

Biointerface Topography for Enhanced Nonviral Gene Delivery

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Substrate-mediated nonviral gene delivery (SMD) represents a burgeoning area of interest in the field of tissue engineering due to its potential to facilitate localized transfection while providing the scaffolding for growing cells and regenerating tissues. Surface modifications such as topographical features can also be incorporated onto the material to improve cell behaviours, including transfection. This approach holds significant promise in the context of vascular tissue engineering, which urgently requires innovative small-diameter vascular graft (SDVG) materials. Previous work has shown that topography, particularly microgrooves, can improve vascular endothelial cell (VEC) response on SDVGs. It is proposed that microRNA-145 (miR-145) delivery can improve vascular smooth muscle cell (VSMC) responses, thus providing a comprehensive platform for improved SDVG outcomes. However, the mechanisms by which topographical features enhance biomaterial-mediated gene delivery remain unclear. Moreover, topographical cues have not been used in combination with miR-145 and their effects on VSMCs are unknown. The aim of this thesis is to address these gaps using chitosan-grafted-polyethyleneimine (CS-PEI) as a nonviral gene carrier. This work is conducted in accordance with the central hypothesis that topography will enhance CS-PEI polyplex delivery in a feature dependent and cell-type specific manner and seeks to provide insight into how topographical cues can be rationally incorporated into biomaterials for enhanced gene delivery to various cell types.

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Dedication

For my parents.

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Chapter 1

Introduction & Literature Review

1.1 Introduction: Substrate-Mediated Nonviral Gene Delivery

Biomaterial-mediated or substrate-mediated nonviral gene delivery (SMD) represents a burgeoning area of interest in the field of tissue engineering due to its potential to facilitate localized transfection while providing the physical scaffolding for growing cells and regenerating tissues [1], [2]. In this approach, a nonviral gene carrier is immobilized, embedded, or coated onto or within substrate via electrostatic interactions or covalent bonds [3], which is then implanted at the site or injury or defect. As the host's cells adhere to and grow along the biomaterial or gene-activated matrix (GAM) *in situ*, they uptake the gene carrier and the exogenous nucleic acid is introduced into the cell via reverse transfection (**Figure 1**). Substrate-mediated gene delivery has emerged to improve transfection efficiency by exposing cells to the nonviral gene carrier in the local microenvironment, thus reducing mass transfer and diffusion limitations. Furthermore, the immobilization of the gene carrier helps to protect the vector from systemic removal, thus enhancing efficacy. Localized delivery also serves to reduce the likelihood of off-target-effects and global immunogenicity and toxicity, as may be observed in the systemic or bolus administration of the nonviral gene carrier. Moreover, one can fine-tune the biophysical and biochemical properties of the scaffold to prime the cells for transfection [4].

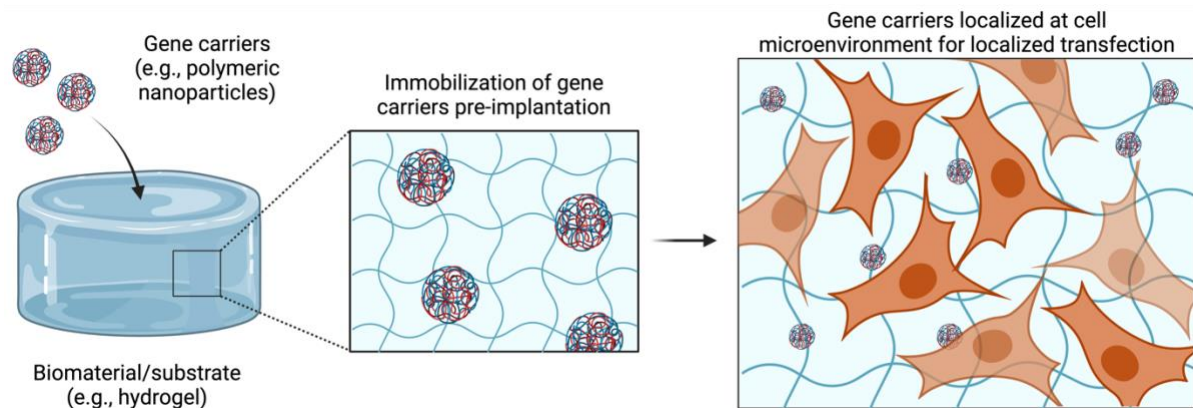


Figure 1. Principles of biomaterial-mediated or substrate-mediated nonviral gene delivery. Gene carriers, such as polymeric nanoparticles, are immobilized onto or within a biomaterial, such as a hydrogel. As the cells grow and migrate along and into the material, the gene carriers are placed directly within their local microenvironment, allowing for specific and targeted transfection. Created with BioRender.com.

An example of a biophysical parameter that can be employed is biointerface topography [5], which can be defined as the three-dimensional arrangement of nano/microstructures that confer surface roughness, curvature, and shape at the cell-material interface. Of particular interest are nano and micropatterns, which consist of ordered, repetitive structures that are rationally designed to mimic the native extracellular matrix (ECM). Biointerface topography can elicit profound effects on cell behavior, and provide mechanical cues to support cell adhesion, thereby improving biocompatibility and biomaterial integration. As will be discussed at length in [Section 1.3](#), such behaviors include adhesion, alignment, migration, and proliferation, which are correlated with enhanced transfection. Moreover, as will be outlined in [Section 1.4](#), topographical cues have been shown to interact with endocytic pathways and may promote the endocytosis of nonviral gene carriers. However, the exact mechanism by which topographical cues promote gene carrier endocytosis remain elusive. Despite this, the incorporation of topographical cues into a GAM presents an opportunity to enhance gene delivery whilst promoting desired cellular responses, thereby achieving a synergistic effect of the genetic and biophysical cues.

The combination of topographical cues and biomaterial-mediated gene delivery offers great promise in the context of small-diameter vascular tissue engineering. Vascular tissue engineering serves to regenerate damaged or diseased blood vessels, which are categorized on the basis of their luminal or internal diameter (i.d.) and range from micro vessels (i.d. < 1mm), small vessels (i.d. < 6mm), and large caliber vessels (i.d. > 6mm). In traditional vascular surgery, a damaged blood vessel is replaced with an autograft or a synthetic polymeric conduit to bypass the damaged vessel and reinstate blood flow, without an emphasis on regeneration of native vascular tissue. In vascular tissue engineering, the focus lies on tissue *regeneration*, which requires the development of vascular constructs that promote the reformation and maturation of the tri-layer structure found in vascular walls. Of particular importance are small-diameter blood vessels, which present a unique challenge in vascular surgery. As will be discussed in [Section 1.2](#), a significant clinical concern is the lack of appropriate materials for small-diameter vascular grafts (SDVGs). Our group has conducted extensive research into the use of biointerface topography for improving SDVG outcomes. Numerous studies have shown that biointerface topography can promote graft endothelialization (see [Section 1.2.2.1](#)), or the formation of an endothelial cell monolayer along the luminal graft surface, thus mimicking native blood vessel structure. However, intimal hyperplasia (IH) remains significant issue. Therefore, the combination of topography and nonviral gene delivery of a nucleic acid that mediates this remodeling response could be utilized to simultaneously promote endothelialization whilst limiting IH.

1.2 Small-Diameter Vascular Tissue Engineering

1.2.1 Introduction

Vascular tissue engineering is at the forefront of regenerative medicine as it serves to combat one of the most pressing clinical concerns of modernity: cardiovascular disease (CVD). The leading cause of global mortality, CVDs accounted for an estimated 19.1 million deaths in 2020, representing 37% of deaths due to noncommunicable diseases [6]. The total economic burden of CVDs was estimated to be \$378.0 billion in the United States alone (12% of the total healthcare expenditures between 2017 and 2018 – greater than any other diagnostic group) [6]. The prevalence and cost of CVDs such as ischemic heart disease, stroke, heart failure, and myocardial infarction are predicted to rise over the next few decades as the global population ages. As such, there is an urgent need for innovation in vascular surgery and CVD treatment.

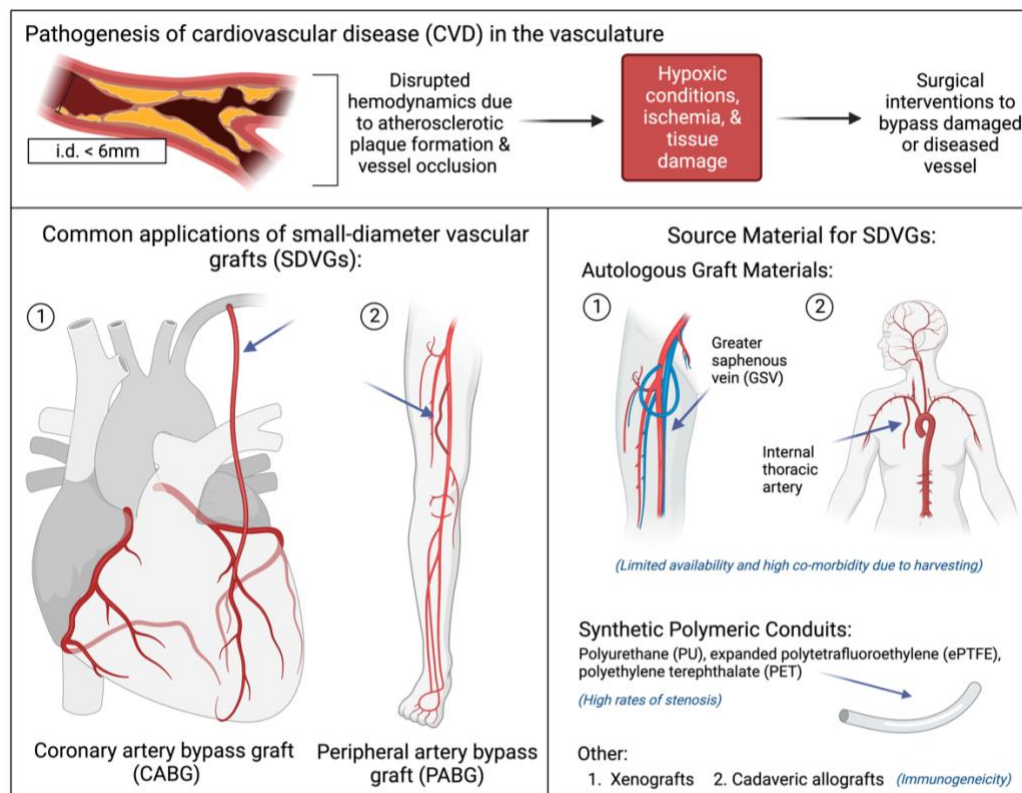


Figure 2. The applications of small-diameter vascular grafts and current graft source materials. The onset of cardiovascular diseases originates in the vasculature, with the formation of an atherosclerotic plaque, which, if stable and sufficiently large, will disrupt hemodynamics and prevent oxygen transport

to downstream tissues. Surgical interventions such as coronary artery bypass graft (CABG) surgeries and peripheral artery bypass graft (PABG) surgeries are often required to restore blood flow. SDVGs can be sourced from autologous vessels, namely the greater saphenous vein (GSV) and the internal thoracic artery, synthetic polymeric conduits, as well as xenografts and cadaveric allografts. The primary limitations associated with each material are denoted in blue. Created with BioRender.com

