stem length is 5 nt.
2.8.4 Accessibility of targeted mRNA sites

The accessibility of mRNA sites for the designed beacons was evaluated. Typically, mRNAs have secondary (folded) structures which can result in regions targeted by the molecular beacon to be hidden and inaccessible. Intracellular signal was detected in one eNOS MB (Figure 2.9A) and two IL-10 MBs (Figure 2.10A) indicating target accessible regions. Also, random-sequence (control) MBs delivered into both treated and non treated HUVECs and HMSCs showed no signal (Figure 2.9A and Figure 2.10A), respectively. This confirms the signal specificity of eNOS and IL-10 MBs since random beacons do not have any complementary mRNA target in human cells. Still, MBs delivered into cells that were not transfected were found to emit significantly lower mean fluorescence intensity (Figure 2.9B and 2.10 B) indicating signal specificity, since HUVECs and HMSCs may express baseline eNOS and IL-10 mRNA levels, respectively. The eNOS mRNA seemed to be localized mostly to a specific side in the perinuclear region in the cytoplasm of HUVECs which is consistent with some studies which have found eNOS mRNA to be sometime associated with the Golgi complex [36]. Dual FRET MB targeting the distal region of eNOS also showed this localization pattern (Figure 2.11). IL-10 expression in HMSCs seemed to be distributed in the perinuclear region and also showed some association with the ribosomal regions (Figure 2.10).
Figure 2.9 Detection of eNOS mRNA region in live HUVECs using eNOS MB. Cells were transfected using 0.5 µg peNOS-polyplexes. After 2d, 300 nM eNOS and random (control) MB were delivered via SLO and cells allowed to reseal for 1h then imaged (A) Fluorescence and bright field image overlap showing FAM MB signals in non transfected and transfected cells, (B) The mean fluorescent intensity ± standard error of the mean, n=40 cells, p< 0.05.
**Figure 2.10** Detection of IL-10 mRNA region in live HMSCs using IL-10 MBs. Cells were transfected using 1 µg pIL-10-polyplexes. After 2d, 400 nM of IL-10 and random (control) MBs were delivered via SLO and cells allowed to reseal for 1h then imaged (A) Fluorescence and bright field image overlap showing FAM MB signals in non-transfected and transfected cells, (B) The mean fluorescent intensity ± standard error of the mean, n=15 cells, p< 0.05.
Figure 2.11 Detection of eNOS mRNA region in live HUVECs using Dual FRET eNOS MB. Cells were transduced at a MOI of 100 for 48h using adenovirus with no gene sequence (control) or eNOS gene sequence (adeNOS). After 2d, a total of 400 nM of beacons were delivered using SLO. Fluorescence and bright field image overlap showing Cy5 MB signals.
2.9 Conclusions

FAM-labelled molecular beacons targeting human eNOS and IL-10 mRNAs were designed, and detected their targets with high specificity and sensitivity. The beacons were found to have high melting temperatures which allowed their use in living cell environments. Importantly, beacons were able to access their intracellular mRNA targets emitting fluorescence signal that was stable for 2-3 h.
2.10 References


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Chapter 2: Design, characterization and evaluation of molecular beacons


CHAPTER 3: MONITORING mRNA EXPRESSION IN LIVING CELLS IN A 3D IN VITRO MODEL

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Monitoring mRNA in Living Cells in a 3D in vitro Model using TAT-peptide Linked Molecular Beacons.


http://pubs.rsc.org/en/content/articlelanding/2011/lc/c1lc20447e
3.1 Introduction
Traditionally the two–dimensional (2D) cell culture system have led to many significant findings in cell and molecular biology, however these systems cannot fully capture the complex and dynamic three dimensional (3D) environment of most living cells[1, 2]. Three dimensional cell culture systems have been shown to better mimic gene expression and other biological processes observed in living organisms[3], thus bridging the gap between 2D cellular assays and animal models of disease[4-6]. The goal therefore of 3D systems is for translation to clinic. Thus, there is a growing need for the development of in vitro 3D cell culture models for assessing newer therapeutics for clinical applications, and mechanisms of human pathology [7].

RNAs have many important functions in living cells from physically conveying and interpreting genetic information, providing structural support for molecular machines, catalyzing chemical reactions, to regulation of gene expression [8]. These functions are controlled through the expression levels and the spatiotemporal distribution of the specific RNA in the cell. However, RNA detection in cells is routinely performed by methods such as reverse transcription-polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH). RT-PCR relatively quantifies gene expression of a cell population from extraction of purified RNA obtained from cell lysates. However, cell lysates give no insight into the spatial and temporal distribution of RNA within the single cell [9]. Furthermore, RNA purification, cDNA synthesis, and other processing techniques performed before PCR analysis can result in loss of RNA transcript.[10, 11] FISH requires fixation of cells. Recent advances in FISH allow for the visualization of gene expression at the single molecule level [12-15]. However, this technique provides very limited temporal resolution of RNA expression [8].

Molecular beacons (MB) technology [16] uses dual–labelled single-stranded antisense oligonucleotide probes that fluoresce on hybridization with target nucleic acids. The probes are `stem-looped-shaped designed with the complementary sequence for the target nucleic acid of interest on the loop while the fluorophore and quencher are held in close proximity by the complementary nature of the 5’ and 3’-ends of the stem. The stem undergoes conformational change on hybridization with complementary target nucleic acids.
The major advantage to MB lies in the ability to monitor, detect and localize specific mRNA expression in live cells that can be visualized in real-time [17]. These probes show high specificity for their targets and have been used to visualize the localization [18], distribution and transport [19], and to monitor specific mRNA expression levels [20, 21] at the single cell level in living cells in two-dimensional (2D) monolayer cell culture.

To accurately detect intracellular RNA molecules, efficient delivery of molecular beacons is critical. There are several techniques used for the intracellular delivery of molecular beacons [8]. TAT peptides have a distinct advantage that these peptides can deliver biomolecules [22] into cells with a relatively quick targeted delivery [23] mechanism with nearly 100% efficiency [24]. Also, the TAT-mediated self-delivery of biomolecules is ideal for an in vitro 3D cell culture system as other methods, such as electroporation, cell membrane permeabilization, and microinjection are not practical.

Probes with DNA backbone are prone to degradation by nuclease [25], and their target RNA are susceptible to degradation by RNase H [26], unlike probes with 2’-O-methyl backbone [27, 28]. Also, phosphorothioate backbone [29], and lock-nucleic acids (LNA) [30] MBs are nuclease resistant. However, backbone chemistry modifications alter the thermodynamics of probe hybridization and should be assessed carefully [31].

In this work, molecular beacons targeting glyceraldehyde-3 phosphate dehydrogenase (GAPDH) mRNA was used. GAPDH is a highly expressed multifunctional cell protein involved in various biological processes such as DNA repair and replication, nuclear RNA transport, translational regulation, microtubule bundling and apoptosis [32]. GAPDH is widely used as a housekeeping gene for gene analysis in PCR and microarray. To down regulate and thus monitor changes in GAPDH mRNA expression, small interfering RNAs (siRNAs), double-stranded nucleic acid molecules capable of silencing gene expression [33] were used. Effectively, siRNAs target sequence specific cytoplasmic mRNAs and initiate their degradation by RNA interference (RNAi).

The purpose of this study was to demonstrate that molecular beacons can monitor change in gene expression in live cells in a 3D scaffolding system. The specific
objectives were to detect and monitor changes in GAPDH mRNA levels in 3D type I collagen scaffold and assess the relationship between RT-PCR and MB detection.

3.2 Materials and methods

3.2.1 Cells and culture conditions

Human foetal foreskin fibroblasts (HFFF2) and HeLa cells were used in this study. HFFF2 were purchased from European collection of cell culture (Salisbury, UK). The cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Sigma) supplemented with 10% foetal bovine serum (FBS; Sigma), containing 100 U/ml of penicillin, 100 mg ml1 streptomycin. The fibroblasts used in this study were between passages 22–26.

3.2.2 Molecular beacon design and synthesis

Unmodified and modified molecular beacons designed in previous studies [35] with 2’-deoxy DNA chemistries were synthesized by Eurofins MWG Operon (AL, USA and Ebersberg, Germany). The GAPDH (glyceraldehyde 3-phosphate dehydrogenase) MBs, as well as a “random” sequence MB (serves as negative control) possessed Cy3 fluorophore at the 5’-end and Black Hole Quencher 2 (BHQ2) at the 3’-end. The 3rd base from the 3’ end of the quencher arm of the MB was modified with a nucleotide dT-amine group linked through a 6-carbon spacer [35]. The beacon design for targeting GAPDH mRNA is shown in Table 3.1.

3.2.3 MB conjugation with TAT-peptide

The MB were conjugated following a protocol previously described [36]. Briefly, 200 µM of sulfo-succinimidyl-4-((N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC, Pierce Biotechnology), a heterobifunctional crosslinker, was reacted for 3 h with dT-amine group of the MB (10 µM) to create maleimide activated MBs. Excess Sulfo-SMCC was removed from the reaction using Amicon® Ultra-0.5 ml centrifugal filter devices (10k MW cutoff; Millipore). The maleimide–activated MBs were reacted overnight at 4 ºC with 20 µM cysteine-modified TAT peptide (Cys-TAT (47-57), Anaspec, Inc). The cysteine modification at the C terminus allowed for direct linkage with the maleimide activated MB. Unreacted TAT peptide/MBs were
removed from the reaction using Amicon® Ultra-0.5 ml centrifugal filter devices.
### Table 3.1 Design of molecular beacons and primers

<table>
<thead>
<tr>
<th></th>
<th>Unmodified molecular beacons</th>
<th>Modified molecular beacons</th>
<th>bp</th>
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</thead>
<tbody>
<tr>
<td><strong>GAPDH</strong></td>
<td>5′-Cy3-CGACGGAGTCCTTTCCACGATACCACGTCG-BHQ2-3′</td>
<td>5′-Cy3-CGACGGAGTCCTTTCCACGATACCACG[AmC6-dT]CG-BHQ2-3′</td>
<td>29</td>
</tr>
<tr>
<td><strong>Random</strong></td>
<td>5′-Cy3-CGACCGCAAGCGCACCGATACGTCG-BHQ2-3′</td>
<td>5′-Cy3-CGACCGCAAGCGCACCGATACG[AmC6-dT]CG-BHQ2-3′</td>
<td>27</td>
</tr>
<tr>
<td><strong>Primers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>18S</strong></td>
<td>5′-GACTCAACACGGGAAAACCTCAC-3’ (forward)</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>5′-TAAGAACGGCCATGCACCAC-3’ (reverse)</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>5′-GTCAGCCGCATCTTTTTTGC-3’ (forward)</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>5′-GCGCCCAATACGACCAAATC-3’ (reverse)</td>
<td></td>
<td>20</td>
</tr>
</tbody>
</table>

The bases that bind to create the stem regions of the molecular beacons are underlined.
3.2.4 Solution MB: target hybridization assay
To determine whether the function of the molecular beacon was altered during peptide conjugation, hybridization assays in solution were performed as previously described [35]. Briefly, 200 nM modified and peptide-linked molecular beacons were each reacted with 1x PBS buffer or 1 µM of complementary DNA (cDNA) target and incubated at 37 ºC for 30 min. The fluorescence intensity was measured using the Safire fluorescence microplate reader (Tecan, Zurich, Switzerland) with excitation wavelength at 525 nm and emission fluorescence signal scanning from 565 to 680 nm wavelength.

3.2.5 Preparation of type I collagen
Collagen type I was extracted in the laboratory from bovine tendons using an acetic acid extraction method previously described elsewhere [34]. The concentration and purity of the collagen was determined using Sircol™ soluble collagen assay and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively. A 5 mg/ml concentration was prepared using 0.5M acetic acid.

3.2.6 Preparation of type I collagen scaffolds
To prepare 1 mg/ml three-dimensional collagen type I scaffolds, 5 mg/ml collagen was diluted on ice with DMEM and the pH adjusted to 7 with 1N NaOH. A 30-50 µl suspension of HFFF2 was incorporated into the neutralized collagen solution to a final concentration of 1.0 x 10^5 HFFF2/ml. Aliquots of the resulting mixture were loaded into multi-well tissue culture plates or 4-well chambered cover glass (NUNC), and incubated at 37ºC for 20 min for scaffold formation. The 3D scaffolds produced were about 1 mm in thickness and were attached to the wells. The top of the scaffolds were covered with antibiotic-free growth medium. Scaffolds were cultured for 18 h before transfection.

3.2.7 Transfection of siRNA
Cells were harvested 18 h before transfection with 0.25% trypsin/EDTA, re-suspended in antibiotics-free growth medium and seeded into collagen scaffolds (see above) or multi-well plates and 4-well chambered cover glass for static monolayer. Transfection of siRNA were performed using Lipofectamine™2000 (Invitrogen)
according to the manufacturer’s instructions. Cells were transfected with 40 to 100 nM Silencer® GAPDH siRNA (Ambion) or 40 nM to 100 nM Silencer® Negative Control #1 siRNA (Ambion) and incubated for 24, 48, or 72 h before analysis. GAPDH mRNA level was monitored by RT-PCR and molecular beacon imaging.

### 3.2.8 RNA extraction and RT-PCR

Static monolayer and 3D collagen scaffolds (960 µl) were prepared in 6-well plates (Sarstedt) at a seeding density of 1.0 x 10⁵ cells per well. Transfected cells were homogenized in 1ml of Tri reagent® (Ambion), the RNA layer extracted using 200 µl of chloroform, and RNA precipitated with 70% ethanol. Total RNA was purified using the RNeasy® Mini kit (Qiagen) and on column treated with DNase-1 (Qiagen). Total RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, DE, US). The quality of the total RNA was determined using the Agilent bioanalyzer 2100 instrument and RNA Nano Chip kit (Agilent) according to manufacturer’s instruction. All RNA samples were of good quality with relative integrity numbers (RIN) greater than 9.0. cDNA was transcribed using Improm-II™ Reverse Transcriptase and random primers from Promega (UK) and 500 ng of total RNA. The 20 µl sample volumes were reverse-transcribed at 25 ºC for 5 min, 42 ºC for 60 min, and 70 ºC for 15 min. All samples were stored at -20 ºC for future use. cDNA was diluted and 1µl of template was reacted with 1 µl each of 3µM GAPDH primers (Table 3.1), 2 µl water and 5 µl Fast SYBR® Green PCR mix (Invitrogen). Human 18S rRNA was used as the normalizing gene (Table 3.1). Each experiment was performed in triplicate. PCR was performed using StepOne Plus Real Time PCR System (Applied Biosystems) under the following conditions: activation at 95º C for 20s; followed by 40 cycles at 95º C for 3s, 60º C for 30s, and dissociation at 95º C for 15 s, 60º C for 60s, and 95º C for 15s.