Surgical interventions are often used to treat these conditions and frequently require the use of vascular grafts to bypass the diseased or defective blood vessel (**Figure 2**). This is exemplified by the estimated 1 million coronary revascularization surgeries performed per annum in the United States alone, 400,000 of which are coronary artery bypass graft (CABG) surgeries. Coronary artery occlusions account for approximately half of CVD mortality, further emphasizing the demand for alternative vascular bypass materials. Traditional vascular graft materials include autologous blood vessels (commonly the saphenous vein (SV) or internal thoracic artery), cadaveric allografts, xenografts, and synthetic polymeric conduits (**Figure 2**). The reader is referred to a recent review by Naegeli et al. for a thorough discussion of these materials [7]. Autografts, particularly the SV, remain the gold standard for bypass surgeries due to the presence of a fully functional endothelium and the natural composition of patients' vessels. However, only ~30% of patients are suitable for surgery, due to reasons of comorbidity, prolonged operating time, and increased chances of pain and infection at the harvesting site. There is evident need for alternative graft materials, which has been met with the application of largely industrial synthetic polymers to vascular tissue engineering. Synthetic polymers used in vascular grafts include expanded polytetrafluorethylene (ePTFE) (commercially known as Gore-Tex®), polyurethane (PU), and polyethylene terephthalate (PET) (commercially known as Dracon®). Despite the enormous progress made in the use of these materials, therein remains several challenges associated with small caliber blood vessels or those with an internal luminal diameter of <6 millimeters. Synthetic small-diameter vascular grafts (sSDVGs) are a key challenge in vascular tissue engineering and are in high demand due to their use in common procedures such as CABG and peripheral artery bypass graft (PABG) surgeries. Crucially, a readily available, off-the-shelf sSDVG must be developed.

Despite the demand, an estimated 75% of sSDVGs are reported to fail within three years of implantation due to complications associated with thrombosis, calcification, aneurysm formation, and intimal hyperplasia (IH). The low long-term patency rates associated with sSDVGs are primarily due to the lack of *in situ* endothelialization as a consequence of the inherent hemoincompatibility of these materials. Endothelialization is the process by which a functional monolayer of endothelial cells (ECs)

forms on the luminal surface, resulting in the formation of an intimal layer reminiscent to that of native vessels [8]. This process is critical for promoting vascular remodeling and ensuring the long-term stability of the graft. Many works have shown that pre-seeding synthetic grafts with ECs improves patency rates and reduces thrombogenicity. Despite these benefits, this process is challenging, laborious, time-consuming, produces limited *in situ* endothelialization, and stifles the development of readily available off-the-shelf sSDVG. Topographical cues can be incorporated onto the surface of a sSDVG to overcome these barriers and represents the focus of this thesis. In this section, the current challenges that sSDVGs will be described to introduce the biological problems we aim to address using biophysical and genetic cues.

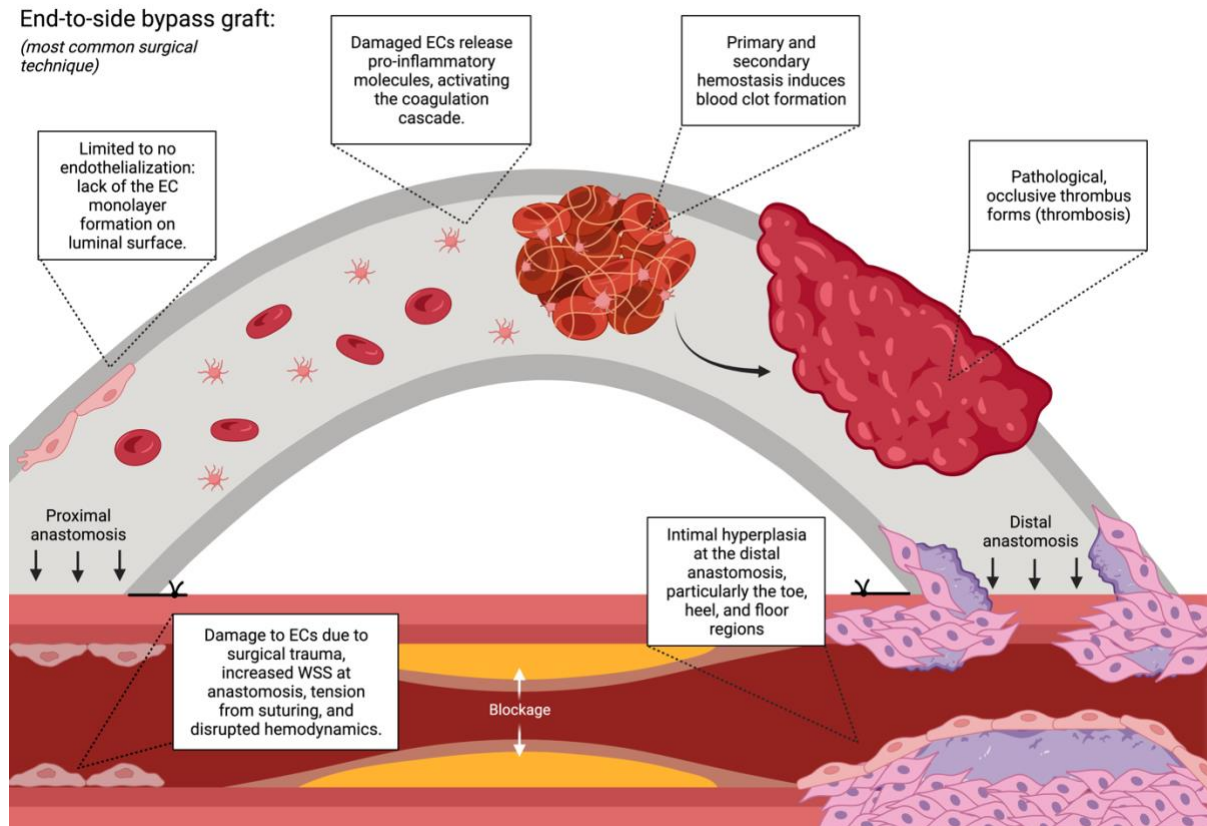


Figure 3. Schematic overview of the current factors that limit the clinical use of synthetic small-diameter vascular grafts, namely limited endothelialization, intimal hyperplasia, and thrombosis. Damage to the host endothelium due to surgical trauma, tension from suturing, and disrupted blood flow the triggers a cascade of injury responses. The lack of graft endothelialization or the colonization of the graft material by host ECs exacerbates the pathological injury responses. The coagulation cascade

is activated, resulting in the formation of a blood clot on the graft surface, which develops further into a thrombus. Additionally, the ECs initiate VSMC phenotypic switching, promoting their adoption of a synthetic migratory phenotype. The VSMCs migrate from the tunica media into the tunica intima and proliferate and produce abundant ECM, a phenomenon known as intimal hyperplasia (IH). Thrombosis and IH block the flow of blood, which has been redirected from the blockage via the end-to-side bypass graft, resulting in restenosis. As such, the graft must be explanted and replaced to prevent ischemia and restore flow. Created with BioRender.com.

1.2.2 Overview of the Current Challenges Facing Small-Diameter Vascular Grafts

The three main factors that lead to sSDVG graft failure are limited endothelialization, intimal hyperplasia, and thrombosis (**Figure 3**). After implantation of the sSDVG, an injury response is triggered due to the surgical procedure and the change in hemodynamics elicits a change in vessel homeostasis [9]. This triggers an injury response, eliciting the host ECs to secrete adhesion factors such as von Willebrand Factor (vWF), platelet tissue factor, fibronectin, coagulation factor and cytokines. As a result, the inflammatory response is activated, leading to the recruitment of monocytes and macrophages. There are several factors that influence the vascular injury response, such as the degree of mechanical mismatch, the suture strength, and so forth. Importantly, the outcome of the vascular response will determine if pathological responses such as IH, chronic inflammation, or atherosclerosis will develop or if vascular remodeling and healing will proceed.

1.2.2.1 Limited Endothelialization

Achieving endothelialization is a crucial factor for the long-term success of vascular grafts. In brief, *in situ* self-endothelialization involves the formation of a monolayer of ECs on the luminal surface via the recruitment, differentiation, and proliferation of endogenous ECs and endothelial progenitor cells (EPCs) from the native intima or the circulating peripheral blood. Three mechanisms of *in situ* endothelialization of sSDVGs have been identified: trans-anastomotic growth, transmural capillarization, and fallout endothelialization. The literature surrounding synthetic vascular graft endothelialization is not straightforward, a fact owed to the complexities of studying endothelialization mechanisms. Further, there are doubts as to the extent at which the three processes occur, both in general and in humans. For a more in-depth discussion the literature, the reader is recommended to the review by Sánchez et al [9].

Trans-anastomotic pannus growth is considered as the growth of the host intima from the anastomosis sites as a consequence of the injury response. Therefore, the endothelium is comprised of ECs from the native artery. Transmural capillarization involves on the migration of capillaries through the graft material, which occurs as a consequence of the angiogenic elements of the foreign body response (FBR). The formation of granulation tissue during the FBR requires neovascularization and involves fibroblasts and macrophages that secrete angiogenic factors. Thus, perigraft tissue forms around the sSDVG and includes capillaries that ultimately migrate through the graft material and towards the lumen. The third mechanism is fallout endothelialization, which involves the homing and adhesion of EPCs from the circulation [9], [10]. EPCs are CD34⁺ mononuclear cells that are capable of adopting EC characteristics. They originate from the bone marrow and are found in very small numbers in the peripheral blood. Chemotactic secreted by host ECs and subsequent immune cells such as platelets post-implantation act as transducers to mobilize EPCs from the bone marrow and into the bloodstream and further promote their homing to the site of injury or neovascularization. Subsequently, the EPCs must be tethered and adhered to the graft surface. Once adhered, the EPCs may differentiate into the mature ECs, a process that is further supported by the peripheral signals produced during the vascular injury response. With this comes the formation of endothelial cell-cell junctions. The EC junctions are composed of tight junctions (claudins, occludins, and junctional adhesion molecules), adherens junctions (EJAs) (vascular endothelial cadherins (VE-cadherin)), and platelet endothelial cell adhesion molecule (PECAM-1 or CD31). The formation of cell-cell contacts at the lateral membrane give rise to an EC monolayer.

1.2.2.2 Intimal Hyperplasia

The pivotal role of intimal hyperplasia (IH) in vascular graft restenosis and failure has been recognized for over 100 years [11]. Since its discovery, significant effort has been dedicated to uncovering the causative factors of IH. The key triggers that have been identified are suture line stress, inherent thrombogenicity of the biomaterial and low biocompatibility, mechanical or compliance mismatch between the graft and the graft, and endothelial damage [12]. An in-depth discussion of these factors is beyond the scope of this thesis, and the reader is recommended the reviews by Lemson et al. [11], Ward et al. [13], and Jeong et al. [14] for further reading on the cellular, biochemical, and biomechanical causes of IH, respectively. Another excellent review by Zhizhou et al. focused on current strategies to circumvent IH in sSDVGs. Briefly, endothelial damage arises as a consequence of tension generated by from suturing and aberrations in local hemodynamics and alterations in wall shear stress

(WSS) due to compliance mismatch, and surgical trauma, which exposes the subendothelial basal lamina, VSMCs, and fibroblasts to the bloodstream.

The damaged ECs then secrete the peptide endothelin, pro-inflammatory cytokines [13], growth factors such as platelet-derived growth factor A and B (PDGF-A, PDGF-B), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF-2), and transforming growth factor β (TGF- β) [15], and anchor proteins such as von Willebrand Factor (vWF), to which platelets adhere. Furthermore, the ECs reduce the release of antiproliferative, vasodilatory molecules such as prostacyclin (PGI₂), heparan sulphate, nitric oxide (NO), and tissue plasminogen activator (tPA). These factors promote the de-differentiation of VSMCs and the pathogenesis of IH.

VSMCs are uniquely plastic and undergo phenotypic switching in response to these signals, thereby exchanging a quiescent, contractile phenotype for a synthetic, migratory phenotype [16], [17]. In adult arteries, VSMCs possess a low proliferative capacity and maintain a contractile status, indicated by the expression of α smooth muscle actin or α -actinin 2 (α -SMA or ACTA-2), calponin, smooth muscle myosin heavy chain II (SMMHC or MYHCII), and smoothelin-B (smooth muscle differentiation specific antigen B). Contractile VSMCs are elongated and spindle-shaped, whereas synthetic VSMCs are characterized by a rounder, cobblestone morphology [16]. The synthetic phenotype is further described by the expression of genes such as, and the upregulation of matrix metalloproteinases (MMPs), which allow them to cleave the surrounding ECM and migrate towards the intima [18]. Furthermore, synthetic VSMCs secrete large amounts of the collagen and proteoglycans (however, not elastin) into the intima, resulting in its expansion. As such, the neointima consists of ~20% of VSMCs and ~60-80% of ECM [11]. Therefore, a principal aim in designing materials that limit IH is to limit VSMC phenotype switching and promote a contractile, quiescent phenotype.

1.2.2.3 Thrombosis

Thrombogenesis occurs as a result of the injury response described in the previous section, ultimately forming an occlusive thrombus in the sSDVG lumen. The lack of an endothelium on the blood-contacting surface to provide antithrombogenic factors, combined with the injury response induced by surgical procedures and disrupted hemodynamics, activates hemostasis and the coagulation cascade [19]–[21]. Hemostasis is classically categorized into two main steps: primary hemostasis and secondary hemostasis. In primary hemostasis, platelets are activated by the exposed ECM and factors released by the ECs upon engraftment, promoting their migration to the site of vascular injury. The

activated platelets aggregate at the injury site by adhering to EC adhesion molecules and the ECM, forming a platelet plug. Secondary hemostasis succeeds platelet formation and involves the proteolytic coagulation cascade. The numerous steps that comprise primary and secondary hemostasis are beyond the scope of this review and the authors recommend the review by Versteeg et al. [20] for further information on the hemostatic system. The coagulation cascade serves to produce an insoluble fibrin mesh that is embedded within and encapsulates the platelet plug. The resulting blood clot serves to arrest bleeding at the injury site and is strengthened and stabilized by the fibrin mesh. Hemostasis is a balance between coagulation and anticoagulation. An imbalance in these forces can drive the formation of a thrombus, or a pathological clot [19], [20]. With limited endothelialization, anticoagulatory signaling (e.g., NO, PGI₂, and tissue factor pathway inhibitor (TFPI)) is reduced, resulting in thrombosis. Therefore, improved endothelialization and EC functionality is crucial for preventing thrombus formation.

1.3 microRNA-145 for Vascular Tissue Engineering Applications

1.3.1 Introduction to microRNA

MicroRNA (miRNA) are small (~22 nucleotides), non-coding RNA molecules that possess post-transcriptional gene silencing activity. miRNAs bind to a complementary region 3' untranslated region (UTR) of their messenger RNA (mRNA) targets by their “seed” sequence, thereby promoting their degradation and repressing translation. Importantly, miRNAs bind with imperfect or partial complementarity. Thus, a single miRNA may have multiple mRNA targets and may mediate the expression of numerous genes. Moreover, a single mRNA may be regulated by numerous miRNAs in a spatiotemporal dependent manner. It is predicted that more than one third of the genome is regulated by miRNAs. As such, it is no surprise that miRNAs are implicated in nearly all key cell processes.

The biogenesis of miRNAs is multi-step (**Figure 4A**). Firstly, miRNAs are transcribed in the nucleus by RNA polymerase II or III from intergenic or intronic regions of the genome, yielding a hairpin structure known as a primary miRNA (pri-miRNA). The pri-miRNA is cleaved via a proteinaceous complex known as the microprocessor, which consists of the nuclease Drosha and the RNA-binding protein DiGeorge syndrome critical region 8 (DGCR8). The resulting precursor miRNA (pre-miRNA) is then exported from the nucleus to the cytoplasm via the Exportin-5 and RanGTP nucleocytoplasmic transporter, where remaining processing and miRNA maturation takes place. The pre-miRNA is then further cleaved via the RNase III endonuclease Dicer to yield a double-stranded

miRNA molecule, consisting of the guide strand and passenger strand. The transient duplex is then unwound, and the guide incorporated into the RNA-induced silencing complex (RISC). Finally, the RISC identifies mRNA targets with complementarity to the seed sequence of the guide strand and cleaves them. However, not all miRNA/mRNA target interactions are repressive. In fact, there are several reports that miRNAs may upregulate expression of certain targets.

1.3.2 microRNA-145 in Vascular Biology & Disease

The pivotal role of microRNA-145 (miR-145) in cardiovascular biology has been energetically explored over the last 15 years. A series of elegant studies performed in 2009 by Cheng et al. [22], Cordes et al. [23], Boettger et al. [24], Elia et al. [25], and Xin et al. [26], provided the foundation for our understanding of the role of miR-145 in vascular development, VSMC plasticity, and CVD. In the following years, both miRNAs have been extensively studied. miR-145 is central to the regulatory gene expression network that governs VSMC differentiation and dedifferentiation [27]–[29].

In humans, miR-145 maps to chromosome 5q32-33 and is a component of the MIR143HG bicistronic unit, or the miR-143/145 cluster. In other terms, both miR-143 and miR-145 are co-transcribed from this locus as a single long non-coding RNA (lncRNA) pri-miRNA transcript, which is then processed to yield distinct miRNAs that bind to different targets [26]. The promoter region of the miR-143/145 contains a highly conserved sequence that possesses binding sites for serum response factor (SRF) and its coactivator myocardin (Mycod), the master regulatory complex of VSMC differentiation [23]. The SRF/Mycod pathway stimulates SMC specific miR-143/miR-145 expression early in development [23], resulting in high expression in the nascent heart. Postnatally, miR-145 expression is present in VSMCs across tissue types (it is, in fact, proportional to the quantity of VSMCs in the tissue) and is concentrated the heart and coronary vessels. The expression of the miR-143/145 cluster is also regulated by cardiac and myocardin-related transcription factors (MRTFs) such as Nk2 transcription factor related locus 5 (Nkx2-5) and growth factors such as transforming growth factor- β (TGF- β) and platelet derived growth factor (PDGF).

To regulate proliferation and differentiation, miR-145 binds to its mRNA targets Krüppel like factor 4 and 5 [22], [30], [31] (KLF4 and KLF5, respectively), Mycod [23], and calmodulin kinase II- δ (CamKII- δ) [23], [32], and Elk-1 (**Figure 4C**). CamKII- δ and Elk-1 are known to interact with SRF to drive the expression of synthetic genes, thus promoting dedifferentiation. Intriguingly, miR-145 binds to the 3' UTR of Mycod and stimulate its expression, creating a positive feedback loop to regulate

its own expression [23]. As cytoskeletal organization, podosome formation, and cellular contractility are also the critical phenotypic switching process, miR-145 targets are also implicated in these processes. For example, Xin et al. found that miR-145 directly targets Sling-shot 2 (Ssh2) phosphatase, which promotes F-actin reorganization by dephosphorylating cofilin, thus increasing cell mobility [26]. The authors also found that miR-145 targets Slit-robo GTPase activating protein (Srgap) 1 and 2, which promote actin depolymerization by interacting with the GTPase Cdc42 [26]. Therefore, miR-145 is also intertwined with the transcriptional networks that govern cytoskeletal assembly and organization. Quintavalle et al. found that miR-145 inhibits podosome formation – a key morphological component of cell migration – through its target fascin, an actin-bundling protein [33]. In their seminal study, Boettger et al. found that the blood vessels of miR-145 null mice were thinner and possessed a much higher proportion of synthetic VSMCs relative to the wild-type [24]. Furthermore, they found that explanted vessels from knockout mice exhibit significantly reduced contractility and a dramatic loss of response to angiotensin II and adrenergic signaling. The authors found that miR-145 directly targets angiotensin-converting enzyme (ACE), and chronic, pharmacological inhibition of ACE can partially restore contractility in knockout (KO) mice. The authors hypothesized that the lack of miR-145 inhibition of ACE results in the aberrant upregulation of membrane-bound ACE, therefore chronically stimulating the VSMCs with angiotensin II, the cleavage product of ACE. According to their model, chronic angiotensin stimulation desensitizes the VSMCs, resulting in the lack of contractile response and loss of blood pressure observed in the miR-145 KO mice. Through these mRNA interactions, miR-145 alone can promote the expression of contractile genes in VSMCs and thereby reverse phenotypic switching.

Due to its central role in mediated VSMC behavior, miR-145 expression and function has been implicated in a range of CVDs, including, atherosclerosis, ischemic stroke, hypertension, and myocardial infarction [27], [34]. Several works have specifically investigated the formation of neointimal lesions during atherosclerosis and after vascular injury. Boettger et al. found that neointimal lesions form in miR-145 KO mice even in the absence of injury [24]. Furthermore, miR-145 has been found to be significantly decreased to near undetectable levels in the neointimal lesions of atherosclerotic plaques in ApoE KO mice [35]. A study by Caruso et al. found that miR-145 was upregulated in VSMCs derived from patients with pulmonary arterial hypertension [36]. More recent studies have examined the utility of miR-145 as a biomarker for CVD. For example, aberrant miR-145 levels are correlated with the incidence and size myocardial infarction [37]. Importantly, low plasma

miR-145 levels are correlated with in-stent restenosis as determined in separate studies by Yuan et al. [38] and Meijao et al. [38]. Overall, miR-145 is a key regulator of not only basic VSMC biology but more complex CVD pathogenesis and presentation.