### 3.2.9 Cellular delivery of molecular beacons via activated SLO

3D collagen scaffolds loaded with HFF2 cells were prepared in 24-well plate at a seeding density of 20,000 cells per well. Scaffolds were incubated for 15 min with serum-free medium (200 µl) containing 0.4 U/ml activated SLO and 1 µM GAPDH or random MB for cell permeabilization and delivery of MB. After removal of SLO medium, complete growth medium (500 µl) was added for 2h before imaging.
3.2.10 Cellular delivery of peptide-linked molecular beacons
Static monolayer and 3D collagen scaffolds were prepared in 4-well chambered cover-glass at a seeding density of 20,000 cells per well. At 48h post transfection, medium was replaced with 150 µl of fresh antibiotic-free medium containing peptide-linked MB at 400 nM final concentration. Cells in monolayer or 3D scaffolds were re-incubated at 37 °C/ 5% CO₂ for 30 min or 1 h, respectively, in the dark to complete MB delivery. Incubation medium was removed and cells or scaffolds were rinsed twice with 1x PBS. Fresh antibiotic-free medium was added and confocal imaging was performed immediately.

3.2.11 3D-image reconstruction
Imaging was performed using the Zeiss Axiovert LSM-510 confocal microscope equipped with 40x C-Apochromat/1.2 W corr objectives (Zeiss). Beacons were visualized with HeNe laser at excitation of 543 nm with signal detection at band-pass filter from 565-615 nm for Cy3. Images were 512 × 512 pixels and acquired with the optical pin hole set at 2.76 Airy units for single plane images and 2.05 Airy units for the Z-stack. The Z-stack image was acquired from 3D collagen scaffold using 1.0 µm optical sections. The Z-stack image was reconstructed to a 3D image for visualization using the ZEN 2009 Light Edition software (Zeiss).

3.2.12 Quantification of imaging data
Mean fluorescence intensity (MFI) for each cell was quantified using Volocity software (Perkin Elmer). Measurements were performed in the single plane for cells in 2D culture and in extended focus view for cells in the 3D scaffold. The extended focus is selected from the image view option of the Volocity software and creates a single image from the brightest-point merge of all the slices in the z-stack (see Appendix A) No Z stack images were acquired in 2D. The Z-stack images acquired in 3D scaffold were reduced to z-stacks containing each individual cell before analysis. To calculate MFI, the outer membrane of the cell was traced using the lasso tool and the selected region measured. This method was performed for negative control and siRNA knockdown cells. The MFI of the background pixel from a
section of the field of view not containing any cells was used for background subtraction.

### 3.2.13 DNA content

The amount of DNA was assessed to determine the cell number post-transfection. The DNA content was determined using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen) assay following manufacturer’s protocol. Briefly, 200 µl volume collagen scaffolds were prepared in 24-well plates. For static monolayer, 10,000 cells/well were seeded into 48 well plates (NUNC). Both systems were rinsed twice with Hanks balanced salts solution (HBSS; Sigma) 48h post siRNA transfection. Scaffolds were dissolved in 50 µl of 1 mg/ml collagenase in DMEM medium for 30 min at 37º C. A 150 or 200 µl volume of double distilled water was added to each test well containing cells or scaffolds respectively, followed by three freeze-thaw cycles at -80º C and room temperature to release cellular DNA. The DNA content was determined using the PicoGreen® assay following manufacturer’s protocol. Fluorescence reading was performed using the Wallac Victor3™ 1420 multilabel counter (Perkin Elmer) at 485 nm excitation and 535 nm emission.

### 3.3 Results and Discussion

Molecular beacons have been successfully delivered in two-dimensional (2D) systems to monitor, detect, and localize specific mRNA expression in living cells at the single cell level. However, to date the use of molecular beacons in three-dimensional (3D) systems has not been reported. To translate this technology into specific clinical targeted applications, it is critical to develop and demonstrate efficacy in a 3D system.

#### 3.3.1 MB delivery to 3D scaffold via reversible permeabilization with SLO

The feasibility of SLO delivery of molecular beacons to 3D scaffolds was evaluated. The 3D scaffold used is relatively thin (~1 mm), has a low concentration of proteins (1mg/ml) and porous (pore size up surrounding cells around 3 µm (Figure 3.1). It was assumed that with this pore size, hindrance during diffusion of SLO (69 kDa) and MB (10 kDa) in scaffolds would be low and similar in magnitude to diffusion in
A resealing time of 1h was sufficient for HFF2 cells in monolayer to recover after SLO delivery of MB before imaging. However, at a time of 2h about 50% of the cells in 3D scaffold show GAPDH MBs signal and was true for non specific signal from control beacon (Table 3.2). While it may be possible to optimize the incubation and resealing times in scaffolds, the major limitation with reversible permeabilization technique was that removal of the pore-forming SLO solution resulted in destruction of the thin, fragile collagen scaffolds. It was thought that the best method to deliver MBs was one in which manipulation of the collagen scaffold was kept to a minimal. Thus, MBs were linked to TAT-peptide which allows self delivery inside cells.

3.3.2 Functionality of TAT- peptide conjugated MB

To demonstrate that conjugation of the modified MB with TAT peptide did not impair beacon function, hybridization assays were carried out in solution in the presence and absence of excess DNA target. The fluorescence intensity scanning the Cy3 emission wavelength (565 to 680 nm) was measured. The in-solution MB: target hybridization assay (red and black curves) for both beacons show a similar emission spectrum, as seen in Figure 3.2. This demonstrates that the functionality of the MB was not affected by conjugation. Furthermore, there is high signal to background ratio on hybridization of peptide-linked MB, as demonstrated by the 10-fold increase in fluorescence intensity at 570 nm.

3.3.3 Self-delivery and specificity of TAT peptide-linked MB

To demonstrate self-delivery and specificity of TAT peptide-linked MB targeting GAPDH mRNA, HeLa cells were first transfected as these cells are easy to transfect and proliferate rapidly in culture. Monolayer HeLa cells were transfected for 24h with 40 nM GAPDH siRNA which degrades cytoplasmic GAPDH mRNA, or 40 nM negative control siRNA which has no mammalian target sequence and thus served as a baseline for which down-regulation of GAPDH mRNA can be assessed. After 30 min incubation with TAT-peptide-linked MB, fluorescence signal was mainly localized close to one side of the nucleus (Figure 3.3). Not surprisingly, this signal was significantly lower in cells treated with GAPDH siRNA. Imaging acquisition
was completed 1-2 h after MB delivery and the random MB showed negligible background signal (Figure 3.3).
Figure 3.1 SEM image of 3D collagen scaffold.
Cells were incorporated into scaffolds for about 24h before preparation for SEM analysis. Protocol can be found in (Appendix III).
<table>
<thead>
<tr>
<th>Test</th>
<th>Signal</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells only</td>
<td>Not detected</td>
<td>1h</td>
</tr>
<tr>
<td>Cells+ GAPDH MB</td>
<td>Not detected</td>
<td>1h</td>
</tr>
<tr>
<td>Cells+ random MB</td>
<td>Not detected</td>
<td>1h</td>
</tr>
<tr>
<td>Cells + SLO delivered GAPDH MB</td>
<td>Detected</td>
<td>1h</td>
</tr>
<tr>
<td>Cells + SLO delivered random MB</td>
<td>Not detected</td>
<td>1h</td>
</tr>
<tr>
<td>Scaffold only</td>
<td>Not detected</td>
<td>2h</td>
</tr>
<tr>
<td>Scaffold + GAPDH MB</td>
<td>Not detected</td>
<td>2h</td>
</tr>
<tr>
<td>Scaffold + random MB</td>
<td>Not detected</td>
<td>2h</td>
</tr>
<tr>
<td>3D scaffold+ GAPDH MB</td>
<td>Not detected</td>
<td>2h</td>
</tr>
<tr>
<td>3D scaffold random MB</td>
<td>Not detected</td>
<td>2h</td>
</tr>
<tr>
<td>3D scaffold + SLO delivered GAPDH MB</td>
<td>Detected</td>
<td>2h</td>
</tr>
<tr>
<td>3D scaffold + SLO delivered random MB</td>
<td>Detected</td>
<td>2h</td>
</tr>
</tbody>
</table>

**Table 3.2** Signal detection in cells and collagen scaffolds in the presence of molecular beacons and the absence or presence of activated Streptolysin O. The period of incubation of the activated SLO and/or MB was 15 min for cells and 30 min for scaffolds. Cells were allowed to reseal for time shown before imaging. Imaging was typically completed within 1-2h. Cells incorporated into collagen scaffolds are termed 3D scaffolds.
Figure 3.2 Emission fluorescence spectrum for 200 nM modified MB and TAT-peptide-linked MB in the presence and absence of 1 µM DNA target.
Figure 3.3 Detection of GAPDH mRNA in transfected HeLa cells. Cells were transfected with 40 nM negative control or GAPDH siRNA for 24h. Fluorescent images of cells after 30 min incubation with 400 nM TAT peptide-linked molecular beacons, specific for GAPDH mRNA (GAPDH MB) or not specific for any mammalian target sequence (Random MB). Single plane images showing Cy3 signal for control and GAPDH knockdown cells and corresponding bright field image overlap.
3.3.4 GAPDH mRNA levels in 2D and 3D cultures using RT-PCR

As RT-PCR is a routinely used technique for mRNA quantification of cell lysates, it was used to validate MB results. The RT-PCR data shown in Table 3.3 confirms GAPDH mRNA knockdown seen in HeLa cells using MB (Figure 3.3).

GAPDH mRNA expression was monitored in transfected HFFF2 cells in 2D and in 3D cultures. To determine the optimal concentration of siRNA and transfection reagent required to achieve knockdown of GAPDH mRNA in HFFF2, transfection using 40 nM and 100 nM siRNA was performed using GAPDH siRNA or negative control siRNA at a single concentration of Lipofectamine™2000 for 24, 48, or 72 h. An untreated control without siRNA was included in each assay to assess non-transfection related phenomena. At the appropriate post–transfection time, the purified cell lysates were analyzed for GAPDH mRNA expression using RT-PCR. Cells transfected with 40 nM GAPDH siRNA for 24 or 48 h showed around 25% and 40%, knockdown, respectively, in GAPDH mRNA levels (Figure 3.4A). From RT-PCR data analysis, 48 h post-transfection with 100 nM GAPDH siRNA (Figure 3.4B) was chosen as the optimal transfection condition as 80% GAPDH mRNA knockdown was seen in HFFF2 cells. These conditions were used for further analysis with 3D collagen scaffolds.

Two methods were used to transfect HFFF2 cells in 3D. First, HFFF2 were embedded into scaffolds and then transfected in this 3D construct. RT-PCR data analysis showed about 35% GAPDH mRNA knockdown at 48 h post transfection with 100 nM GAPDH siRNA (Figure 3.5). This is significantly less than what is seen in 48 h transfection in 2D (Figure 3.4B). To demonstrate that this decrease in transfection efficiency is due to the inefficient delivery of siRNA molecules through the tight matrix of the collagen scaffold, a second transfection method was investigated. siRNA transfection is transient and may last up to 7 days depending on the cell proliferation rate; HFFF2 proliferates slowly hence the cellular monolayer was transfected for 24 h, followed by incorporation of the transfected cells into collagen scaffolds for another 24 h before performing RT-PCR. This second method resulted in about 80% GAPDH mRNA knockdown (Figure 3.5), which is similar to transfection in 2D.
### Table 3.3 GAPDH mRNA levels remaining in siRNA transfected HeLa cells.

Cells were transfected with 40 nM negative control or GAPDH siRNA for 24h before RT-PCR analysis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative % GAPDH mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 nM negative control siRNA</td>
<td>100</td>
</tr>
<tr>
<td>40 nM GAPDH siRNA</td>
<td>8 ± 2</td>
</tr>
</tbody>
</table>

± Standard error of the mean
Figure 3.4 GAPDH mRNA levels in siRNA knockdown HFFF2 cells in 2D culture systems. Cells were transfected with (A) 40 nM or (B) 100 nM negative control or GAPDH siRNA. At the various post transfection time points (24, 48, or 72 h) cell lysates were extracted and analyzed for GAPDH mRNA levels using RT-PCR. The percentages of GAPDH mRNA expression relative to the control ± standard error of the mean are shown.
Figure 3.5 GAPDH mRNA levels in siRNA knockdown HFFF2 cells in 3D collagen scaffolds.

Cells were transfected with 100 nM negative control or GAPDH siRNA. At 48h post transfection, cell lysates were extracted and analyzed for GAPDH mRNA levels using RT-PCR. Cells were either transfected directly in 3D scaffold for 48 h, or cells were transfected for 24 h in 2D then seeded into 3D scaffolds for another 24 h before analysis. The percentages of GAPDH mRNA expression relative to the control ± standard error of the mean are shown (n=3 for each).
3.3.5 GAPDH mRNA levels in 2D and 3D cultures using MB

To evaluate the results from RT-PCR, we delivered 400 nM GAPDH specific TAT-peptide linked MB to HFFF2 cells in 2D and 3D. A random TAT-peptide linked MB with no known mammalian target sequence was included as the negative control beacon. At 48 h post transfection, HFF2 cells were incubated for 30 min with 400 nM peptide-linked ‘random’ MB or 400 nM peptide–linked GAPDH MB. Molecular beacon signal was detected with GAPDH specific TAT-peptide linked MB, while negligible signal was observed with random beacon (Figure 3.6A) 1-2h after MB delivery. This demonstrates specificity of GAPDH peptide linked MB for GAPDH mRNA targets. The localization pattern for GAPDH mRNA in HFFF2 cells appears distributed throughout the cell cytoplasm and excludes the nucleus. Furthermore, GAPDH MB signal seemed to decrease in GAPDH knockdown HFFF2 cells compared to control cells which indicate transfection had occurred. Using one-way ANOVA, comparison of the MFI of the single plane image data from the 2D culture showed that fluorescence intensity level in control cells was significantly different to signal from GAPDH knockdown cells (Figure 3.6B).

Cells were transfected directly in collagen scaffolds or transfected in 2D for 24 and incorporated into collagen scaffolds for another 24 h. At 48h post transfection, 3D constructs were incubated with TAT-peptide linked MB for 1h then imaged as no signal was detected in the cells after 30 min incubation. Direct transfection of cells within the scaffolds appeared to be less efficient compared to 2D with no obvious difference between GAPDH mRNA signal localization and intensity in control and GAPDH knockdown cells as seen in Figure 3.7. This is a limitation with a qualitative approach where a small change in signal intensity may not be obvious to the eye. Image analysis of the data may help elucidate the difference. On the other hand, for cells transfected in 2D for 24 and incorporated into collagen scaffolds for another 24 h, GAPDH mRNA signal in control cells is distinct and distributed throughout the cytoplasm while the signal in GAPDH knockdown cells is low and located close to the nucleus (Figure 3.8). The signal emitted from random peptide linked MB in 3D scaffolds was low 2-3 h after MB delivery.
Figure 3.6 GAPDH mRNA expression in post-transfected HFF2 cells in 2D cell culture system using 400 nM GAPDH specific TAT peptide-linked molecular beacons. Cells were transfected with 100 nM negative control or GAPDH siRNA for 48h. (A) The beacons signals distributed throughout the cytoplasm, but excluded the nucleus. The Cy3 signal is seen for each cell. (B) Mean fluorescence intensity ± standard error of the mean of control cells (n=33) versus GAPDH knockdown cells (n= 28), p < 0.05.
Figure 3.7 Molecular beacon detection of incomplete knockdown of GAPDH mRNA in HFF2 cells transfected directly in 3D collagen scaffolds. Cells in scaffolds were transfected with 100 nM negative control or GAPDH siRNA. At 48h post transfection, cell were incubated for 1h with 400 nM TAT peptide-linked molecular beacons targeting GAPDH mRNA (GAPDH MB) or not targeting any mammalian sequence (Random MB). Single plane images showing Cy3 signal for control and GAPDH knockdown cells and corresponding bright field image overlap.
Figure 3.8 Molecular beacon detection of GAPDH mRNA knockdown in HFF2 cells transfected in 2D and seeded into 3D collagen scaffolds. Cells were transfected with 100 nM negative control or GAPDH siRNA for 24h, harvested and seeded into collagen scaffolds for another 24h. At 48h post transfection, cells were incubated for 1h with 400 nM TAT peptide-linked molecular beacons targeting GAPDH mRNA (GAPDH MB) or not targeting any mammalian sequence (Random MB). Single plane images showing Cy3 signal for control and GAPDH knockdown cells and corresponding bright field image overlap.
3.3.6 DNA content
HFFF2 cells were transfected for 48 h using 2 µg/ml of Lipofectamine™2000 to deliver 100 nM of siRNA. To ensure that the transfection conditions maintained an acceptable level of cell viability, the DNA content of HFFF2 in 2D and 3D was analyzed using the PicoGreen® assay, as an indicator of cell number post transfection. The DNA content from transfected cells was compared to untreated control after 48h. Transfection with GAPDH siRNA reduces GAPDH mRNA and protein levels, producing slow growth rates and reduced proliferation in most cell types. Although this was more evident in 2D cultures (Figure 3.9A), than in 3D (Figure 3.9B), there was no significant difference between cell numbers in transfected and untreated cells in 2D or 3D, using one way ANOVA data analysis.

3.3.7 Spatial distribution of cells within the scaffold
The location of cells within the 3D constructs may influence spatiotemporal gene expression levels (discussed in Chapter 1). To visualize the spatial location of mRNA in cells in 3D, a Z-stack image of a 140 µm thick section from the bottom of the scaffold was performed. The 3D view of the Z-stack is shown in Figure 3.10A. MB signals in HFFF2 cells embedded in the 3D collagen scaffold show the spatial distribution of cells within the scaffold.
Mean fluorescence intensity level in GAPDH knockdown cells in the 3D scaffold was significantly different to signal from control cells using ANOVA one-way statistical analysis of the image data (Fig. 3.10B).

3.3.8 Comparison of GAPDH mRNA levels RT-PCR vs. MB
Comparison of GAPDH mRNA detection techniques for HFF2 cells transfected first in 2D then seeded into 3D collagen scaffolds showed that the average GAPDH mRNA knockdown in cells based on MFI is 72% (Figure 3.10B). This value agrees closely to the 78% calculated from RT-PCR (Figure 3.5).
Figure 3.9 DNA content of HFFF2 cells after 48h transfection. (A) 2D culture system and (B) 3D collagen scaffolds. (n=3 for each).
Figure 3.10 A

<table>
<thead>
<tr>
<th>Cell</th>
<th>Optical slice depth (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell 1</td>
<td>14-22</td>
</tr>
<tr>
<td>Cell 2</td>
<td>29-35</td>
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<tr>
<td>Cell 3-6</td>
<td>31-41</td>
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<td>44-55</td>
</tr>
<tr>
<td>Cell 9</td>
<td>94-101</td>
</tr>
<tr>
<td>Cell 10</td>
<td>106-118</td>
</tr>
</tbody>
</table>
Figure 3.10 B

**Figure 3.10** Three dimensional view of spatial distribution of HFFF2 seeded into collagen scaffold with detection of GAPDH mRNA using 400 nM peptide-linked MB. (A); Cells distributed within a 140 µm section of the scaffold. The optical slices containing the numbered whole cell (1-10) in the 140 µm section is indicated in the legend. (B); Image analysis data showing mean fluorescence intensity (MFI) from control and GAPDH mRNA knockdown cells. Values shown are MFI ± standard error of the mean, p < 0.05, n=15 cells
3.4 Conclusions

For the first time the use of TAT-peptide conjugated MB to monitor mRNA in a 3D \textit{in vitro} system has been reported. Specifically, we have shown that TAT-peptide linked molecular beacons can monitor GAPDH mRNA expression in 3D type I collagen scaffold and delivery can be completed with fast kinetics (~1h). Furthermore, spatial distribution of cells in 3D can be visualized. MB technology can be used to detect mRNA abundance in cells in 3D, but it is necessary to perform quantification with another complementary technique such as RT-PCR. Molecular beacons show a clear advantage over RNA FISH in providing spatial and temporal location of mRNA in living cells within a 3D cell culture system without the need to physically section the sample. This research was carried out using MB with 2’-deoxy backbone chemistries, but there are various chemistry modifications that can be utilized each with its pros and cons [38].

Probe stability is essential for long term monitoring of gene expression inside living cells and to distinguish the true signal of the RNA targets from background signal. To address this problem, MB probes were design with nuclease-resistant backbone chemistries that limit susceptibility to degradation by intracellular nucleases. Further, recently developed variants of MB such as ratiometric biomolecular beacons [39] and Dual FRET MB [21] have shown improved sensitivity over conventional MB and could potentially be adopted for live cell RNA imaging in 3D. Understandably, successful detection of target mRNA by MB is hindered by a combination of factors such as target accessibility, probe specificity for target, efficiency of probe delivery inside cells, and the sensitivity of the imaging technique used.
3.5 References


CHAPTER 4: MONITORING THE EFFICACY OF DUAL GENE DELIVERY VIA 3D COLLAGEN SCAFFOLDS
4.1 Introduction
Cardiovascular diseases are the leading cause of death in the Western world and accounts for more than 17 million deaths globally (WHO 2012). Cell-based therapies have been investigated to promote tissue regeneration, but proved to be challenging due to cell death, low retention of cells at the site, and poor integration of cells with the native tissue [1]. Gene therapy is emerging as a potential therapeutic approach to address the challenges of cell-based strategies [2], with local gene transfer being a more effective therapy [3]. Targets routes for cardiovascular gene therapy include veins, arteries, myocardium, skeletal muscle of the lower limbs, and the liver.

An appropriate gene therapy approach for cardiovascular pathogenesis may require multiple genes to enhance therapeutic outcome by modulating inflammatory response and angiogenesis in a controlled manner. Interleukin 10 (IL-10) is a potent multifunctional cytokine produced by a variety of cells [4]. It plays a crucial role in vivo in the attenuation of immune and inflammatory responses [5]. On the other hand, endothelial nitric oxide synthase is an inducible gene expressed in vascular endothelial cells (e.g. HUVECs) and few other cells [6, 7]. It is an enzyme that catalyses the conversion of the amino acid L-arginine to L-citrulline to produce nitric oxide (NO), a potent vasodilator and mediator of angiogenesis and arteriogenesis [8]. NO has multiple biological functions and plays an important role in cardiovascular homeostasis [9, 10].

Intracellular delivery of the genetic materials is the main challenge to specific and efficient gene therapy. There are two delivery systems available for gene transfer, viral and non-viral. Generally, non-viral vectors do not transfer gene material as efficiently as viral vectors [11]. However, non-viral vectors, typically plasmids, are considered safer as they generally exhibit lower toxicity and lower immune responses and do not integrate into the genome [12]. Among the agents used form complexes with plasmid DNA and facilitate cellular uptake and transfection, Superfect™, formulated from partially degraded (fractured) dendrimers, is one of the optimal [13]. Fractured dendrimers rupture endosomes to allow the escape of the plasmid DNA from degradation. Dendrimers are a class of polymers consisting of highly branched 3D macromolecules usually presenting well define sizes and structures. The terminal groups exhibit high surface area presenting multiple sites for
attachment of the plasmids. These properties make dendrimers potential carrier candidates for gene delivery.

Plasmid DNA provides transient gene expression. However, sustained gene expression can be facilitated using biomaterial scaffolds which have the potential to maintain an effective level of the plasmid for the required time [14]. Methods in which the plasmid DNA complexes are entrapped or encapsulated within the scaffold generally release complexes via the process of diffusion and scaffold degradation [15]. The loaded scaffold comes into close contact with target cells or tissues and enables localized delivery of the DNA that is released in a controlled and sustained manner. Collagen-based scaffolds have been employed for both non-viral and viral therapeutic gene delivery [16].

Based on the review literature Chapter 1, molecular beacons can provide a spatiotemporal pattern of mRNA expression in living cells in real time within a short period of time. Thus, MB technology may represent a quick and informative method to visualize the efficacy of gene-transfer from 3D biomaterial scaffolds. MB can provide some info into localization pattern of mRNA expression levels in the individual cells transfected over a period of time.

In this study a gene therapy model for cardiovascular tissue engineering was evaluated for efficacy. A dual gene release collagen-based scaffold loaded with pIL-10 polypelexes and peNOS polypelexes encapsulated into microspheres was used to transfect HUVECs and HMSCs. The therapeutic efficacy of the system over time was monitored for eNOS and IL10 expression at 2, 7, and 14d using RT-PCR and molecular beacon technology. The MB designed in Chapter 2 were used

4.2 Materials and methods

4.2.1 Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and cultured in endothelial basal medium (EBM-2) supplemented with the EGM-2 bullet kit (Lonza). Human mesenchymal stem cells (HMSCs) were obtained from the Regenerative Medicine Institute, National University of Ireland, Galway and were maintained in MEM α, medium (Gibco) supplemented with 10% research grade FBS
4.2.2 Molecular beacon design and synthesis
Molecular beacons labelled at the 5’-end with FAM fluorophore and the 3’-end with BHQ 1 and targeting a human eNOS or IL-10 mRNA were designed in Chapter 2. The sequences are shown in Table 4.1.

4.2.3 Preparation of polyplexes
A human eNOS gene sequence encoded into a pcDNA3 vector containing the CMV promoter was a kind donation from Dr. Karl McCullagh (Regenerative Medicine Institute, National University of-Ireland-Galway, Ireland). The plasmid encoding human IL-10, pORF-hIL10, was purchased from InvivoGEN (CA, USA). Gaussia luciferase plasmids (GLuc; New England Biosciences, Ipswich, USA) were Cy3-labeled using a Cy3 labeling kit (Mirus, Madison, USA). Plasmids were combined with Superfect® (3mg/ml, Qiagen) to form polyplexes at ratio between Superfect® and plasmid DNA (µL:µg) of 3:1. The molar ratio of the nitrogen (N) of SuperFect® to the phosphate (P) of pDNA (N: P ratio) influences transfection efficiency and cytotoxicity and thus needs to be optimized for each cell type and the experimental system. A 6 µl volume of Superfect® was mixed with 2 µg of plasmid DNA and allowed to form polyplexes for 5-10 min at room temperature.

4.2.4 Fabrication of microspheres loaded with polyplexes
Hollow collagen microspheres were fabricated in our laboratory by PhD student, Shane Browne using a template method described elsewhere [17-20]. The microspheres were prepared from 1 µm polystyrene beads, cross-linked using pentaerythritol poly(ethylene glycol) ether tetrusuccinimidyl glutarate (4S-PEG), and the polystyrene core was dissolved to create hollow spheres by washing with tetrahydrofuran (THF). Plasmid (2 µg) eNOS polyplexes were encapsulated into the 1 µm hollow. The spheres were sterilized by the addition of 250 µl absolute ethanol and centrifuged at 13,000 rpm for 5 min. The supernatant was discarded.
4.2.5 Fabrication of dual gene releasing collagen constructs

Collagen type I was extracted in the laboratory from bovine tendons using an acetic acid extraction method previously described elsewhere [21]. A 5 mg ml\(^{-1}\) concentration was prepared using 0.5M acetic acid. To prepare 4 mg ml\(^{-1}\) type I scaffolds, 5 mg ml\(^{-1}\) collagen was diluted on ice with 10X PBS, the pH adjusted to 7.0 using 2N NaOH. Solutions containing 2 µg pIL10 polyplexes were added to 2 µg peNOS-polypelexes encapsulated collagen microspheres before adding 100 µl of collagen solution and mix gently by pipetting.