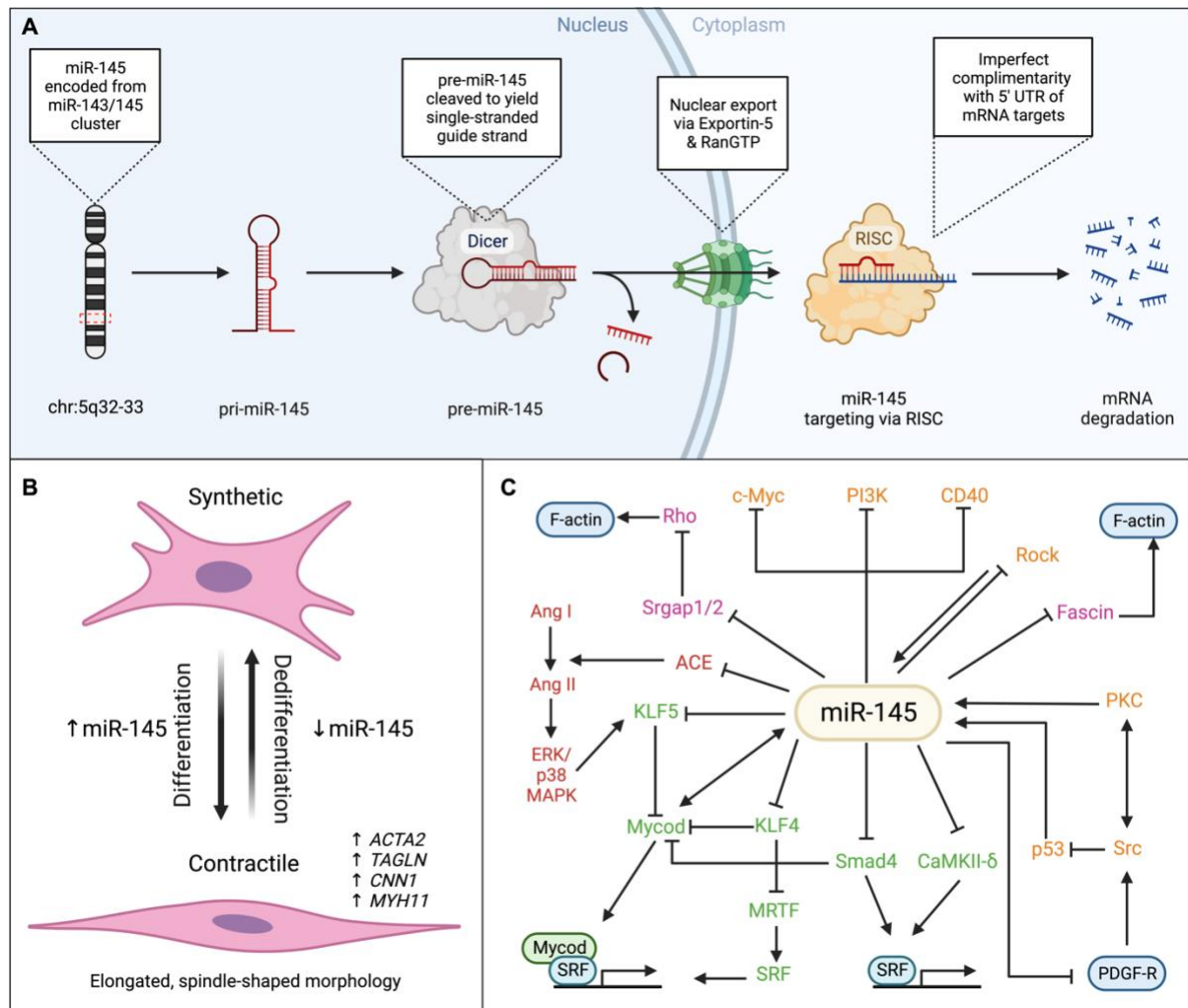


Figure 4. The central role of miR-145 in vascular smooth muscle cell biology. (A) The biogenesis of miR-145, beginning with its transcription from the miR-143/145 cluster located on the q arm of chromosome 5. The resulting pri-miR-145 transcript, a long hairpin loop, undergoes processing in the nucleus by Dicer and is exported into the cytoplasm as a single stranded guide strand. Upon association with the RISC, the mature miRNA can target mRNA targets for degradation. (B) The role of miR-145 in mediating phenotypic switching, or the differentiation and dedifferentiation of VSMCs from and to a synthetic, fibroblasts like phenotype. (C) The integral role of miR-145 in the transcriptional networks

that govern VSMC phenotype. In green are the mRNA targets involved in synthetic and contractile gene expression. In red, the mRNA targets that play a key role in mediating VSMC contractility and response to vascular tone. In pink, the mRNA targets involved in actin remodelling and podosome formation, thus migration. In orange, the mRNA targets involved in proliferation. Created with BioRender.com.

1.3.3 Applications of microRNA-145 in Vascular Biomaterials

The therapeutic potential of miR-145 has been explored since the discovery of its role in neointimal hyperplasia formation. Cheng et al. and Elia et al. both found that miR-145 overexpression is sufficient to reduce IH after angioplasty [22] and balloon-injured arteries [25] in rat and mouse models respectively. Based on these reports, recent efforts have focused on the development of innovative vascular graft and stent materials that incorporate miR-145-based gene therapies. Biomaterial-mediated delivery is utilized to achieve targeted delivery of miR-145 to VSMCs. Such works include those of Ohnaka et al., 2014 [39], Zhou et al., 2017 [40], Cui et al., 2018 [41], Nishio et al., 2019 [42], Wen et al., 2019 [43], and Wen et al., 2020 [44].

Ohnaka et al. and Nishio et al. utilize autologously derived vein grafts from rabbits for miR-145 gene therapy [39], [42]. In these works, the authors extract the vein graft and then transfect native cells with either plasmid DNA encoding miR-145 or miR-145 mimics encapsulated in polymeric nanoparticles, respectively. Both studies have shown a reduction neointimal lesion formation, as well as an increase in miR-145, α -SMA, and MHC expression in host VSMCs 2 and 4 weeks after implantation of the treated graft. Furthermore, the authors show an increase in the Ki-67 positive or proliferative VSMCs in the control graft compared to the treated graft. Nishio et al. also show a reduction in the inflammatory phenotype, demonstrating a decrease in the inflammatory phenotype, showing a reduction in the expression of CD40 and nuclear factor kappa B (NF- κ B) [42]. Ohnaka et al. provided early insight into not only the profound effects of miR-145 in attenuating IH, but also the capacity of pDNA encoding miRNA to mediate cell responses. Zhou et al., Cui et al., Wen et al., 2019, and Wen et al., 2020 utilize a synthetic material for miR-145 SMD [40], [41], [43], [44]. In these studies, the authors develop multilayered electrospun sSDVGs with polymeric nanoparticles containing miR-145 embedded in the inner graft layers. The authors show exceedingly promising controlled release of miR-145, with the release extending beyond 50 days in multiple cases [40], [41], [43]. In the self-same studies, the proportion of contractile VSMCs was significantly higher on the miR-145 grafts,

indicated by increased α -SMA, myocardin. However, as demonstrated in Cui et al., miR-145 overexpression alone was insufficient to increase EC coverage of the graft. In a subsequent study by Wen et al., 2020, miR-126 was delivered in conjunction with miR-145 [41]. This miRNA plays a key role in angiogenesis and was selected with the aim of encouraging endothelialization. The synergy of the two miRNAs promoted both endothelialization and the contractile phenotype [44]. As such, a key conclusion from these studies is that miR-145 overexpression from a vascular graft can modulate VSMC behavior to reduce IH yet is inadequate in promoting endothelialization. Therefore, miR-145 offers enormous potential when utilized in combination with additional biochemical and biophysical cues.

1.4 Cationic Polymers for Nonviral Gene Delivery

Despite their high efficacy, the safety concerns surrounding the use of viral vectors have halted their progression. Alternative methods for gene delivery have long been sought after to overcome the shortfalls of viral vectors. Nonviral gene delivery platforms can be divided into two categories: cationic lipid-based and cationic polymer-based. The principles of nucleic acid delivery are relatively conserved across the two methods, and result in the formation of *lipoplexes* and *polyplexes*, respectively [45]. In both cases, the cationic charge on the molecule is used to form electrostatic interactions with anionic DNA or RNA and condense it into particles. Lipid-based nonviral gene delivery systems are comprised of amphiphilic lipid molecules with a hydrophobic tail and a hydrophilic amine head with a permanent positive charge [46]. Polymeric systems are considered an attractive source of nonviral gene carriers due to their facile chemistry, cost-effectiveness, ability to be synthesized from both natural and synthetic sources, and safe toxicity profiles. An advantage of polymeric systems is that they are entirely soluble in aqueous solution as they lack a hydrophobic group and have the capacity to condense nucleic acids into relatively small particles. Although nonviral gene delivery platforms offer improved safety, they suffer from poor transfection efficiency, a technical hurdle that must be overcome to facilitate their transfection from the bench to bedside. Thus, there is major impetus to improve the efficacy of nonviral gene delivery systems.

A polymer that has gained significant traction over the last two decades is polyethyleneimine (PEI), which was first used as a gene carrier in 1995 [47]. PEI can be synthesized to yield two geometrical forms, branched PEI (BPEI) and linear PEI (LPEI), which are used at molecular weights of 25 kDa and 22 kDa respectively. BPEI contains primary, secondary, and tertiary amines whereas

LPEI possesses only secondary amines. The capacity of PEI to efficiently condense nucleic acids and its relatively high transfection efficiency is owed to the large number of amines found in the polymer. Moreover, only a small fraction (10-15%) of these amines are protonated at physiological pH, which confers its high buffering capacity. PEI-based polyplexes are believed to primarily internalized by clathrin-mediated endocytosis, which is a form of receptor-dependent endocytosis. This mechanism of endocytosis is activated by the interaction between PEI polyplexes and the negatively charged proteoglycans found at the surface of the cell. Other reports have postulated that PEI enters the cell via direct membrane penetration and caveolae-mediated endocytosis, both of which are non-receptor dependent. Upon entry into the cell, the polyplexes enter the endolysosomal system, which determines their intracellular fate. A key intracellular barrier in nonviral transfection is escaping from the endolysosomal system to avoid exocytosis or degradation in the lysosomes and enter the cytoplasm to begin the journey to their target site. The high buffering capacity of PEI facilitates its release from the endosomes via a phenomenon known as the “proton sponge effect”. As mentioned, PEI possesses a high buffering capacity, and upon entry into the acidic endolysosomal compartments, undergoes protonation. This drives the ATP-dependent influx of protons and chloride, causing an osmotic balance. The subsequent influx of water causes the endosome to swell. Protonation also introduces conformational changes in the polyplex, resulting in free PEI chains that intercalate with the endolysosomal membrane and form nanopores in the bilayer. The combination of swelling and the formation of pores promotes the rupture of the endosome, releasing the polyplex into the cytoplasm. Here, the PEI must dissociate from the nucleic acid, likely through competitive interactions with anionic molecules found in the cytosol or in the nucleus itself. While the positive charge on PEI is critical for its transfection efficiency, it comes at the cost of reduced viability. As first introduced by Godbey et al., PEI-induced cytotoxicity occurs in two stages. The initial stage of cell death, which occurs within hours of exposure, is caused by free PEI and is the result of perturbations in the membrane. The second stage of cell death arises within days and is caused by PEI-induced mitochondrial dysfunction. In summary, while PEI has great potential as an efficient nonviral gene carrier, it faces several limitations due to toxicity.