4.2.6 Plasmid DNA release studies

Single gene loaded collagen constructs (see above) were prepared using Cy3 labelled GLuc-polypelexes. Briefly, 100 µl of collagen solution was mixed with Cy3 labelled GLuc-polypelexes or Cy3 labelled GLuc-polypelexes encapsulated into microspheres and pipetted into 48-well plates (Nunc). After gelling for 30 min at 37°C/5% CO\(_2\), 300 µl of HBSS medium was added and plates were re-incubated and protected from light. Medium was replaced at time points from 1-14d and collected medium was kept at -20 °C until the final time point. A standard curve was prepared with quantification of plasmid DNA polypelexes released from the scaffold measured at excitation of 528 nm and emission ranging from 570-610 nm using the FLx800 Fluorescence Microplate Reader (BioTek, U.K).

4.2.7 Gene delivery via 3D collagen scaffolds

Human MSCs and HUVECs were seeded into 8-well chambered cover glass (Nunc), 24-well glass bottom plates (MatTex Corporation, MA, USA) or 6-well plates the day before gene delivery. The Nunc 8-well chambered cover glass provided superior optical property. The medium was removed and 25 (1µg) or 100 (4µg) µl of dual gene polypelexes loaded or “empty” collagen mixture were added to each well and incubated for 30 min at 37°C/5% CO\(_2\) for gel formation. Untreated cells and cells treated with the “empty” collagen mixture without the polypelexes served as controls. After gelling, complete medium was added to each well and cells cultured for 2, 7, and 14d before analysis for eNOS and IL-10 expression using RT-PCR and molecular beacon technology. The media was changed every 2-3days for HUVECs and every 4 days for HMSCs.
4.2.8 Molecular beacon detection of eNOS and IL10 mRNA
HMSCs and HUVECs were seeded into 24-well glass bottom plates and 8-well chambered cover glass, respectively, at a density of 1 x 10⁴ cells per well. At 2, 7 and 14d post transfection via 1 µg dual gene loaded collagen scaffolds (see above), cells were analysed for transcription of eNOS and IL-10. Molecular beacons targeting eNOS or IL-10 mRNA were delivered to cells via reversible permeabilization using Streptolysin O (SLO) as previously described [22]. Briefly, 2U/ml SLO (Sigma) was activated with 5 mM TCEP (Sigma) in HBSS (without calcium or magnesium, Sigma) for 30 min at 37°C. Cells were incubated for 10 min with 100 µl of serum-free medium containing 0.2U/ml SLO and 300 nM molecular beacons. Cells were allowed to reseal for 1h at 37°C before confocal imaging.

4.2.9 RNA extraction and RT-PCR
HMSCs and HUVECs were seeded into 6-well plates at a density of 1 x 10⁵ cells/well before transfection via 4 µg-dual gene loaded collagen scaffolds (see above). Total RNA was isolated using 1 ml of Tri reagent® (Ambion) and purified using the RNeasy® Mini Kit (Qiagen) and on-column treated using DNase I (Qiagen). The purity and quantity of the RNA was determined using NanoDrop 1000 spectrophotometer (NanoDrop Technologies, DE, US). cDNA was obtained using 500 ng of total RNA and the Improm-II™ Reverse Transcription system with random primers (Promega, UK). Real-time PCR was performed using StepOne Plus Real Time PCR system (Applied Biosystems) and Fast SYBR Green PCR kit (Applied Biosystems). Relative quantification of eNOS and IL10 was performed using the comparative CT method and human GAPDH as the internal standard. IL10 primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov), while previously published sequences for eNOS [23] and GAPDH [24] were used (Table 4.1). Each experiment was performed in triplicate.
Table 4.1 Probes and primers design

<table>
<thead>
<tr>
<th>Molecular beacons</th>
<th>5'-3' sequence</th>
</tr>
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<tbody>
<tr>
<td>eNOS</td>
<td>FAM-CACCGTGTAGTACTGGTTGATGAAACGGTG-BHQ1</td>
</tr>
<tr>
<td>IL-10</td>
<td>FAM-CGCAGGGGAAGAAAATCGATGCCTGCG-BHQ1</td>
</tr>
<tr>
<td>Random</td>
<td>FAM-CGACGCAGACAAGCGCACCAGATACGTCG-BHQ1</td>
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</tbody>
</table>

**Primers**

<p>| | |</p>
<table>
<thead>
<tr>
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</tr>
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<tbody>
<tr>
<td>eNOS_forward</td>
<td>CTGAGAGACCAGCAGAGATACCAC</td>
</tr>
<tr>
<td>eNOS-reverse</td>
<td>CTGAAGCTCTGGGTCTCTGAT</td>
</tr>
<tr>
<td>IL10-forward</td>
<td>TGAGGCTACGCCGCTGTCAT</td>
</tr>
<tr>
<td>IL10-reverse</td>
<td>TTCTTCACCTGGCTCCAGCCT</td>
</tr>
<tr>
<td>GAPDH-forward</td>
<td>GTCAGCCGCACTCTTTTTCG</td>
</tr>
<tr>
<td>GAPDH-reverse</td>
<td>GCGCCCAATACGCCAAAAATC</td>
</tr>
</tbody>
</table>

Molecular beacon stem is indicated in **bold caps** and the **underlined** bases were shared with the probe on hybridization.
4.3 Results and discussion

One of the aims of advanced biomaterials constructs is to deliver therapeutic molecules to cells in controlled and sustained manner. In particular cases where multiple genes are to be delivered, delayed release of one gene may be required. Our model sought to deliver IL-10, an anti-inflammatory cytokine, to cells followed by release of eNOS which promotes angiogenesis. An illustration of the experimental system is shown in Figure 4.1.

4.3.1 Characterization of the collagen scaffold delivery system

In order for the therapeutic gene to be effective it has to be functional at the target site and remain there for the required period. Thus the release profile for polyplexes in the scaffold was monitored for 14 days. The ability of collagen scaffold system to provide both sustained and delayed delivery was assessed using Cy3-labelled pGLuc polyplexes. A sustained released was observed for polyplexes loaded directly into collagen scaffold which displayed an initial release of about 50ng on day 1, followed by ~125 ng/day from days 2-8 and 60 ng/day from days 9-14. Total pDNA released from collagen scaffolds at day 14 ranged from 60-65%, as shown in Figure 4.2. Polyplexes encapsulated into collagen microspheres displayed a delayed release (<1 ng) over the first 2 days, followed by sustained released of ~18ng on day 3, ~75ng/day from days 4-8 and ~30ng/day from days 9-14. Total pDNA released from collagen microspheres at day 14 ranged from 45-50%, as shown in Figure 4.2.
Cells were seeded the day before gene transfer. A collagen scaffold loaded with IL-10 polyplexes and eNOS polyplexes encapsulated into collagen microspheres coats a portion of the cell monolayer. Polyplexes are released from by diffusion and degradation of the collagen (4mg/ml) scaffold and collagen microspheres. Collagen microspheres were cross-linked to exhibit delayed the degradation process. IL-10 and eNOS mRNA expression levels were evaluated at 2, 7 and 14d using RT-PCR and molecular beacons.
Figure 4.2 A representative plasmid DNA release over time in HBSS from polypelexes loaded collagen scaffold. 2µg of plasmid DNA polypelexes were loaded directly into the scaffold (Collagen scaffold) or encapsulated in microspheres then loaded into the scaffolds (Microspheres). Media was changed at the time point indicated and the amount of Cy3 signal from the DNA quantified and calculated as a percent of the total DNA loaded. Data show mean ± standard deviation (n=3).
4.3.2 Temporal expression levels of IL-10 and eNOS using RT-PCR

The efficiency of gene transfer from the dual-gene scaffolds was examined using primary HMSCs and HUVECs. Two controls were prepared with each experiment. The “No scaffold” control (cells not treated with the scaffold or polyplexes) was performed in order to assess scaffold related phenomena, while the “Empty scaffold” control (treated with the collagen scaffold not loaded with polyplexes) was performed to evaluate baseline mRNA levels. The IL-10 and eNOS mRNA expression in “No scaffold” controls were similar to “Empty scaffold” controls. To observe changes due to dual gene-transfer from the scaffolds loaded with both the 2µg polyplexes, IL-10 and eNOS mRNA expression levels were calculated relative to the day 2 “Empty scaffold” control. Differences in IL-10 mRNA expression levels were observed between the two cell types. HMSCs transfected with dual-gene scaffolds showed about 3-fold increase in IL-10 mRNA levels at day 7, relative to day 2, while day 14 levels were similar to day 2 levels (Figure 4.3A). Similarly, and relative to day 2, eNOS mRNA expression levels in the transfected cells were increased to about 4-fold at day 7 and to about-2-fold by day 14 (Figure 4.3B).

On the other hand, transfected HUVECs showed IL-10 mRNA levels at day 2 that were slightly higher (1.5-fold) than days 7 and 14 levels (Figure 4.4A). This is probably due to lower concentrations of IL-10 polyplexes available to cells due to twice as much media changes compared to HMSCs, lower transfection efficiency, or post-transcriptional regulation. It appears that diffusion is the prominent process of release of the IL-10 polyplexes from the non-cross-linked collagen scaffold. The eNOS mRNA levels in transfected HUVECs were increased by 7-fold at day 7 (relative to day 2) and levels had decreased at day 14 to about-2-fold that seen in day 2 (Figure 4.4 B). The slower release of polyplexes from cross-linked collagen microspheres may have contributed to the fold increase seen at day 7. In both HMSCs and HUVECs the levels of eNOS and IL-10 mRNA were significantly lower in empty scaffold controls which contained no polyplexes which may indicate quiescent levels of the mRNA in these cells [25, 26].
Figure 4.3 RT-PCR data showing the fold changes in (A) IL-10 and (B) exogenous eNOS mRNA levels in HMSCs at 2, 7 and 14 d after dual-gene delivery. Cells were treated with collagen scaffold loaded with 2 µg each of plasmid IL-10 polyplexes and plasmid eNOS polyplexes encapsulated into microspheres (Dual gene scaffold). Control cells were treated with collagen scaffold containing no polyplexes or microspheres (Empty scaffold control). The fold changes in eNOS and IL-10 mRNA expression levels relative to 2d control ± standard error of the mean are shown (n = 3 for each). Media was changed every 4 days.
Figure 4.4 RT-PCR data showing the fold changes in (A) IL-10 and (B) exogenous eNOS mRNA levels in HUVECs at 2, 7 and 14d after dual-gene delivery. Cells were transfected with collagen scaffold loaded with 2 µg each of plasmid IL-10 polyplexes and plasmid eNOS polyplexes encapsulated into microspheres (Dual gene scaffold). Control cells were treated with collagen scaffold containing no polyplexes or microspheres (Empty scaffold control). The fold changes in eNOS and IL-10 mRNA expression levels relative to 2d control ± standard error of the mean are shown (n = 3 for each). Media was changed every 2-3 days.
4.3.3 Spatiotemporal expression pattern of IL-10 and eNOS using MB

To evaluate the spatiotemporal efficacy of IL-10 and eNOS gene-transfer to HMSCs and HUVECs molecular beacons were delivered to the cells using SLO mediated delivery. In this experiment 0.5µg each of IL-10 and encapsulated eNOS polyplexes were loaded into the scaffold and delivered to cells (~10,000) in monolayer. “No scaffold” controls and “Empty scaffold” controls were prepared to evaluate collagen scaffold related phenomena. The presence of the collagen scaffold seemed to slightly increase baseline levels of IL-10 and eNOS mRNA expression in HUVECs more so than in HMSCs. IL-10 mRNA signals were observed in control and transfected HUVECs cell, and transfected cells seemed to exhibit increased signal intensities compared to control cells. Changes in signal intensity between days 2 to 14 were not evident with localization pattern that seems to be mainly perinuclear (Figure 4.5A). No signal was observed with the control (random) beacon which has no targets inside cells, suggesting the specificity of the IL-10 MB. The signal intensity from eNOS MB in transfected HUVECs showed low signal at day 2 which increased by day 7 and 14 (Figure 4.5B). Signal localization for eNOS mRNA appears to be perinuclear and at times concentrated to one region (probably associated with the Golgi) of the cell which is more evident at day 14.

IL-10 and eNOS MB signals from transfected HMSCs appeared more intense than controls (Figure 4.6A & Figure 4.6B). Signal intensities in HMSCs varied at the different time points with low signal at day 2 and higher signal intensities at day 7 and 14. In HMSCs, the localization pattern for eNOS mRNA appeared distributed throughout the cytoplasm, while IL-10 showed localization in the perinuclear and ribosomal regions.

4.3.4 Comparison of RT-PCR and molecular beacons technology

Both RT-PCR and MB data for IL-10 mRNA expression corresponded well with each other. The sequence of the eNOS plasmid used for gene-transfer has 85% homology with a human eNOS mRNA which made the selection of primers that detected total eNOS impossible. The MBs were design to detect total (endogenous and exogenous) eNOS and IL-10. The RT-PCR primers were specific for either plasmid (exogenous) eNOS or total IL-10 mRNA. Both RT-PCR and MB data
demonstrated low levels of eNOS mRNA at the day 2 time point and increases in mRNA expression at day 7.
Chapter 4: Monitoring the efficacy of dual gene delivery via 3D collagen scaffolds

Figure 4.5 A
Figure 4.5 Detection of total IL-10 (A) and total eNOS mRNA (B) in HUVECs using 300 nM MBs. Fluorescence signal from IL-10 (A) and eNOS (B) MBs at 2, 7 and 14d. Cells were transfected with the collagen scaffold loaded with 2 µg each of plasmid IL-10 polyplexes and plasmid eNOS polyplexes encapsulated into microspheres (Dual gene scaffold). Control cells were not treated with the scaffold or polyplexes (No scaffold), treated with the collagen scaffold not loaded with polyplexes (Empty scaffold). Media was changed every two to three days.
Chapter 4: Monitoring the efficacy of dual gene delivery via 3D collagen scaffolds

Figure 4.6 A
Figure 4.6 Detection of total IL-10 (A) and total eNOS (B) mRNA in HMSC using 300 nM MBs. Fluorescence signal from IL-10 (A) and eNOS (B) MBs at 2, 7 and 14d. Cells were transfected with the collagen scaffold loaded with 2 µg each of plasmid IL-10 polyplexes and plasmid eNOS polyplexes encapsulated into microspheres (Dual gene scaffold). Control cells were not treated with the scaffold or polyplexes (No scaffold), treated with the collagen scaffold not loaded with polyplexes (Empty scaffold). Media was changed every four days.
4.4 Conclusions

DNA-polyplexes encapsulated into collagen microspheres or collagen scaffolds remained functional and were able to transfect cells. Further, encapsulation of eNOS DNA polyplexes into cross-linked collagen microspheres resulted in delayed release of this gene to the cells. This was evident from eNOS MB images which showed low FAM fluorescence signal at day 2 in both transfected HUVECs and HMSCs, confirmed by RT-PCR data which showed the lowest eNOS mRNA expression at day 2. IL-10 mRNA expression for HUVECs remained at a relatively constant level, while that of HMSCs was increased to a maximum at day 7 decreasing to day 2 levels by day 14. The decrease in mRNA expression at day 14 could be explained by decreased concentration of polyplexes available to transfect an increased cell population (doubling time about 4d) and the transient expression of plasmid transfection (transfected cells undergo mitosis and dilution occurs as plasmid DNA is not passed on to daughter cell, or is degraded). However, the collagen scaffolds (4mg/ml) loaded with dual genes can provide sustained delivery of these genes for up to 14d as noted by the 50-1400 fold increases in mRNA levels from day 2-14 when compared to scaffold only control.