Several strategies have evolved to improve the cytotoxicity of PEI polyplexes. A well-studied approach is “charge shielding”. To reduce the positive charge on the polyplex surface, a polysaccharide or hydrophobic moiety is conjugated to the polymer which acts as a shield to the positive surface charge. Chitosan (CS) is a cationic polyaminosaccharide that has been explored for charge shielding due to its

biocompatibility and biodegradability. As a cationic polymer, CS can form polyplexes with nucleic acids, but presents a pronounced reduction in transfection efficiency compared to polymers like PEI. CS is obtained from the N-acetylation of chitin, a highly abundant biopolymer. Unlike PEI, CS does not possess a high buffering capacity and experiences limited endosomal escape – the primary cause of its low transfection efficiency. As such, conjugates of CS and PEI have arisen to couple the favorable biocompatible and charge-shielding properties of CS with high buffering capacity of PEI to limit cytotoxicity while maintaining transfection efficiency. In this thesis, a copolymer formed by the grafting of CS onto a PEI scaffold is explored. First described in Lin et al., 2018 [48], this copolymer, herein referred to as CS-PEI, is formed from the succinated CS and high molecular weight BPEI. The succinyl group attached to chitosan acts as a linker to improve the engraftment efficiency. The CS-PEI described in Lin et al. showed high efficacy both *in vitro* and *in vivo* for the delivery of pDNA encoding insulin for the attenuation of diabetes in a mouse model. This report emphasizes the use of CS to improve the toxicity of PEI and suggests that it has significant potential in miRNA therapeutics due to its favourable intracellular binding kinetics.

1.5 Mechanism of Nonviral Gene Carriers: Endocytosis

For the therapeutic nucleic acid to reach its target site (e.g., the cytosol or nucleus), it must transverse the plasma membrane, escape the endosomal system, avoid degradation by cytosolic nucleases, undergo transport across the nuclear membrane, and undergo translation or integration into the host genome. The rate-limiting step for nonviral gene delivery is the internalization or endocytosis of the gene carrier [49]. Endocytosis is the process by which cells engulf extracellular macromolecules and solutes into membrane bound vesicles. The vesicles are formed from the cellular plasma membrane, which invaginates to wrap around the extracellular material and then buds off intracellularly. Several endocytic pathways have been identified and current knowledge defines five major types (**Figure 5**): clathrin-mediated endocytosis (CME), fast endophilin-mediated endocytosis (FEME), clathrin-independent/dynamin-independent carrier endocytosis (CLIC/GEEC), macropinocytosis, and phagocytosis. The latter is a cell-specific process and is found only in phagocytes, which pathogens and apoptotic remains are ingested. As such, it will not be discussed here.

Macropinocytosis, or fluid-phase endocytosis (FPE) is a low-energy, receptor-independent, and non-specific process by which extracellular fluid and its solutes are uptaken into the cell. This process is dependent on actin polymerization and occurs at sites of membrane ruffling. Contrastingly,

CME, the best understood mechanism, is a receptor-mediated endocytosis (RME) pathway and requires interactions between the gene carrier and membrane-bound receptors. Receptor activation and clustering recruits adaptor and accessory proteins to clathrin-coated pits within the plasma membrane and promotes clathrin nucleation into curved lattices. Progressive clathrin polymerization supports the constriction and fission of the vesicle from the plasma membrane, which is pinched off via dynamin activity. The resulting clathrin-coated vesicle is released into the cell, where it travels to its destination. FEME is a similarly receptor-dependent pathway and specifically internalizes receptors as its cargo. Instead of clathrin, FEME depends on the enrichment of endophilins at the plasma membrane yet still requires dynamin for vesicular fission. Finally, CLIC/GEEC is a receptor-independent pathway that is mediated by the oligomerization of the cargo on the extracellular surface, invaginating the membrane from the outside. Gene carriers, specifically nanoparticles, are believed to interact predominantly with the CME pathway, yet are also known to be internalized through FPE. While caveolae, nanoscale, flask-shaped pits in the plasma membrane, are also implicated in endocytosis, yet their function remains uncertain. Pivotal to all endocytic pathways is membrane bending and invagination, or the induction of negative membrane curvature inwards. This process involves the formation of networks of structural proteins which drive the formation of the vesicle, which is followed by scission from the membrane.

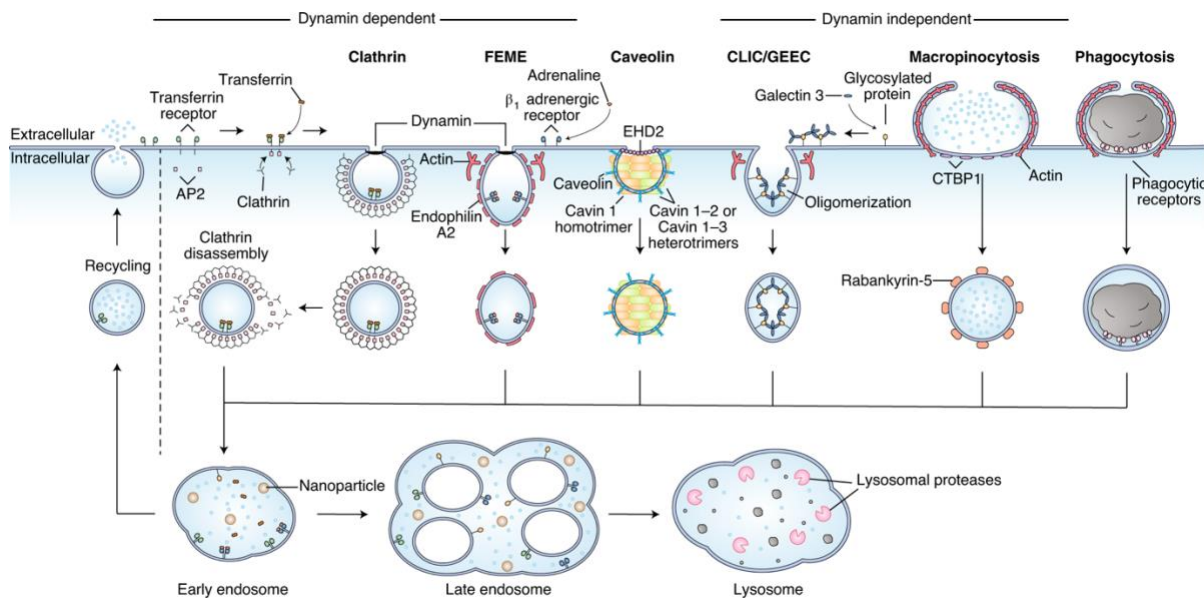


Figure 5. Mechanisms of nanoparticle endocytosis and entry into the endolysosomal system. Reprinted with permission from Nature Nanotechnology, 16, 266–276, Joshua J. Rennick et al., “Key principles

and methods for studying the endocytosis of biological and nanoparticle therapeutics”. Copyright © 2021, Springer Nature Limited.

Gene carriers exploit endocytic pathways, predominantly CME, to transverse the plasma membrane. The intracellular fate of the nanoparticles is largely determined by the mechanism of endocytosis, including the rate of endocytosis and quantity of material internalized. Moreover, the physicochemical properties of the nanoparticle (size, shape, surface chemistry and charge) influence the route of endocytosis [50]. Furthermore, as will be discussed subsequently, the physical properties of the microenvironment modulate the endocytic phenotypic and may influence nanoparticle internalization. As such, understanding the mechanism and efficiency of nanoparticle endocytosis is of vital importance in designing gene delivery platforms.

1.6 Topographical Features for Tissue Engineering: Overview

An array of topographical patterns has emerged to modulate cell behavior. Biointerface topography can be categorized as either *isotropic* or *anisotropic*. Isotropic patterns are those that do not have a direction vector and include pillars, wells, holes, pits, pores, cones, and so forth. Alternatively, anisotropic topographies create a surface with direction. These patterns include gratings, grooves, ridges, etc. A key tool to systematically screen several topography geometries and dimensions is the MultiARChitecture (MARC) chip, which is a grid of numerous topographies of that allows for rapid and simultaneous analysis of topography-induced behaviours under identical experimental conditions. [51]. The MARC chip has been used to uncover a range of effects of topography on cell behavior and is shown in **Figure 6**.

A range of fabrication methods can be employed to pattern such topographies on varying materials. Although they will not be reviewed here at length, the reader is recommended to the reviews by Cui et al. [52] and Tenje et al.[53] for further insight into surface patterning methods. Additionally, the authors suggest the review by Ahn et al.[54] for a recent, in-depth analysis of the engineering and fabrication of hierarchal topographies specifically. Briefly, biointerface topography can be produced via inducing surface roughness or by patterning techniques. Only the latter will be discussed in this work. Patterning techniques include photolithography (UV lithography)[55], soft lithography[56]–[58], stereolithography (SLA)[59], [60], microcontact printing (μ CP), nanoimprint lithography (NIL)[61], 3D printing (inkjet based, extrusion based, and vat polymerization based)[62], and scanning-probe based techniques such as laser micromachining.

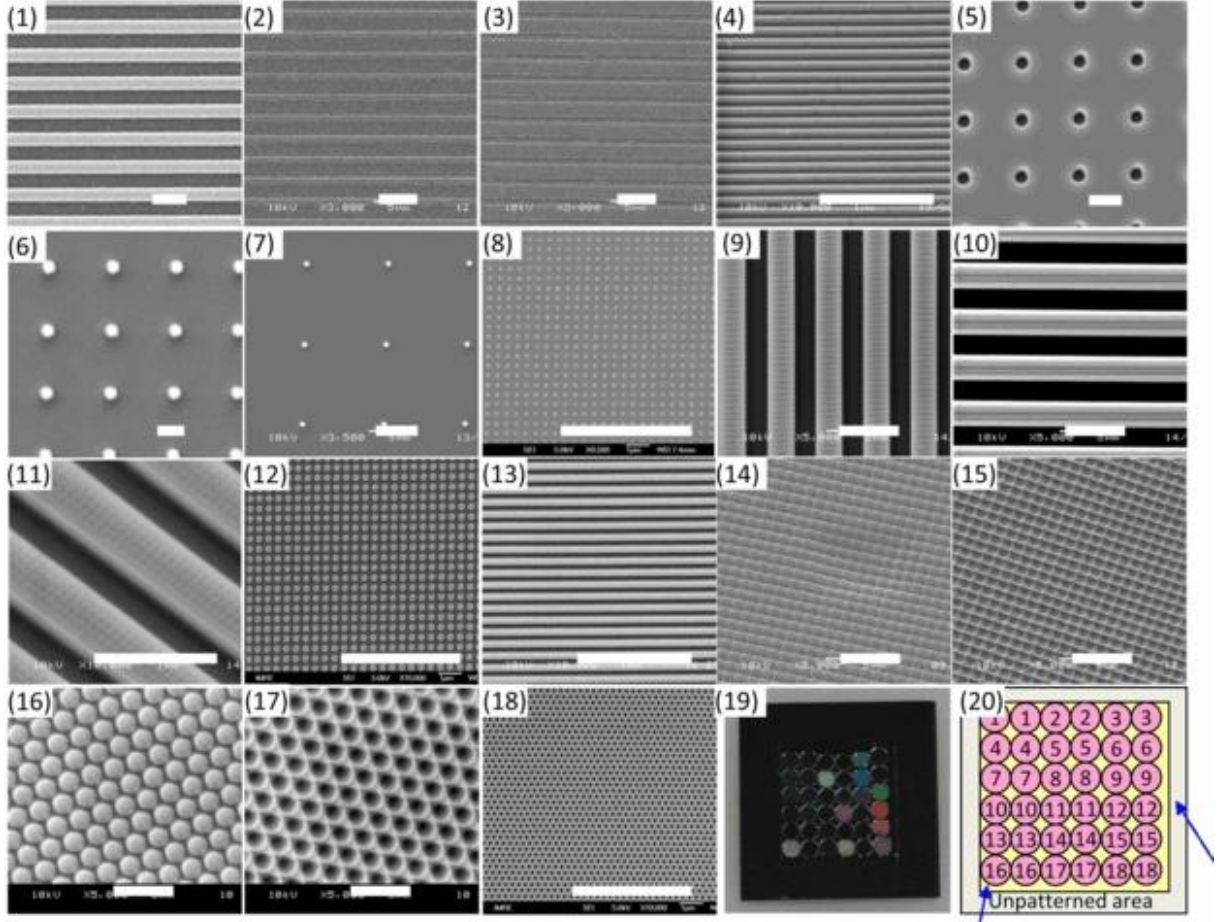


Figure 6. A selection of topographical patterns from the MultiARChitecture (MARC) chip fabricated by Moe et al., 2012 [51]. Topographical patterns may be anisotropic, as seen in patterns 1-4 or isotropic as exemplified by the pillars in patterns 6 and 7. Additionally, hierarchal topographical patterns have been developed, as in the case of pattern 9 and 11. The MARC chip allows for the systematic screening of a range of topographical features, as shown in the photograph in panel 19. Reprinted with permission from Small, 8: 3050-3061, Moe et al., “Microarray with Micro- and Nano-topographies Enables Identification of the Optimal Topography for Directing the Differentiation of Primary Murine Neural Progenitor Cells”. Copyright © 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

The term “contact guidance” is used to describe the range of behaviors that are induced by topographical features and is most well-understood in the context of anisotropic topographies. As discussed in Leclech & Barakat, 2021, the predominant model for topography-driven cellular alignment and elongation (the principal behaviors observed on anisotropic features) is based on focal adhesions

(FAs) [63]. In this model, anisotropic topography guides the growth of cell protrusions such as lamellipodia and filopodia and the formation and elongation of FAs in the direction of the topography while restricting their growth in the orthogonal direction. The lateral constraints placed upon cellular protrusions and stress fibers generates anisotropic stresses within the cell. Anisotropic stress and tension drives the formation of F-actin fibers (stress fibers) in the direction of the topography, resulting in cellular alignment and elongation along the topography axis. The FA-based model of topography-mediated cell alignment places FAs as the principal mechanosensing components. In accordance with this model, FA formation and cytoskeletal rearrangement is believed to underpin the behaviors observed on topography. Other models have been proposed, such as topography-induced membrane curvature, yet these have not been as thoroughly explored. In this model, curvature-sensing proteins such as BAR domain containing proteins detect membrane curvature induced by topography and activate actin cytoskeleton reorganization [64]. Overall, it is clear that a range of cellular behaviors can be activated in response to topography through various sensing mechanisms.

1.7 Topographical Features for Enhanced Endocytosis

The potential connections between biointerface topography, endocytosis, and nonviral gene delivery were proposed over decade ago in the review by Adler & Leong [5]. Since this publication, numerous studies have sought to explore the link between enhanced nanoparticle endocytosis and biophysical cues, namely stiffness and topography [65], [66]. The prevailing consensus suggests that increased substrate stiffness alters endocytosis by altering membrane tension and cell spreading area [66]–[68]. As shown in Huang et al., stiffer substrates resulted in increased uptake, which the authors propose is owed to alterations in myosin II and actin cytoskeleton function and organization, which promote increased cell spreading. A subsequent study by Lee et al. further evaluated these results and observed cell-type specific responses to stiffness, with epithelial cells and macrophages showed enhanced internalization on stiff and soft substrates, respectively. The interplay between stiffness and topography-sensing mechanisms provides insight into the mechanoresponses that mediate endocytosis in various microenvironments.

Several studies [69]–[71] support the conjecture posed in both Adler & Leong and Mantz & Pannier that substrate topography mediates integrin activation, FA formation, cytoskeletal reorganization and assembly, and cellular spreading to promote the endocytic phenotype. For example, a landmark study [69] from our lab has shown that on 2 μ m micropillars COS-7 and human

mesenchymal stem cells (hMSCs) form actin-rich cytoskeletal structures at the top of the pillars and exhibited enhanced macropinocytosis. Teo et al. suggest that macropinocytosis was initiated at the actin-rich structures, which modulated internal cellular tension and activated the family of Rho GTPases that facilitate actin remodelling during FPE [69]. The authors also showed that Lipofectamine™ 2000 mediated transfection was highest on nanopillars, suggesting CME was activated by these features. Several groups have examined the effect of fibrous topography on nonviral gene delivery. For example, Yau et al., 2015 examined the effects of varying nanofiber diameter on small-interfering RNA (siRNA) gene silencing activity using TKO as the transfection reagent [72]. The authors showed that decreasing the nanofiber diameter from 1.3µm to 300nm not only enhanced the transfection efficiency but altered the endocytosis pathway from clathrin-mediated endocytosis to macropinocytosis. Furthermore, the smaller diameter nanofibers increasing the retention of the lipoplexes. Teo et al. and Yau et al. both highlight the importance of feature dimensions in determining the endocytic pathway that is activated, and that anisotropic and isotropic topographies will modulate the endocytic phenotype in different ways. Others have examined transfection on anisotropic features outside of fibers, such as Ge et al. [73] and Wang et al. [74]. The former demonstrated increased transfection on microgrooves, yet this phenomenon was not found to be correlated with increased cell spreading nor elongation – parameters that have been previously considered critical for topography-mediated endocytosis [73]. This conclusion suggests additional factors are involved in the enhanced endocytosis observed on topographical features.

More recent studies have investigated the relationship between membrane curvature-sensing proteins, topography-induced membrane curvature, and enhanced endocytosis [64], [75]–[81]. As described in Lou et al., these works have shown that topographical cues, specifically isotropic nanostructures (e.g., nanopillars [79], [81] and nanocones [77], [80]) deform the plasma membrane, which wraps around the feature. As such, topography induces regions of high membrane curvature at the cell-material interface. Membrane-curvature sensing proteins detect the induced curvature and may activate intracellular signalling pathways such as endocytosis. Importantly, nanotopographies may induce nanoscale membrane curvatures within the range of membrane bending observed in the early stages of endocytosis. The degree of membrane curvature induced by the topographical feature is thus dependent on the dimensions of the structure. These studies have explored how intrinsic curvature-sensing mechanisms such as BAR domain containing proteins detect and respond to exogenous membrane curvatures. For example, the seminal work of Zhao et al., demonstrated that the F-BAR

protein FCHo1, the N-BAR protein Amphiphysin, and the ENTH domain-containing protein epsin1 localize to the top of nanopillar structures at regions of high membrane curvature [81]. The BAR and ENTH domains present in these proteins are utilized to detect membrane curvature. These proteins are also involved in CME and are critical for late-stage endocytosis. The authors also investigated a range of non-curvature sensitive CME proteins such as clathrin, dynamin, and the endocytic adaptor protein AP2, and found that they also localize at the top of the nanopillar. Moreover, Zhao et al. demonstrated that the frequency of CME protein localization is increased on the regions of topography-induced curvature, suggesting endocytic events are increased on pre-curved areas. Furthermore, the authors showed that rate of CME is increased on the nanostructures. Zhao et al. demonstrated that the dynamics of clathrin assembly is increased, and the lifetime of the coated pits is significantly decreased on the nanopillars [81]. Lou et al. and Zhao et al. hypothesize that is due to topography-induced membrane bending, which reduces the energy required for membrane bending on a planar substrate, therefore reducing the energetic cost of CME [64], [81]. However, this study requires further investigation, particularly with various topography geometries and membrane curvatures.

Chapter 2

Thesis Scope & Aims

2.1 Gaps in Knowledge & Hypothesis

The overarching objective of this thesis is to investigate the application biointerface topography in enhancing the efficiency of nonviral gene delivery. Specifically, this work will examine potential mechanisms by which topographical cues interact with endocytic pathways and which features of substrate topography play a significant role in transfection. This thesis will also conduct preliminary, *in vitro* evaluation of the combined effects of topographical cues and microRNA gene therapy on vascular cell behaviour. The gene carrier utilized in this thesis is the cationic polymer chitosan-grafted-polyethyleneimine (CS-PEI). The central hypothesis of this thesis is that biointerface topography will enhance CS-PEI-mediated gene delivery in a feature-dependent and cell-type specific manner.

2.2 Research Scope & Thesis Aims

To address the aforementioned gaps and limitations, the aims of this thesis are as follows:

Aim 1: *To screen and identify for biointerface topography features that enhance CS-PEI/pEGFP transfection and endocytosis in COS-7 and human umbilical vein endothelial cells (hUVECs).*

The physicochemical characterization of different CS-PEI/pEGFP weight ratios ($\mu\text{g}/\mu\text{g}$) will be conducted alongside analyses of cytotoxicity and transfection efficiency to determine the optimal N/P ratio for transfection in COS-7 and hUVECs. The MARC chip will be used in combination with the optimal N/P ratio to screen for and identify topographical patterns that increase the endocytosis of CS-PEI/pEGFP nanoparticles. Identified patterns will be further analysed using flow cytometry, morphometric analysis, and principal component analysis (PCA).

Aim 2: *To determine the combined effects of CS-PEI/miR-145 transfection and topography on HUVECs and human umbilical artery smooth muscle cells (hUASMCs).*

The optimal N/P ratio for CS-PEI/miR-145 transfection in hUASMCs and hUVECs will be evaluated. Proliferation and viability assays will be used to determine the optimal dose of miR-145. Behavioral studies and analysis of contractile markers will be performed in hUASMCs in the presence of $2\ \mu\text{m} \times 2\ \mu\text{m}$ microgratings or microgrooves and miR-145 overexpression.

Chapter 3 Topographical Features for Enhanced Nonviral Gene Delivery

3.1 Introduction & Background

Nonviral gene delivery platforms have emerged to overcome the limitations of viral vectors and have widespread applications in tissue engineering and regenerative medicine. Transfection, the delivery of exogenous genetic material (a transgene) to a cell of interest, is commonly achieved via cationic lipid-based or cationic polymer-based gene carriers. In both cases, the positively charged groups interact with anionic nucleic acids through electrostatic interactions and condense the DNA or RNA, resulting in the formation of lipoplexes and polyplexes, respectively. The residual positive charge on surface of the lipoplex or polyplex facilitates their interaction with the plasma membrane, triggering their internalization or endocytosis. While nonviral methods are generally much safer than viral vectors, they suffer from unsatisfactory transfection efficiency. A nonviral gene carrier must overcome several extracellular and intracellular barriers to achieve expression of its genetic cargo. However, the rate limiting step of transfection remains traversing the plasma membrane. Thus, there is an impetus to both understand and modulate the endocytosis of nonviral gene carriers to enhance their efficacy.