It was demonstrated that molecular beacons are able to monitor changes in mRNA levels at various time points in the presence of a 3D gene carrier scaffold, and the results were validated using RT-PCR.

4.5 References


CHAPTER 5: SUMMARY AND FUTURE STUDIES
5.1 Summary

In Chapter 2, the focus was on developing molecular beacons targeting therapeutic mRNAs of interest in cardiovascular tissue engineering. To achieve this aim, FAM-labelled molecular beacons targeting IL-10 and eNOS mRNA were designed and characterized in homogeneous solution then evaluated in living cells to investigate whether optical imaging of the respective mRNA was possible. These probes were introduced into cells via a reversible permeabilization technique using low concentration (0.2U/ml) of activated Streptolysin O, which forms pores in the plasma membranes that can be resealed by incubating cells for 1h in medium containing foetal bovine serum. The MBs were designed using a method that utilized the software Mfold, OligoWalk and BLAST. Mfold software was used to predict the secondary structures and the single stranded regions of the mRNAs of interest, while OligoWalk was used to select regions of the mRNA that bind tightly to the MB, and BLAST was used to ensure uniqueness of the selected sequence. The eNOS MB was designed with a 6-base-pair stem, while the IL-10 MBs were designed with a 5-base-pair stem.

To prove that the MBs designed were highly sensitive, the beacons were allowed to hybridize to target DNA, and demonstrated an increased in signal intensity with increasing DNA concentrations. To further demonstrate sensitivity of the beacons, the signal: background noise ratio of the beacons in the presence of target DNA was shown to be high and coincided with the high quenching efficiency of the fluorophore–quencher (FAM–BHQ1) pair selected.

Furthermore, characterization to evaluate the specificity and thermodynamic stability of the eNOS and IL-10 MB showed that the beacons can discriminate between targets over a range of temperatures and underwent correct phase transition characteristics typical of molecular beacons.

Using Streptolysin O mediated delivery, it was demonstrated that the IL-10 and eNOS MBs could specifically image endogenous (and exogenous with the same sequence) mRNAs in living cells by the increase of fluorescence signal seen compared to no signal in control (non target in mammalian cells) MB. This was further confirmed by the significant increases in mean fluorescent signal calculated in transfected relative to non transfected cells. Assessment of the intracellular signals in the cytoplasm of HUVECs
showed eNOS mRNA to be localized mainly to one side in the perinuclear region, while IL-10 mRNA in HMSCs appeared distributed in the perinuclear region.

The purpose of the study in Chapter 3 was to develop a molecular beacon detection platform for monitoring changes in transcription gene expression levels in living cells embedded into a 3D collagen scaffolding system. To achieve this goal, this study utilize a previous molecular beacon sequence specific for GAPDH mRNA, that have been successfully delivered in two-dimensional (2D) systems to monitor, detect, and localize this mRNA expression in living cells at the single cell level. The 3D scaffolding system developed had a thickness of ~1mm (based on the volume of collagen that resulted in attachment of the scaffolds to the corners of the wells) and collagen type I concentration of 1mg/ml (the lowest concentration of collagen that formed a stable gel when embedded with cells).

Using reversible permeabilization with SLO to deliver the MB proved to be a challenge as removal of the pore-forming SLO solution resulted in destruction of the thin, fragile collagen scaffolds. To overcome this limitation, TAT-peptide mediated delivery of the MB was performed. A stable linkage was formed between the MB and TAT-peptide and allowed for self delivery of the beacons. Also, delivery of TAT-peptide linked MB to 3D scaffold showed that a time of 1h was required before signal detection, and imaging can be completed within 1-2h after beacon delivery.

Comparison of the hybridization signal emitted by un-conjugated MB and TAT-peptide linked MB demonstrated conjugated MBs were functional. To validate that TAT-peptide linked MB were sensitive (able to detect different levels of mRNA expression) and specific for imaging mRNA in cells within the collagen scaffold, transfection was performed using siRNA to knockdown GAPDH mRNA levels. MB targeting of GAPDH mRNA in cells showed that direct transfection of siRNA into the 3D scaffold was not as efficient as transfection in 2D followed by incorporation of the cells into the collagen scaffold which was demonstrated in cells of the latter showing lower GAPDH mRNA expression (RT-PCR) and emitted lower signal intensities (MB technology). In fact, results showed that the mean fluorescent intensities (MFI) of GAPDH knockdown cells were significantly different from that of the negative control cells. Further, it was
demonstrated that 3D image analysis for MFI of the MB signal in the whole cell corresponded closely to results of RT-PCR. However, MB technology detected mRNA abundance in cells in 3D, but it is necessary to perform quantification with another complementary technique such as RT-PCR.

The final stage of this research (Chapter 4) was aimed at the evaluation of the efficacy of a dual-gene therapy model for cardiovascular tissue engineering. This gene therapy model consisted of a dual gene releasing 3D collagen scaffold loaded with pIL-10 polyplexes and peNOS polyplexes encapsulated into microspheres. The therapeutic efficacy of the system over time was monitored for eNOS and IL10 expression at 2, 7, and 14d using RT-PCR and MB technology. The FAM -molecular beacons designed to target therapeutic genes (Chapter 2) were utilized to monitor the spatiotemporal expression pattern of IL-10 and eNOS mRNA in gene transfected cells. The MBs were delivered using reversible permeabilization with SLO.

To validate the capability of the 3D scaffold to provide both a sustained and delayed release of polyplexes, in vitro release studies were performed over a 14 day period. Results showed that polyplexes loaded directly into collagen scaffold or encapsulated into microspheres and loaded into collagen scaffold exhibited sustained released of up to day 14, while the release of polyplexes encapsulated in microspheres was delayed with low levels being release for up to 2 days.

To demonstrate that IL10 and eNOS polyplexes released from the scaffold remained functional, the genes were loaded into the scaffold and the expression levels monitored at 2, 7 and 14d showed increased in mRNA expression (RT-PCR data) and stronger fluorescence signal (MB technology) in transfected cells relative to controls (cells not treated with polyplexes). Furthermore, encapsulation of eNOS DNA polyplexes into cross-linked collagen microspheres resulted in delayed release of this gene to the cells as demonstrated by the low MB signal seen at day 2 in both transfected HUVECs and HMSCs and confirmed with RT-PCR data which showed the lowest eNOS mRNA expression at day 2.

Maximum fold increase in IL-10 and eNOS mRNA expression levels occurred at day 7 in HMSCs as demonstrate by results of RT-PCR and stronger MB fluorescence signal at
this time point. On the contrary, IL-10 mRNA expression levels in HUVECs were relatively similar at days 2, 7 and 14. This observation seems to indicate the effect of frequent media changes, or lower transfection efficiency of polyplexes in HUVECs. Results of RT-PCR and MB for eNOS mRNA expression in HUVECs at day 14 do not necessarily agree, but it should be noted that expression of endogenous (native) and exogenous (plasmid) mRNA were measured by MB while only exogenous levels were measured in RT-PCR.

Typically, lower mRNA expression levels of both genes were seen in transfected cells at day 14, though these levels were much higher than those of controls.

It was demonstrated that molecular beacons are able to monitor changes in mRNA levels at various time points, in the presence of 3D scaffolding gene carrier system and the results complement those of RT-PCR.

5.2 Future studies

Based on the limitations of and challenges encountered during this research study, there are several areas that can be considered for future work.

5.2.1 Modified molecular-beacons backbone chemistries

In this study, molecular beacons with a DNA backbone were used. These were limited to short term analysis (~3h) in living cells in 3D collagen scaffolds due to non-specific signal in the control MB. Also, diffusion rates of MB in 3D scaffold may be increased which in turn may increase the time before confocal imaging could be performed from the bottom up. So, MB signal in cells at the top of the scaffold may be increased due to probe degradation which can make evaluation of gene expression due to spatial distribution inaccurate.

Inside cells, DNA MBs are prone to degradation by endonucleases and non-specific interactions with single nucleotide proteins, and probe-target mRNA hybrids may be degraded by Ribonuclease H. Thus, more stable MB designs can overcome these limitations. Modification of the backbone chemistry can render the MB resistant to nucleases, prevent probe-RNA hybrid degradation by Ribonuclease H, as well as provide long term imaging of the mRNAs. This can enable monitoring of gene
expression in the same experimental system over time, as well as increase the time available for image processing. Live cell imaging using MB with modified backbone chemistries such as 2’-O-methyl RNA [1, 2] or locked nucleic acid (LNA) [3] has been performed.

Another advantage of backbone chemistry modification is the possibility to increase the melting temperature of the MB and thus the affinity for target mRNA. Thus, it is possible to design MB with shorter probe lengths [4].

### 5.2.2 Molecular beacon-quantum dot hybrid system

Molecular beacons labelled with organic dyes can detect changes in mRNA expression in living cells in 3-dimensional collagen scaffold. However, the labelling of the MB with quantum dots (QDs) has many advantages over organic dyes. QDs are semiconductors nanoparticles typically 2-10 nm in size [5, 6] that have been shown to be more photo-stable (100 fold) and emit brighter fluorescence signals (20 folds) than organic dyes [7]. Also, QDs have broad excitation spectra and narrow tuneable emission spectra which allow for multiplexing [8]. These properties can result in longer imaging time, as well as rapid and highly sensitive detection.

As a proof of concept, GAPDH MBs conjugated to carboxyl CdTe QDs were delivered via SLO to detect intracellular GAPDH mRNA in HUVECs. The gelatinized carboxyl CdTe QDs (2.5 nm) used for this study were synthesized in our lab by PhD student Olga Gladkovskaya, following a previously described method [8]. The QDs, with an emission wavelength of 540 nm, were conjugated to MBs following a slight modification of the method described elsewhere [9], and outlined in Appendix III-I. GAPDH molecular beacons were labelled at the 5’-end with an amine group and the 3’-end with BHQ-1. Preliminary results show that the MB–QD conjugates emitted bright signals, and were specific for their targets (see Figure 5.1).
Figure 5.1 GAPDH mRNA detection in HUVECS. 540QD-GAPDH or Random (control) molecular beacons (MB) were delivered to cells in microfluidic chambers (0.6cm²) using activated Streptolysin O (0.2U/ml) mediated delivery. After recovery for 1h, cells were imaged using confocal microscopy and imaging completed after 2h. Signal collected using a Plan-Apochromat 63X/1.4 oil DIC objective and laser excitation at 488 and emission at 530 for 540QD MB. Fluorescence and bright field image for overlap for each cell is shown.
5.3 Conclusions
The ultimate aim of this research was to develop a detection platform using molecular beacons for monitoring spatiotemporal changes in transcription gene expression in cells associated with three-dimensional biomaterial constructs. The general principle of the proposed research was to use two different tissue engineering approaches, one utilizing a cell-embedded and the other a gene delivery scaffolds based on collagen type I a natural extracellular matrix protein that when gelled \textit{in vitro} can result in relatively transparent gels. The next objective then was to design and deliver molecular beacons that allow the detection of mRNA expression of living cells using confocal microscopy imaging. It was hypothesized that MB can monitor changes in mRNA expression in cells embedded into 3D \textit{in vitro} collagen scaffolds by targeting a constitutively expressed gene, GAPDH (Chapter 3). Furthermore, MB can monitor the efficacy of gene therapy to cells transfected via 3D \textit{in vitro} collagen scaffolds gene carriers by targeting therapeutic genes (IL-10 and eNOS) of cardiovascular importance (Chapter 4). The findings of this research indicated that molecular beacons with DNA backbone can be used to monitor the spatiotemporal expression pattern of cells associated with 3D collagen scaffolds. However, conjugation to a TAT-peptide represents a faster and more practical delivery technique to introduce MB into cells embedded in 3D \textit{in vitro} scaffolds than reversible permeabilization with SLO, while either technique is suitable for cells seeded outside scaffolds. Also, DNA molecular beacons are prone to degradation and non-specific interactions inside cells which limit their use for imaging in living cells to a few hours (about 2-3 h after molecular beacon delivery). Furthermore, optical limitation were encountered with confocal microscopy for imaging the 3D scaffolds of increasing thickness and collagen concentration due to increases in light scattering which make focusing and localization of mRNA difficult in the presence of already low beacon signals.
5.4 References


APPENDIX I: SUPPLEMENTAL DATA
Figure S.1 Schematic of the method used in this study for fluorescence image analysis of cells in single plane (2D) and 3D (z-stack). Single plane images were collected when cell fluorescence was at maximum intensity. Image was analyzed similar to extended focus view, but cell average fluorescence intensity per pixel were analysed.
NM_000572.2| Homo sapiens interleukin 10 (IL10), mRNA

ACACATCAGGGCTTGTCTCTGCAAAACCAAGACAGACATTTGCAAAAAAGAGGCTACATCACAGCTC
AGCACTGCTCTGTCCCTGTGCTACTCGAGTGGGTAGGCCCAAGCCACCAGGGGCAAGTCTTCTGAG
AACAAGCTGCACCCTCCCCAGGCAACCTGCTAATGCTCTGAGATCTCCGAGATGCTCTATCACAGAG
TGAGAGCTTCTTCTTTCAAATAGAGGCTACTGGGTTGCCAAGCCTGTCTGAGATGATCCAGTTTTACCTGGGAGGAGGTGATGCCCCAAGCTGAGAA
CCAAGACCAGACATC
AAGGCGCATGTGAACTCCCTGGGGGAGAACCTGAAGACCCTCAGGCC
TGAGGCTACGGCGCTGT
CATCGATTTCTTCCC
TGTGAAAACAAGAGCAAGGCCGTGGAGCAGGTGAAGAA
TGCCTTTAATAAGCTCCAAGAGAAGCCATCCTACAAAGCCATTGAGTGAGTTTGACATCTTCAT
CAACTAC
ATAGAAGCTACATGACAATGAAGATACGAAACTGAGACATCAGGGTGGCGACTCTATAGACTCTAGGAC
ATAAAATTAGAGGCTCTCCAAAATCGGATCTGGGCTCTGGGATAGCTGACCCAGCCCCTTGAGAAACCTTA
TTGACTCTCTCTTTATAGAATATTTATTTACCTGCTGATACCCTCAACCCCATTCTATTTATTATTTACGTGCTCTCTGGAAGTTGAG TTGAAACGGC
ACAGATTTAGAAAGAAGGCAAATTATAATTTTTTTCAATATTTATTATTTTCACCTGTTTTTTAGAGATCCAGTTTTTACATAGTAGGAAGATTTTTCA
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GGGGAGCCAACAGAAGCTTCCATTCCAAGCTGCCACGCTTTCTAGCT
GTTGAGCTGGTTTTCCTGACACTCCCTCCTCTAATTTATCTTGCTCCTGGGGCTTTCCCTAACATAATTTTAATTTTAATTTTAACTGTTTTTACATTGTT
TCTGCAGTGGTGGCAGGCTGACATTTTGTTCTTATATTAGAGGCT
GTTGGGAATGGCCTGCTTCTTGCTTCATAGCAGATTATTTTGAATAAATA
AATGTATCTTATTTGACATC

MB 1

MB 2

MB 3

MB 4
Human eNOS GeneArt (plasmid sequence)

ATGGGCAACCTGAGAAGCGTGGCCAGCAGCGCTGGGCTGGGCTGGGGCTGGGGCTCGGCCTCGG

ACTGTGCAGGCAGAGGCAACCCTGACACCCCTGGGGGCTGGGGCTGGGGCTCGGCCTCGG

ACTGTGCAAGCAGGGCCCTGCCACCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCGCCTCGG

ACTGTGCAAGCAGGGCCCTGCCACCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCGCCTCGG

ACTGTGCAAGCAGGGCCCTGCCACCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCGCCTCGG

ACTGTGCAAGCAGGGCCCTGCCACCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCGCCTCGG

ACTGTGCAAGCAGGGCCCTGCCACCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCGCCTCGG

ACTGTGCAAGCAGGGCCCTGCCACCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCGCCTCGG

ACTGTGCAAGCAGGGCCCTGCCACCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCGCCTCGG

ACTGTGCAAGCAGGGCCCTGCCACCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCGCCTCGG

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ACTGTGCAAGCAGGGCCCTGCCACCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCGCCTCGG

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ACTGTGCAAGCAGGGCCCTGCCACCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCGCCTCGG

ACTGTGCAAGCAGGGCCCTGCCACCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCGCCTCGG

ACTGTGCAAGCAGGGCCCTGCCACCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCGCCTCGG

ACTGTGCAAGCAGGGCCCTGCCACCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCGCCTCGG

ACTGTGCAAGCAGGGCCCTGCCACCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCGCCTCGG

ACTGTGCAAGCAGGGCCCTGCCACCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCCCCCCTGCCGCCTCGG

MB 2

MB 3
CCTGTACCCTGAGACAGGCCCTGACCTTCTTCCTGGACATCACCAGCCCCCCTAGCCCCCAGCTGCTGCGG
CTGCTGAGCACCCTGCGAGGAGGAGGAGACAGAATCAGCCAGAGCACAGATCCTAGAGGAGTGGAAGTGGTTCAGATGCCCCACCCTGCTCGAAGTGCTGGAGCAGTTCCCCAGCGTGGCCCTGCCAGCCCCACTGCTGCTGACCCAGCTGCCTCTGCTGCTG
CAGCCAGCCTGCTGCTGACCCAGCTGCCTCTGCTGCTG
AGCACCCACCACCAGAGATCCACCTGACCGTGGCCGTCCTGGCCTACAGAACCCAGGACGGCCTGGGACCTGCACTACGGCGTGTGTAGCACCTTGGCTAGCCAGCCAGCTGAAAGGCGAGGCTGCACGACATCGAGAGCAAAGGCCTGCAGCCTACCC
CTATGACCCTGTGTTTGTGGCTGCAGATGCTCCAGCTGAGACCTACCTATACAGGAGCTGAGAATGCCAGCACCCACCCCGGCGAGATCCACCTGACCGTGGCCGTCCTGGCCTACAGAACCCAGGACGGCCTGGGACCTGCACTACGGCGTGTGTAGCACCTTGGCTAGCCAGCCAGCTGAAAGGCGAGGCTGCACGACATCGAGAGCAAAGGCCTGCAGCCTACCC
ATCAGGAGAGGGGCCACATGT
TGTTGTGCTGCCAGCAGCATGCTGACCCTTGCTGAGAGGCGCCGTGCCTTG
GGCCTTCGATCCCCCTGGCAGCGATACCAACAGCCCCTGATG
Homo sapiens nitric oxide synthase 3 (endothelial cell) (NOS3), transcript variant 1, mRNA

ATGGGCAACTTTGAGAGCGAGGCAGCTGGCCACCCTTGCGGCGTGCTGCGCTGCTT
GGCTGCAGGCTGAGTCAGGCCCGGGACTTCATCAACCAGTACTACAGCTCCATTAAGAGGA
AGCGGCTCCAGCCACGAGCTGGCCATCCCGACAGCGTCTCAAGAGTTGAGGGGACCATCGGAGAGCGAGCTGGTGCTGGTGTAACCAGCACATTTGGGAATGGGGATCCCCCGGAGAATGGAGAGCTTTGCAGCTGCCTGATGGAGATGTCCGCCCCTACAACAGCTCCCCTCGGCCGGAACAGCACAA
GAGTTATAAGATCCGCTTCAACAGCATCTCCTGCTCAGACCCACTGGTGTCCTCTTGGCGGCGGAAGAGGAAAGGAGTCCAGTAACACAGACAGTGCAGGGGCCCTGGGCACCCTCAGGTTCTGTGTGTTCGGGCTCGGCTCCCGGGCATACCCCCACTTCTGCGCCTTTGCTCGTGCCGTGGACACACGGCTGGAGGA
ACTGGGCGGGGA
GCGGCTGCTGCAGCTGGGCCAGGGCGACGAGCTGTGCGGCCAGGAGGAGGCCTTCCGAGGCTGGGCCCAG

The eNOS MB sequence is specific for eNOS mRNA variants 2-4 as well.
APPENDIX II: LIST OF MATERIALS AND REAGENTS
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<thead>
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<th>Supplier</th>
<th>Materials and reagents</th>
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<tr>
<td>Abcam, Cambridge, UK</td>
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<tr>
<td></td>
<td>Anti-VCAM1 polyclonal antibody</td>
</tr>
<tr>
<td></td>
<td>Goat polyclonal Secondary antibody-FITC</td>
</tr>
<tr>
<td>Ambion USA</td>
<td>TRI reagent</td>
</tr>
<tr>
<td></td>
<td>siRNA</td>
</tr>
<tr>
<td></td>
<td>Nuclease-free water</td>
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<tr>
<td></td>
<td>20X SSC</td>
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<tr>
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<td>-----------------------------------------------------</td>
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<td>Molecular beacons</td>
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<td>Oligonucleotide primers</td>
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<td>USA</td>
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<td></td>
<td>MgCl₂</td>
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<tr>
<td></td>
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<td>Sodium hydroxide</td>
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<tr>
<td></td>
<td>Trypan blue</td>
</tr>
<tr>
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APPENDIX III: PROTOCOLS
**A. Molecular beacon hybridization assay**

*Note: Carry out this protocol under the bio-safety cabinet in cell culture. Protect beacons from light.*

1. Add 2 µl of 100 µM MB to 98 µl of nuclease-free PBS (1x PBS), pH 7.4. Final molecular beacon concentration is 2 µM.
2. Add 2 µl of 100 µM target DNA solution to 38 µl of 1x PBS. Final DNA concentration is 5 µM. Target DNA is complementary to the loop end of the molecular beacon of interest.
3. In 6-wells of a black 96-well plate, add 90 (well 1), 89.5 (well 2), 89 (well), 88 (well 4), 85 (well 5), and 80 (well 6) µl of 1x PBS to the respective wells.
4. Add 0 (well 1), 0.5 (well 2), 1 (well 3), 2 (well 4), 5 (well 5), and 10 (well 6) µl of 5µM target DNA to the respective wells.
5. Add 10 µl of 2 µM molecular beacon solution to each well and incubate for 1h at 37 °C. Final concentrations of 200 nM MB and 0, 25, 50, 100, 250, 500 nM DNA respectively.
7. Analyse data. The background signal from beacon only well (0 nM DNA) is subtracted from all 6 samples. The 6 corrected values are plotted for DNA concentration versus fluorescence intensity.

**B. Activation of Streptolysin O**

*Note: Carry out this protocol under the bio-safety cabinet in cell culture.*

1. Prepare 500 mM (tris(2-carboxyethyl)phosphine (TCEP) in sterile nuclease-free water. Dissolve 14.3 mg TCEP in 100 µl water. This solution is stable for about 3-4 weeks at 2-8°C.
2. Prepare 25,000 U/ml SLO in cold sterile nuclease-free water. Store 20 µl aliquots at -20 °C. Do not refreeze. Note: An SLO solution will lose ~50% activity within 10 days when stored at 2-8 °C.
3. Thaw and dilute 20 µl stock SLO using 480 µl of water. Final concentration is 1,000 U/ml.
4. Add 10 µl of TCEP to 988 µl HBSS (without calcium or magnesium ions as calcium inactivates SLO activity). Final concentration of 5 mM TCEP.
5. Add 2µl of 1000U/ml SLO to 5mM TCEP and incubate at 37°C for 30 min. Final concentration 2U/ml SLO in 5mM TCEP. (SLO is loses its activity in oxygen and is reactivated by TCEP).

C. Molecular beacon delivery using streptolysin O

Note: Turn lights off in bio-safety cabinet and protect beacons from light.
1. Dilute 10 µl of activated SLO with 89 µl serum-free medium (PBS, HBSS, or serum-free medium typical for cell being used) and add 2 µl of 15 µM MB. Final concentrations of 0.2U/ml SLO in 300 nM MB.
2. Add 100 µl of SLO-MB solution/well to cover cells cultured in 8-well chambered coverglass.
3. Incubate cells at 37°C/5% CO2 for 10 min to allow cell permeabilization and beacon delivery.
4. Immediately remove permeabilization medium and rinse cells gently 2 times with 150-250 µl of complete culture medium.
5. Add 250 µl of complete culture medium and incubate cells at 37°C/5% CO2 for 1 h to allow cell recovery.
6. Replace medium with fresh medium.
7. Image cells using confocal microscope equipped with 63X objective at the appropriate excitation/emission. (e.g., FAM beacons, 488/530, Cy3 beacons, 543/565-615, Cy5 beacons, 633/670).

D. Molecular beacon conjugation to TAT peptide
1. Dissolve 1 mg sulfo-SMCC in 300 µl H2O (8mM) and use immediately.
2. Add 20 µl of 100 µM MB to 175 µl nuclease-free water.
3. Add 5µl of 8mM SMCC to beacon solution. Final concentration is 200 µM SMCC in 10 µM MB.
4. Wrap in foil and leave for 3h to react @RT.
5. Add 200 µl 1x PBS to MB-SMCC solution
6. Transfer the 400 µl solution to a 0.5 ml Amicon centrifugal device, 10k MWCO.
7. Centrifuge for 15-30 min @ 14,000 x g, 4°C (~50-70 µl solution remains).
8. Add 200 µl 1x PBS to the concentrate.
9. Centrifuge for 15-30 min @ 14,000 x g, 4°C (~50-70 µl solution remains).
10. Repeat steps 8 and 9.
11. Add 350 µl 10x PBS to the concentrate & carefully aspirate up and down.
12. Transfer solution to a 1.5 ml tube.
13. Add 16 µl of 500 µM cys-TAT peptide. Final concentration is 20 µM.
14. Cover with foil and react overnight at 4°C mixing.
15. Transfer reaction ~400 µl solution to a new Amicon device.
16. Centrifuge for 15-30 min @ 14,000 x g, 4°C.
17. Add 350 µl H2O to the concentrate and repeat centrifugation.
18. Add 350 µl 1x PBS to the concentrate and repeat centrifugation.
19. Collect concentrate-turn filter device upside down in new micro centrifuge tube–by centrifugation for 2 min at 1,000 x g.
20. Determine concentration nucleic acid(ssDNA) using Nanodrop 1000.
21. Store @ -20 oC (stable 2-3 weeks).

E. TAT-peptide molecular beacons delivery into 3D collagen scaffolds (constructs)
1. Prepare 400 nM final concentration of TAT-peptide linked molecular beacons in 150 µl serum free medium.
2. Carefully remove medium from constructs.
3. Add the prepared TAT-peptide linked molecular beacons solution to constructs.
4. Incubate constructs at 37°C/5% CO2 for 60 min in dark.
5. Carefully remove medium.
6. Rinse constructs twice with 1x PBS(nuclease-free).
7. Add fresh serum-free medium.
8. Immediately perform confocal imaging of constructs.
9. Perform z-stack and single plane imaging using 40x and 63x objectives, respectively.
10. Perform image analysis to quantify signal.
F. Reverse Transcription (RT) to prepare cDNA

10. Prepare RT reaction mix (1) and (2) for each sample using Promega RT reagents as follows:

1. RT reaction mix for 10.6 µl
   - RNase-free water: (9.6 µl – x µl)
   - RNA sample (0.5-1µg): x µl
   - Random Hexamer (50µM): 1.0µl

2. RT reaction mix for 9.4µl
   - 5x RT buffer: 4.0µl
   - 25mM Magnesium chloride: 2.4µl
   - DeoxyNTPs mixture: 1.0µl
   - RNase Inhibitor (20U/µl): 1.0µl
   - Reverse Transcriptase (50U/µl): 1.0µl

2. Prepare reaction mix (1) for each sample in 200 µl eppendorfs tubes.
3. Place the eppendorfs in the thermal cycler and run program as follows:

   Step 1: 5 minutes at 70°C    Removes secondary structure of RNA and primers.
   Step 2: 2 min at 4°C.