Substrate-mediated gene delivery offers numerous advantages to bolus delivery of nonviral gene carriers and has enormous potential in the field of tissue engineering and regenerative medicine. In SMD, the gene carrier is immobilized on the biomaterial surface via nonspecific adsorption or covalent attachment and may be uptaken by cells as they grow along and within the material. SMD places the gene carrier directly in the cellular microenvironment, allowing for localized delivery to regenerating tissues and reducing the dose of the transgene needed. This also reduces the susceptibility of lipoplexes and polyplexes to undergo degradation and aggregation in the serum as well as mass transport barriers to delivery to target cells. An additional advantage of SMD is the ability to integrate biochemical and biophysical cues onto the material surface to prime the cells for transfection. Examples of biophysical stimuli include substrate stiffness and topographical cues. Previous works have shown that modulating substrate stiffness and incorporating topographical features can be used to promote In this chapter, the use of topographical cues to enhance SMD are explored using a polymeric, nonviral gene carrier.

A polymer-based gene delivery system that has been extensively explored over the last two decades is polyethyleneimine (PEI), owed to its relatively high transfection efficiency. PEI contains a large number of positively charged amines, which interact with and condense nucleic acids to small particles. Importantly, only a small fraction (10-15%) of these amines are protonated at physiological pH, which confers this polymer a strong buffering capacity. This underpins the so-called “proton sponge effect” which drives the endolysosomal escape and entry of PEI polyplexes into the cytoplasm. The positive charge on PEI polyplexes also facilitates direct membrane penetration or its interaction with negatively charged proteoglycans on the cell surface, thereby triggering endocytosis. The transfection efficiency of PEI gene carriers comes at the cost of high toxicity, which is due to the positive charge. To shield the positive charge of the polyplexes, polysaccharides or hydrophobic molecules may be grafted onto PEI. The most common agent used for cationic shielding is chitosan (CS), a polysaccharide derived from chitin. CS and PEI conjugates have been developed to improve cell viability while maintaining transfection efficiency. Lin et al., 2018 developed a chitosan-grafted-polyethyleneimine (CS-PEI) copolymer using branched PEI (BPEI) and succinated CS [48]. The succinyl groups on the CS act as a linker for conjugation to bPEI. The authors achieved relatively high levels of transfection while maintaining cell viability *in vitro*. In this work, the authors also demonstrated the utility of the polymer *in vivo* via oral administration. Their data recommend CS-PEI as a gene carrier system for further investigation and potential use in SMD.

In this chapter, the use of topography to enhance CS-PEI transfection is explored. Two cell types of interest are evaluated. Firstly, COS-7: a cell line generated from CV-1 African green monkey fibroblasts and commonly used in transfection studies. Secondly, human umbilical vein endothelial cells (hUVECs), which are primary cells and notoriously difficult to transfect [82]. hUVECs have a low proliferation rate, short lifespan, and high sensitivity to transfection reagents. However, hUVECs are a key cell type for modelling cardiovascular diseases and investigating endothelium function *in vitro*. In this chapter, the MultiARChitecture (MARC) chip (**Figure 8**) is used for high-throughput screening of 41 different topographical features with varying geometries (Table 3) [83] using both COS-7 and hUVECs. The MARC chip allows for high-throughput evaluation of topographical features on cellular responses with identical experimental parameters. To evaluate the effects of varying topographical features, pDNA encoding enhanced green fluorescent protein (pEGFP) is used as the reporter gene. Patterns that result in significantly higher transfection compared to the unpatterned control are selected for further analysis.

3.2 Materials & Methods

3.2.1 CS-PEI Synthesis

The CS-PEI copolymer was synthesized by Dr. Huiyi Liang at Columbia University in the lab of Professor Kam Leong and is described in Lin et al., 2018 [48]. The properties of CS-PEI copolymer utilized in both Chapter 3 and Chapter 4 are outlined in **Table 1**. These features are used to calculate the mass of the CS-PEI required to obtain a specific N/P ratio relative to the mass of nucleic acid to be delivered.

Feature	Number
Acetylated Units	2842
Deacetylated Units	127.75
Succinated Units	3781.4
PEI units	60092.14
Mn	66843.29
n(Ionizable N)	576.7
n(Ionizable N)/Mn (polymer)	0.008627642
n(P)/Mn	0.003
N/P when mass ratio = 1:1	2.875880785

Table 1. Features of the CS-PEI copolymer.

3.2.2 Plasmid DNA Isolation and Purification from *E. Coli*

Nutrient agar plates are prepared by autoclaving agar dissolved in sterile deionized (DI) H₂O at 121°C for 15 minutes. Liquid agar is allowed to cool to 55°C, upon which kanamycin is added to achieve a final concentration of 50µg/mL. 25 mL of agar is added to each plate, which is sealed with parafilm and stored at 4°C until use (up to two months). Kanamycin resistant, *E. coli* DH5-α (Invitrogen™) are streaked on the agar plate under sterile conditions and incubated at 37°C overnight. Subsequently, a single colony is added to 100 mL of sterile, tryptic soy broth liquid media to prepare a liquid culture. After overnight incubation, pEGFP N1 is isolated using the QIAGEN plasmid mini-prep kit according to protocol. In all cases, 5 mL of overnight culture is used per miniprep. If to be used in zeta potential and particle size measurements, pDNA is eluted in ultrapure, DPEC-treated DI H₂O. If to be used in transfection studies, pDNA is eluted in TE buffer, Tris-EDTA 1X solution, pH 8.0 (Fisher Scientific). pDNA is then quantified using the NanoDrop ND-1000 spectrophotometer. Two measurements are made for each sample. Stock pDNA solutions of 500ng/µL in TE buffer are prepared for transfections.

3.2.3 CS-PEI/pEGFP Polyplex Formation & Transfection

The lyophilized CS-PEI is resuspended in sterile 1X PBS and briefly vortexed under sterile conditions. If aggregates of CS-PEI are observed, the solution is passed through a 0.2µm filter. A stock solution of 1.65 µg/µL is prepared and aliquoted. Stock solution of pDNA with a concentration of 500 ng/µL is prepared in TE buffer. The formulations for each N/P ratio for transfection in a 48-well plate are shown in **Table 2**. To prepare the CS-PEI polyplexes, various masses (dictated by the N/P ratio) of CS-PEI are added to a constant mass of pDNA. For transfections a 48-well plate, the mass of CS-PEI is determined according to the N/P ratio. The necessary volume of CS-PEI to provide an adequate mass to achieve the desired N/P ratio is based on the stock concentration (1.65 µg/µL in **Table 2**). The resuspended CS-PEI is diluted in sterile, room temperature 1X PBS to achieve a final volume of 22µL. The pDNA stock is diluted in sterile, room temperature 1X PBS to achieve a final volume of 11µL in a separate microcentrifuge tube. The diluted CS-PEI is added to the diluted pDNA in 5µL increments. Upon the addition of CS-PEI, the CS-PEI/pDNA solution is vortexed for 10 seconds. The final solution is gently spun down and incubated for 15 minutes at room temperature, before resuspension in transfection or complete media for COS-7 and HUVECs respectively. The media is then gently aspirated and replaced with the polyplex solution. Transfection is evaluated based on EGFP fluorescence 24 hours after transfection. For transfection in a 24-well plate, 1µg of pDNA is used (from a stock of 0.5µg of pDNA in TE buffer). As such, 2µL of 500ng/µL pDNA stock is added to 9 µL of 1X PBS. The composition of the transfection media is outlined in Section 3.2.5.

N/P	CS-PEI (µg)	[CS-PEI] (µg/µL)	CS-PEI (µL)	PBS (µL)	pDNA (µL)	PBS (µL)	Total Polyplex (µL)	Media (µL)
10	3.478	3.48	1.1	20.9	1	10	33	167
15	5.216	9.64	1.6	20.4	1	10	33	167
20	6.955	12.86	2.2	19.8	1	10	33	167
40	13.909	25.71	4.3	17.7	1	10	33	167

Table 2. Sample formulations for a CS-PEI/pEGFP transfection performed in a 48-well plate. The mass of CS-PEI is determined based on N/P ratio; the higher the N/P ratio the greater the mass needed. The concentration of CS-PEI is determined based on the total volume (200µL), the mass of pDNA, and the mass of CS-PEI used to form the polyplexes.

3.2.4 Zeta Potential & Particle Size Measurements

The lyophilized CS-PEI is resuspended in sterile DI H₂O and vortexed briefly until fully dissolved. CS-PEI resuspended in DI water is stored at -80°C and kept for up to 1 month. Polyplexes are formed at N/P ratios, 10, 15, 20 and 40 with pDNA resuspended in ultrapure DPEC-treated DI H₂O. 4µg of pDNA is used for each N/P ratio and technical replicate. For zeta potential measurements, a final volume of 750µL is prepared. For particle size measurements, a final volume of 1 mL is prepared. Until measurements are conducted samples are kept at 4°C; measurements are taken with haste to prevent degradation of the sample in DI H₂O. Immediately before measurements, samples are diluted to achieve the final volume and vortexed at maximum speed for 30 seconds. For both zeta potential and particle size measurements $n = 4$. Three measurements were made for each replicate.

3.2.5 Cell Culture

COS-7 are maintained DMEM with High Glucose, L-Glutamine, without pyruvate (Wisent BioProducts) with 10% fetal bovine serum (FBS) (Wisent BioProducts) and 1% penicillin-streptomycin (PenStrep, 10,000 U/mL) in T-25 flasks and passaged at 90% confluency. To thaw HUVECs, 5 mL of complete media is added to the Primary human umbilical vein endothelial cells (HUVECs) are obtained from HUVECs (passages 6-11) are maintained in Vasculife® VEGF Endothelial Media (Lifeline Cell Technologies) and passaged at 80% cell confluency. To thaw HUVECs, 4.5mL of the Vasculife® VEGF Endothelial Media is incubated at 37°C and 5% CO₂ with 500µL (10%) of FBS for 1 hour. Upon thawing in the water bath, the cells are resuspended in 3mL of the equilibrated media in a dropwise fashion, and then returned directly to the T-25 flask. Within 24 hours of thawing, the media is aspirated and replaced to remove excess DMSO. Every 48 hours 3mL of the media is removed and replaced with fresh media. HUVECs are passaged at a lower confluency compared to the COS-7 cells as they are subject to contact inhibition: cell proliferation is strongly inhibited once the cells reach high confluency. COS-7 transfection media consists of DMEM with 10% FBS; antibiotics are excluded from the transfection media to improve viability. Contrastingly, the gentamycin and amphotericin B are maintained in the HUVEC transfection media due to issues with contamination.

3.2.6 Cytotoxicity Studies

Cells are seeded in a tissue-cultured treated polystyrene flat bottom 96-well plate at a density of 1,000 cells/cm². Cells are seeded in four replicates per treatment. Furthermore, the outer wells of the 96-well plate are filled with 100µL of 1X PBS to reduce evaporation. Polyplexes are prepared as per Section

3.2.4 yet resuspended in 367µL to achieve a final volume of 400µL. 100µL of the polyplex solution is added to each replicate well in the 96-well plate. 24 hours after transfection, the transfection media is removed and then replaced with 90µL of the full maintenance media using a multichannel pipette. 10µL of prewarmed alamarBlue™ Cell Viability Reagent (Invitrogen™) is added to each well and then incubated for 1.5 hours protected from light at 37°C and 5% CO₂ prior to measurement with the BioTek Synergy HTX microplate reader (Agilent Technologies). The absorbance is measured at 570nm and 600nm. Every 24 hours, this procedure is repeated to evaluate changes in viability over time. 2µg of CS-PEI and 500ng of pDNA are used as negative controls. Media with no cells is used to control for background absorbance. The percentage (%) viability is calculated according to the following equation:

$$\% \text{ viability} = \frac{(\epsilon_{OX})\lambda_2 A \lambda_1 - (\epsilon_{OX})\lambda_1 A \lambda_2 (\text{test well})}{(\epsilon_{OX})\lambda_2 A^\circ \lambda_1 - (\epsilon_{OX})\lambda_1 A^\circ \lambda_2 (\text{untreated positive control})}$$

Where $\lambda_1 = 570\text{nm}$, $\lambda_2 = 600\text{nm}$, $(\epsilon_{OX})\lambda_1 = 80,586$, $(\epsilon_{OX})\lambda_2 = 117,216$, A = absorbance of the test well, and A° = absorbance of the untreated, positive control.