4. Prepare reaction mix (2) for each sample in new 200 µl eppendorfs tubes.
5. Transfer denatured RNA/primer reaction mix (1) to reaction mix (2). Final volume 20 µl.
6. Place the eppendorfs in the thermal cycler and run program as follows:

   Step 1: 5 minutes at 25°C    Annealing of primer to target RNA sequence
   Step 2: 60 minutes at 42°C    Reverse transcription.
   Step 3: 15 minutes at 70°C    Inactivation of Reverse transcriptption.

7. Remove eppendorfs from instrument and store at -20°C until further analysis.
G. Real Time PCR:

7. Prepare \( m(3n+2) \) reaction mixture for each gene of interest (\( m \)) per \( n \) samples as follows: (for example, if there are 4 samples and 2 genes of interest (housekeeping gene (GAPDH) and test gene (eNOS)) then reaction mixture needed = \( 2(3\times4 +2) = 28 \times \), which is = \( 28 \times 8 \mu l = 224 \mu l \)

\[
\begin{align*}
\text{Fast SYBR Green} & \quad 5 \mu l \\
\text{RNase-free water} & \quad 1\mu l \\
3\mu M \text{ forward primer} & \quad 1\mu l \\
3\mu M \text{ forward primer} & \quad 1\mu l
\end{align*}
\]

7. Add 8 \( \mu l \) of reaction mixture to each well of a 96-well optical plate.

8. Add 2 \( \mu l \) cDNA to wells in triplicates. Run 1 no template control replacing cDNA template with nuclease-free-water for each gene of analysed.

9. Cover plate with optical film and centrifuge very briefly to remove bubbles from bottom of wells.

10. Run on StepOne Real Time PCR System with the following settings:

\[
\begin{align*}
\text{Step 1:} & \quad 20 \text{ seconds at } 95^\circ C \\
\text{Step 2} & \quad 40 \text{ cycles of } 3 \text{ s at } 95^\circ C \text{ followed by } 30 \text{ s at } 60^\circ C \\
\text{Step 3} & \quad 15 \text{ s at } 95^\circ C \text{ followed by } 60 \text{ s at } 60^\circ C \text{ then } 15 \text{ s at } 95^\circ C
\end{align*}
\]

11. Results are presented as CT (threshold cycle) values and indicate the cycle at which the fluorescence from the amplified cDNA is registered above a fixed background fluorescence.

12. Calculations are carried out to determine relative quantification of mRNA expression of the genes of interest normalized to the housekeeping control. Quantity of mRNA in target (treated sample) is expressed relative to a calibrator (typically a “no treatment” sample) and the amount of RNA relative to the calibrator is calculated as \( 2^{\Delta\Delta CT} \), where \( \Delta\Delta CT \) is the difference between CT (target) - CT (control) of sample and CT (target) - CT (control) of calibrator.
H. Scanning electron microscopy (SEM) for collagen scaffolds

1. Rinse cell-seeded scaffolds (constructs) with 0.1M phosphate buffer, pH 7.2.
2. Fix constructs with 2.5% glutaraldehyde for 2-3 hours at room temperature.
3. Remove constructs from fixing solution and rinse with phosphate buffer.
4. Dehydrate constructs in 50% ethanol solution for 5 minutes at 4°C.
5. Remove ethanol and replace with fresh ethanol, and dehydrate for a further 5 minutes at 4°C.
6. Remove previous solution and repeat step 4 and 5 using 75% ethanol.
7. Remove previous solution and repeat step 4 and 5 using 80% ethanol.
8. Remove previous solution and repeat step 4 and 5 using 90% ethanol.
9. Remove previous solution and repeat step 4 and 5 using 100% ethanol.
10. Remove solution and add hexamethyldisilazane (HMDS) for 30 minutes (use glassware!).
11. Allow constructs to air dry in fume hood.
12. Place constructs on carbon pads and attach to SEM stubs.
13. Gold coat constructs and view under SEM.

I. Carboxyl quantum dot conjugation to molecular beacons

1. Dissolve 1 mg EDC in 175 µl nuclease free H2O, use only fresh EDC solutions.
2. Transfer 2µl (100 pmol) of 50 uM Qdots into small glass vial.
3. Add 10 ul (300 nmol) of 30 mM EDC to Qdots solution. Mix by shaking.
4. Incubate at room temperature for 5 min.
5. Add 360 µl PBS. Mix by shaking.
6. Add 20 ul (2 nmol) of 100 uM MB to EDC-Qdots solution. Mix by shaking.
7. Wrap in foil and incubate for 2h at room temperature to react, on a shaker with gentle shaking.
8. Transfer Qdot-MB solution to a Amicon centrifugal filter.
9. Centrifuge at 7000g for 15 min
10. Resuspend retenate with 400 ul PBS (discard flow through).
11. Repeat step 7 and 8 twice.
12. Resuspend the final retenate in 200 ul PBS.
APPENDIX IV: PUBLICATIONS & CONFERENCE PROCEEDINGS
Appendices

Journal publications


Submitted articles


Conference proceedings


METHOD

Monitoring mRNA in living cells in a 3D in vitro model using TAT-peptide linked molecular beacons

Jennifer Claire Alexander,a Abhay Pandit,a Gang Bao,b David Connollyc and Yury Rocherc

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There is a growing need for the development of in vitro 3D cell culture models for assessing newer therapeutics for clinical applications and mechanisms of human pathology. Molecular beacons have been successfully delivered in two-dimensional (2D) systems to monitor, detect, and localize specific mRNA expression in living cells at the single cell level. However, to date the use of molecular beacons in three-dimensional (3D) systems has not been reported. To translate this technology into specific clinical targeted applications, it is critical to develop and demonstrate efficacy in a 3D system. For the first time the use of TAT-peptide conjugated molecular beacons to monitor mRNA in a 3D in vitro system has been reported.

Introduction

Traditionally the two-dimensional (2D) cell culture system has led to many significant findings in cell and molecular biology, however these systems cannot fully capture the complex and dynamic three dimensional (3D) environment of most living cells. Three dimensional cell culture systems have been shown to better mimic gene expression and other biological processes observed in living organisms, thus bridging the gap between 2D cellular assays and animal models of disease. The goal therefore of 3D systems is for translation to clinic. Thus, there is a growing need for the development of in vitro 3D cell culture models for assessing newer therapeutics for clinical applications, and mechanisms of human pathology.

RNAs have many important functions in living cells from physically conveying and interpreting genetic information, providing structural support for molecular machines, catalyzing chemical reactions, to regulation of gene expression. These functions are controlled through the expression levels and the spatiotemporal distribution of the specific RNA in the cell. However, RNA detection in cells is routinely performed by methods such as reverse transcription-polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH). RT-PCR relatively quantifies gene expression of a cell population from extraction of purified RNA obtained from cell lysates. However, cell lysates give no insight into the spatial and temporal distribution of RNA within the single cell. Furthermore, RNA purification, cDNA synthesis, and other processing techniques performed before PCR analysis can result in loss of RNA transcript. In situ FISH requires fixation of cells. Recent advances in FISH allow for the visualization of gene expression at the single molecule level. However, this technique provides very limited temporal resolution of RNA expression.

Molecular beacons (MB) technology uses dual-labelled single-stranded antisense oligonucleotide probes that fluoresce on hybridization with target nucleic acids. The probes are 'stem-looped-shaped designed with the complementary sequence for the target nucleic acid of interest on the loop while the fluorophore and quencher are held in close proximity by the complementary nature of the 5' and 3'-ends of the stem. The stem undergoes conformational change on hybridization with complementary target nucleic acids.

The major advantage to MB lies in the ability to monitor, detect and localize specific mRNA expression in live cells that can be visualized in real-time. These probes show high specificity for their targets and have been used to visualize the localization, distribution and transport, and to monitor specific mRNA expression levels at the single cell level in living cells in two dimensional (2D) monolayer cell culture.

To accurately detect intracellular RNA molecules, efficient delivery of molecular beacons is critical. There are several techniques used for the intracellular delivery of molecular beacons. TAT peptides have a distinct advantage that these peptides can deliver biomolecules into cells with a relatively quick targeted delivery mechanism with nearly 100% efficiency. Also, the TAT-mediated self-delivery of biomolecules is ideal for an in vitro 3D cell culture system as other methods, such as electroporation, cell membrane permeabilization, and microinjection are not practical.

Probes with DNA backbone are prone to degradation by nucleases, and their target RNA are susceptible to degradation...
by RNase H, unlike probes with 2′-O-methyl backbone. Also, phosphorothioate backbone and locked-nucleic acids (LNA) MBs are nuclease resistant. However, backbone chemistry modifications alter the thermodynamics of probe hybridization and should be assessed carefully.

In this work, molecular beacons targeting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA is used. GAPDH is a highly expressed multifunctional cell protein involved in various biological processes such as DNA repair and replication, nuclear RNA transport, translational regulation, microtubule bundling and apoptosis. GAPDH is widely used as a housekeeping gene for gene analysis in PCR and microarray. To downregulate and thus monitor changes in GAPDH mRNA expression, small interfering RNAs (siRNAs), double-stranded nucleic acid molecules capable of silencing gene expression, were used. Effectively, siRNAs target sequence specific cytoplasmic mRNAs and initiate their degradation by RNA interference (RNAi).

The purpose of this study was to demonstrate that molecular beacons can monitor change in gene expression in live cells in a 3D scaffolding system. The specific objectives were to detect and monitor changes in GAPDH mRNA levels in 3D type I collagen scaffold and assess the relationship between RT-PCR and MB detection.

Materials and methods

Cells and culture conditions

Human foetal foreskin fibroblasts (HFF2) and HeLa cells were used in this study. HFF2 were purchased from European collection of cell culture (Salisbury, UK). The cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Sigma) supplemented with 10% foetal bovine serum (FBS; Sigma), containing 100 U/ml of penicillin, 100 μg ml⁻¹ streptomycin. The fibroblasts used in this study were between passages 22–26.

Preparation of type I collagen

Collagen type I was extracted in the laboratory from bovine tendons using an acetic acid extraction method previously described elsewhere. The concentration and purity of the collagen was determined using Sircol™ soluble collagen assay and sodium dodecyl sulphate-polyacrylamide scaffold electrophoresis (SDS-PAGE), respectively. A 5 mg ml⁻¹ concentration was prepared using 0.5M acetic acid.

Preparation of type I collagen scaffolds

To prepare 1 mg ml⁻¹ three-dimensional collagen type I scaffolds, 5 mg ml⁻¹ collagen was diluted on ice with DMEM and the pH adjusted to 7 with 1N NaOH. A 30–50 μl suspension of HFF2 was incorporated into the neutralized collagen solution to a final concentration of 1.0 × 10⁶ HFF2/ml. Aliquots of the resulting mixture were loaded into multi-well tissue culture plates or 4-well chambered cover glass (NUNC), and incubated at 37 °C for 20 min for scaffold formation. The 3D scaffolds produced were 1 mm in thickness and were attached to the wells. The top of the scaffolds were covered with antibiotic-free growth medium and cultured for 18 h before transfection.

Transfection of siRNA

Cells were harvested 18 h before transfection with 0.25% trypsin/EDTA, re-suspended in antibiotic-free growth medium and seeded into collagen scaffolds (see above) or multi-well plates and 4-well chambered cover glass for static monolayer. Transfection of siRNA was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were transfected with 40 to 100 nM Silencer® GAPDH siRNA (Ambion) or 40 nM to 100 nM Silencer® Negative Control #1 siRNA (Ambion) and incubated for 24, 48, or 72 h before analysis. GAPDH mRNA level was monitored by RT-PCR and molecular beacon imaging.

RNA extraction and RT-PCR

Static monolayer and 3D collagen scaffolds (960 μl) were prepared in 6-well plates (Sarstedt) at a seeding density of 1.0 × 10⁶ cells per well. Transfected cells were homogenized in 1ml of Tri reagent® (Ambion), the RNA layer extracted using 200 μl of chloroform, and RNA precipitated with 70% ethanol. Total RNA was purified using the RNeasy® Mini kit (Qiagen) and on column treated with DNase I (Qiagen). Total RNA was quantified using a NanoDrop spectrophotometer (Nanodrop Technologies, DE, US). The quality of the total RNA was determined using the Agilent bioanalyzer 2100 instrument and RNA Nano Chip kit (Agilent) according to manufacturer’s instructions. All RNA samples were of good quality with relative integrity numbers (RIN) greater than 9.0. cDNA was transcribed using Imprint-ITM Reverse Transcriptase and random primers from Promega (UK) and 500 ng of total RNA. The 20 μl sample volumes were reverse-transcribed at 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 15 min. All samples were stored at −20 °C for future use. cDNA was diluted and 1 μl of template was reacted with 1 μl each of 3′M GAPDH primers (sense primer: 5′-GGCTA-GCCGATCTTCTTGTGC-3′; and antisense primer: 5′-GGGCTTTATA-GGACCAATC-3′), 2 μl water and 5 μl Fast SYBR® Green PCR mix (Invitrogen). Human β2 RNA was used as the normalizing gene (sense primer: 5′-GAACCATCAACGGGAAAACCTCAG-3′; and antisense primer: 5′-GTTACAAGGCACCATGACACAC-3′). Each experiment was performed in triplicate. PCR was performed using StepOne Plus Real Time PCR System (Applied Biosystems) under the following conditions: activation at 95 °C for 20s; followed by 40 cycles at 95 °C for 3s, 60 °C for 30s, and dissociation at 95 °C for 15 s, 60 °C for 60s, and 95 °C for 15s.

Molecular beacon design and synthesis

Modified molecular beacons designed in previous studies with 2′-deoxy DNA chemistries were synthesized by Eurofins MWG Operon (AT, USA and Ebersberg, Germany). The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) MBs, as well as a “random” sequence MB (serves as negative control) possessed Cy3 fluorophore at the 5′-end and Black Hole Quencher 2 (BHQ2) at the 3′-end. The 3′ base from the 3′ end of the quencher arm of the MB was modified with a nucleotide thymine group linked through a 6-carbon spacer. The beacon design for targeting GAPDH is 5′-Cy3-CGACGGAGTCCTTCACCGATACCA CG(AM6s-(D7)CG-BHQ2)3′.

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and that for the random beacon is 5'-Cy3-CGACGCGACAAGCGCACCGATAAC(AnC6-dT)CG-BHQ2-3'. The bases that bind to create the stem regions of the molecular beacons are in italic.

**MB conjugation with TAT-peptide**

The MB were conjugated following a protocol previously described. Briefly, 200 μM of sulfo-succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC, Pierce Biotechnology), a heterobifunctional crosslinker, was reacted for 3 h with dT-amine group of the MB (10 μM) to create maleimide activated MBs. Excess Sulfo-SMCC was removed from the reaction using Amicon® Ultra-0.5 ml centrifugal filter devices (10k MW cutoff; Millipore). The maleimide-activated MBs were reacted overnight at 4 °C with 20 μM cysteine-modified TAT peptide (Cys-TAT (47-57), AnaSpec, Inc). The cysteine modification at the C terminus allowed for direct linkage with the maleimide activated MB. Unreacted TAT peptide/MBs were removed from the reaction using Amicon® Ultra-0.5 ml centrifugal filter devices.

**Solution MB: target hybridization assay**

To determine whether the function of the molecular beacon was altered during peptide conjugation, hybridization assays in solution were performed as previously described. Briefly, 200 nM modified and peptide-linked molecular beacons were each reacted with 1x PBS buffer or 1 μM of complementary DNA (cDNA) target and incubated at 37 °C for 30 min. The fluorescence intensity was measured using the Safire fluorescence microplate reader (Tecan, Zurich, Switzerland) with excitation wavelength at 525 nm and emission fluorescence signal scanning from 565 to 680 nm wavelength.

**Cellular delivery of peptide-linked molecular beacons**

Static monolayer and 3D collagen scaffolds were prepared in 4-well chambered cover-glass at a seeding density of 20,000 cells per well. At 48h post transfection, medium was replaced with 120 μl of fresh antibiotic-free medium containing peptide-linked MB at 400 nM final concentration. Cells in monolayer or 3D scaffolds were re-incubated at 37 °C, 5% CO2 for 30 min or 1 h, respectively, in the dark to complete MB delivery. Incubation medium was removed and cells or scaffolds were rinsed twice with 1x PBS. Fresh antibiotic-free medium was added and confocal imaging was performed immediately.

**3D-image reconstruction**

Imaging was performed using the Zeiss Axiovert LSM-510 confocal microscope equipped with 40x C-Apochromat/1.2 W corr objectives (Zeiss). Beacons were visualized with HeNe laser at excitation of 543 nm with signal detection at band-pass filter from 565-615 nm for Cy3. Images were 512 × 512 pixels, and acquired with the optical pin hole set at 2.76 Airy units for single plane images and 2.05 Airy units for the Z-stack. The Z-stack image was acquired from 3D collagen scaffold using 1.0 μm optical sections. The Z-stack image was reconstructed to a 3D image for visualization using the ZEN 2009 Light Edition software (Zeiss).

**Quantification of imaging data**

Mean fluorescence intensity (MFI) for each cell was quantified using Velocity software (Perkin Elmer). Measurements were performed in the single plane for cells in 2D culture and in extended focus view for cells in the 3D scaffold. The extended focus was selected from the image view option of the Velocity software and creates a single image from the brightest-point merge of all the slices in the z-stack. No Z stack images were acquired in 2D. The Z-stack images acquired in 3D scaffold were reduced to z-stacks containing each individual cell before analysis. To calculate MFI, the outer membrane of the cell was traced using the lasso tool and the selected region measured. This method was performed for negative control and siRNA knockdown cells. The MFI of the background pixel from a section of the field of view not containing any cells was used for background subtraction.

**DNA content**

The amount of DNA was assessed to determine the cell number post-transfection. The DNA content was determined using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen) assay following manufacturer’s protocol. Briefly, 200 μl volume collagen scaffolds were prepared in 24-well plates. For static monolayer, 10,000 cells/well were seeded into 48 well plates (NUNC). Both systems were rinsed twice with Hanks balanced salts solution (HBSS, Sigma) 48h post siRNA transfection. Scaffolds were dissolved in 50 μl of 1 mg ml−1 collagenase in DMEM medium for 30 min at 37 °C. A 150 or 200 μl volume of doubly distilled water was added to each test well containing cells or scaffolds respectively, followed by three freeze-thaw cycles at −80 °C and room temperature to release cellular DNA. The DNA content was determined using the PicoGreen® assay following manufacturer’s protocol. Fluorescence reading was performed using the Wallac Victor™ 1420 multilabel counter (Perkin Elmer) at 485 nm excitation and 535 nm emission.

**Results and discussion**

Molecular beacons have been successfully delivered in two-dimensional (2D) systems to monitor, detect, and localize specific mRNA expression in living cells at the single cell level. However, to date the use of molecular beacons in three-dimensional (3D) systems has not been reported. To translate this technology into specific clinical targeted applications, it is critical to develop and demonstrate efficacy in a 3D system. To demonstrate that conjugation of the modified MB with TAT peptide did not impair beacon function, hybridization assays were carried out in solution in the presence and absence of excess DNA target. The fluorescence intensity scanning the Cy3 emission wavelength (565 to 680 nm) was measured. The in-solution MB target hybridization assay (red and black curves) for both beacons show a similar emission spectrum, as seen in Fig. 1. This demonstrates that the functionality of the MB was not affected by conjugation. Furthermore, there is high signal to background ratio on
hybridization of peptide-linked MB, as demonstrated by the 10-fold increase in fluorescence intensity at 570 nm.

To demonstrate self-delivery and specificity of TAT peptide-linked MB targeting GAPDH mRNA, HeLa cells were first transfected as these cells are easy to transfect and proliferate rapidly in culture. Monolayer HeLa cells were transfected for 24h with 40 nM GAPDH siRNA which degrades cytoplasmic GAPDH mRNA, or 40 nM negative control siRNA which has no mammalian target sequence and thus served as a baseline for which down-regulation of GAPDH mRNA can be assessed. After 30 min incubation with TAT-peptide-linked MB, fluorescence signal was mainly localized close to one side of the nucleus (image not shown). Not surprisingly, this signal was significantly lower in cells treated with GAPDH siRNA. Imaging acquisition was completed 1-2 h after MB delivery and the random MB showed negligible background signal (images not shown).

To validate these results with a routinely used technique for mRNA quantification of cell lysates, RT-PCR was performed using 24h post-transfected HeLa cells. The RT-PCR data shown in Table 1 confirm GAPDH mRNA knockdown seen in MB imaging data.

GAPDH mRNA expression was monitored in transfected HFF2 cells in 2D and 3D cultures. To determine the optimal concentration of siRNA and transfection reagent required to achieve knockdown of GAPDH mRNA in HFF2, transfection using 40 nM and 100 nM siRNA was performed using GAPDH siRNA or negative control siRNA at a single concentration of Lipofectamine 2000 for 24, 48, or 72 h. An untreated control without siRNA was included in each assay to assess non-transfection related phenomena. At the appropriate post-transfection time, the purified cell lysates were analyzed for GAPDH mRNA expression using RT-PCR. Cells transfected with 40 nM GAPDH siRNA for 24 or 48 h showed around 25% and 40% knockdown, respectively, in GAPDH mRNA levels (data not shown). From RT-PCR data analysis, 48 h post-transfection with 100 nM GAPDH siRNA (Fig. 2A) was chosen as the optimal transfection condition as 80% GAPDH mRNA knockdown was seen in HFF2 cells. These conditions were used for further analysis with 3D collagen scaffolds.

Two methods were used to transiently transfected HFF2 cells in 3D. First, HFF2 were embedded into scaffolds and then transfected in this 3D construct. RT-PCR data analysis showed about 35% GAPDH mRNA knockdown at 48 h post-transfection with 100 nM GAPDH siRNA (Fig. 2B). This is significantly less than what is seen in 48 h transfection in 2D (Fig. 2A). To demonstrate that this decrease in transfection efficiency is due to the inefficient delivery of siRNA molecules through the tight matrix of the collagen scaffold, a second transfection method was investigated. siRNA transfection is transient and may last up to 7 days depending on the cell proliferation rate; HFF2 proliferates
Fig. 4 GAPDH mRNA expression in post-transfected HFFF2 cells in 2D cell culture system using 400 nM GAPDH specific TAT peptide-linked molecular beacons. Cells were transfected with 100 nM negative control or GAPDH siRNA for 48h. (A) The beacon signals distributed throughout the cytoplasm, but excluded the nucleus. The Cy3 signal is seen for each cell. (B) Mean fluorescence intensity ± standard deviation of control cells (n = 33) versus GAPDH knockdown (n = 28) cells, p < 0.05.

slowly hence the cellular monolayer was transfected for 24 h, followed by incorporation of the transfected cells into collagen scaffolds for another 24 h before performing RT-PCR. This second method resulted in about 80% GAPDH mRNA knockdown (Fig. 2B), which is similar to transfection in 2D.

HFFF2 cells were transfected for 48 h using 2 µg ml⁻¹ of Lipofectamine™2000 to deliver 100 nM of siRNA. To ensure that the transfection conditions maintained an acceptable level of cell viability, the DNA content of HFFF2 in 2D and 3D was analyzed using the PicoGreen® assay, as an indicator of cell number post transfection. The DNA content from transfected cells was compared to untreated control after 48 h. Transfection with GAPDH siRNA reduces GAPDH mRNA and protein levels, producing slow growth rates and reduced proliferation in most cell types. Although this was more evident in 2D cultures (Fig. 3A), than in 3D (Fig. 3B), there was no significant difference between cell numbers in transfected and untreated cells in 2D or 3D, using one way ANOVA data analysis.

To evaluate these results from RT-PCR, we delivered 400 nM GAPDH specific TAT-peptide linked MB to HFFF2 cells in 2D and 3D. A random TAT-peptide linked MB with no known mammalian target sequence was included as the negative control beacon. At 48 h post transfection, HFFF2 cells were incubated for 30 min with 400 nM peptide-linked ‘random’ MB or 400 nM

Fig. 5 Detection of GAPDH mRNA in 3D collagen scaffolds using 400 nM TAT peptide-linked molecular beacons. Single plane images showing Cy3 signal for control and GAPDH knockdown cells and corresponding bright field image overlap.
peptide-linked GAPDH MB. Molecular beacon signal was detected with GAPDH specific TAT-peptide linked MB, while negligible signal was observed with random beacon (Fig. 4A) 1–2 h after MB delivery. This demonstrates specificity of GAPDH peptide linked MB for GAPDH mRNA targets. The localization pattern for GAPDH mRNA in HFF2 cells appears distributed throughout the cell cytoplasm and excludes the nucleus. Furthermore, GAPDH MB signal seemed to decrease in GAPDH knockdown HFF2 cells compared to control cells which indicate transfection had occurred. Using one-way ANOVA, comparison of the MFI of the single plane image data from the 2D culture showed that fluorescence intensity levels in control cells was significantly different to signal from GAPDH knockdown cells (Fig. 4B).

To perform transfection in 3D, cells were transfected in 2D and at 24 h post-transfection incorporated them into collagen scaffolds for another 24 h. At 48 h post-transfection, 3D constructs were incubated with TAT-peptide linked MB for 1h. As shown in Fig. 5, GAPDH mRNA signal in control cells is distinct and distributed throughout the cytoplasm while the signal in GAPDH knockdown cells is low and located close to the nucleus. The signal emitted from random peptide linked MB in 3D scaffolds was low (Fig. 5) 2–3 h after MB delivery.

To visualize the spatial location of mRNA in cells in 3D, a Z-stack image of a 14 μm thick section from the bottom of the scaffold was performed. The 3D view of the Z-stack is shown in Fig. 6A. MB signals in HFF2 cells embedded in the 3D collagen scaffold show the spatial distribution of cells within the scaffold. ANOVA one-way statistical analysis of the image data showed that mean fluorescence intensity level in GAPDH knockdown cells in the 3D scaffold was significantly different to signal from control cells (Fig. 6B). Based on mean fluorescence intensity analysis, the GAPDH mRNA knockdown in cells in the 3D scaffold is 72%. This value agrees closely to the 78% calculated from RT-PCR data.

Conclusions

For the first time the use of TAT-peptide conjugated MB to monitor mRNA in a 3D in vitro system has been reported. Specifically, we have shown that TAT-peptide linked molecular beacons can monitor GAPDH mRNA expression in 3D type I collagen scaffold and delivery can be completed with fast kinetics (~1 h). Furthermore, spatial distribution of cells in 3D can be visualized. MB technology can be used to detect mRNA abundance in cells in 3D, but it is necessary to perform quantification with another complementary technique such as RT-PCR. Molecular beacons show a clear advantage over RNA FISH in providing spatial and temporal location of mRNA in living cells within a 3D cell culture system without the need to physically section the sample. This research was carried out using MB with 2'-deoxy backbone chemistries, but there are various chemistry modifications that can be utilized each with its pros and cons.28

Probe stability is essential for long term monitoring of gene expression inside living cells and to distinguish the true signal of the RNA targets from background signal. To address this problem, MB probes were designed with nuclease-resistant backbone chemistries that limit susceptibility to degradation by intracellular nucleases. Further, recently developed variants of MB such as ratiometric biomolecular beacons29 and Dual FRET MB30 have shown improved sensitivity over conventional MB and could potentially be adopted for live cell RNA imaging in 3D. Understandably, successful detection of target mRNA by MB is hindered by a combination of factors such as target accessibility, probe specificity for target, efficiency of probe delivery inside cells, and the sensitivity of the imaging technique used.

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Notes and references