3.2.7 MARC Chamber Fabrication

The fabrication of the MARC chip has been previously described [83]. The patterns present on the MARC chip are outlined in **Table 3**. Briefly, a MARC chip is prepared as a PDMS-based double replicate of the silicon master (using a 5:1 ratio of elastomer base to curing agent to improve demolding). The chip is silanized with ~25µl of 100% trichloro(1H,1H,2H,2H-perfluorooctyl)silane (silane, Millipore Sigma) in a desiccator. The desiccator is placed under a vacuum for 30 minutes to promote the evaporation of silane into chamber. The silanized chambers are then gently washed in 0.01% Triton-X 100 in DI H₂O first on the patterned side and then on the unpatterned side of the sample. The surfactant is gently removed from the sample by blowing gently with air, and, if necessary, gently washed with DI H₂O. Once the MARC chip has been washed with the surfactant, it may be gently cleaned using DI H₂O or used directly to prepare a PDMS replicate.

The MARC chip is used to produce the MARC chamber (MARC-C), which allows for a confined seeding area. The preparation of the MARC-C from the MARC chip is shown in **Figure 7**. PDMS is prepared by mixing 10 parts (of the elastomer base with 1 part of the curing agent (10:1 ratio). The elastomer base and curing agent are thoroughly mixed by altering the mixing motion at 20 second intervals for 5 minutes. The PDMS mixture is then degassed under vacuum for 15-20 minutes, poured over the MARC chip mold, and degassed for an hour under vacuum. The PDMS is then cured overnight

at 60°C before being carefully demolded. The resulting chamber is cut to fit inside the well of a 6-well plate. The LEXT microscope is used for optical profilometry of individual patterns. Prior to cell culture, 6 replicates of the mold are prepared to remove excess silane.

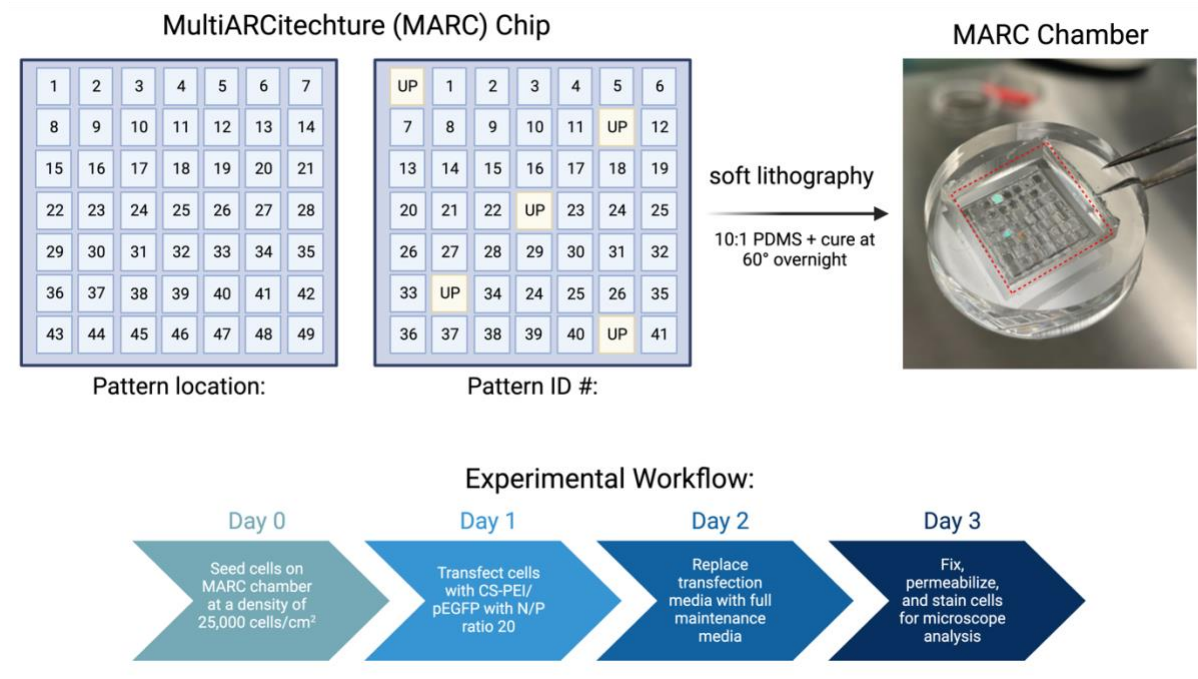


Figure 7. Experimental approach for screening the effects of topography on CS-PEI/pEGFP endocytosis using the MARC-C. The location of the patterns as outlined in Table 4 are shown schematically by location number and by ID number. The MARC chip is used to fabricate the chamber (shown in the image), which has a defined seeding area (outlined with the red dashed line) and can fit securely in a 6-well plate. A timeline for the overall experimental workflow with the MARC-C is shown below, from Day 0 (seeding) to Day 3 (fixation and analysis).

Position	ID	Topography Geometry	Topography dimensions [diameter or width x pitch (center to center) x height or depth]
1	UP	Unpattern	-
2	1	Gratings	2 μm $\times$ 4 μm $\times$ 2 μm
3	2	Gratings	2 μm $\times$ 3 μm $\times$ 80 nm
4	3	Gratings	1 μm $\times$ 3 μm $\times$ 120 nm
5	4	Gratings	250 nm $\times$ 500 nm $\times$ 250 nm
6	5	Pillars (Square array)	10 μm $\times$ 10 μm $\times$ 10 μm
7	6	Pillars (Square array)	2 μm $\times$ 12 μm $\times$ 2 μm

8	7	Pillars (Square array)	$2\ \mu\text{m} \times 4\ \mu\text{m} \times 2\ \mu\text{m}$
9	8	Pillars (Square array)	$500\ \text{nm} \times 10\ \mu\text{m} \times 500\ \text{nm}$
10	9	Pillars (Square array)	$250\ \text{nm} \times 500\ \text{nm} \times 250\ \text{nm}$
11	10	Pillars (Square array)	$1\ \mu\text{m} \times 7.5\ \mu\text{m} \times 1\ \mu\text{m}$
12	11	Convex lens (Hexagonal array)	$1.8\ \mu\text{m} \times 2\ \mu\text{m} \times 700\ \text{nm}$
13	UP	Unpattern	-
14	12	Convex lens (Square array)	$800\ \text{nm} \times 1\ \mu\text{m} \times 300\ \text{nm}$
15	13	Pillars (Square array)	$2\ \mu\text{m} \times 12\ \mu\text{m} \times 700\ \text{nm}$
16	14	Pillars (Square array)	$2\ \mu\text{m} \times 12\ \mu\text{m} \times 500\ \text{nm}$
17	15	Pillars (Square array)	$2\ \mu\text{m} \times 12\ \mu\text{m} \times 450\ \text{nm}$
18	16	Pillars (Square array)	$2\ \mu\text{m} \times 12\ \mu\text{m} \times 350\ \text{nm}$
19	17	Pillars (Square array)	$2\ \mu\text{m} \times 12\ \mu\text{m} \times 200\ \text{nm}$
20	18	Pillars (Square array)	$500\ \text{nm} \times 10\ \mu\text{m} \times 300\ \text{nm}$
21	19	Pillars (Square array)	$500\ \text{nm} \times 10\ \mu\text{m} \times 200\ \text{nm}$
22	20	Pillars (Square array)	$500\ \text{nm} \times 10\ \mu\text{m} \times 100\ \text{nm}$
23	21	Pillars (Square array)	$250\ \text{nm} \times 500\ \text{nm} \times 120\ \text{nm}$
24	22	Concave lens (Hexagonal array)	$1.8\ \mu\text{m} \times 2\ \mu\text{m} \times 700\ \text{nm}$
25	UP	Unpattern	-
26	23	Concave lens (Square array)	$800\ \text{nm} \times 1\ \mu\text{m} \times 300\ \text{nm}$
27	24	Hierarchical micron with gratings $\perp$ nano gratings	Primary topography: $2\ \mu\text{m} \times 4\ \mu\text{m} \times 2\ \mu\text{m}$ Secondary topography: $250\ \text{nm} \times 500\ \text{nm} \times 150\ \text{nm}$
28	25	Hierarchical micron gratings with // nano gratings	Primary topography: $2\ \mu\text{m} \times 4\ \mu\text{m} \times 2\ \mu\text{m}$ Secondary topography: $250\ \text{nm} \times 500\ \text{nm} \times 150\ \text{nm}$
29	26	Hierarchical micron gratings with nano pillars	Primary topography: $2\ \mu\text{m} \times 4\ \mu\text{m} \times 2\ \mu\text{m}$ Secondary topography: $250\ \text{nm} \times 500\ \text{nm} \times 250\ \text{nm}$
30	27	Bumps	$300\ \text{nm} \times 300\ \text{nm} \times 200\ \text{nm}$
31	28	Cones	$270\ \text{nm} \times 300\ \text{nm} \times 350\ \text{nm}$
32	29	Inverse cones	$270\ \text{nm} \times 300\ \text{nm} \times 350\ \text{nm}$
33	30	V-gratings	$2\ \mu\text{m} \times 2\ \mu\text{m} \times 1.5\ \mu\text{m}$
34	31	V-gratings	$500\ \text{nm} \times 500\ \text{nm} \times 350\ \text{nm}$
35	32	U-gratings	$2\ \mu\text{m} \times 2\ \mu\text{m} \times 700\ \text{nm}$
36	33	U-gratings	$500\ \text{nm} \times 500\ \text{nm} \times 200\ \text{nm}$
37	UP	Unpattern	-

38	34	Gratings	$10\ \mu\text{m} \times 20\ \mu\text{m} \times 10\ \mu\text{m}$
39	24	Hierarchical micron gratings with $\perp$ nano gratings	Primary topography: $2\ \mu\text{m} \times 4\ \mu\text{m} \times 2\ \mu\text{m}$ Secondary topography: $250\ \text{nm} \times 500\ \text{nm} \times 150\ \text{nm}$
40	25	Hierarchical micron gratings with $//$ nano gratings	Primary topography: $2\ \mu\text{m} \times 4\ \mu\text{m} \times 2\ \mu\text{m}$ Secondary topography: $250\ \text{nm} \times 500\ \text{nm} \times 150\ \text{nm}$
41	26	Hierarchical micron gratings with nano pillars	Primary topography: $2\ \mu\text{m} \times 4\ \mu\text{m} \times 2\ \mu\text{m}$ Secondary topography: $250\ \text{nm} \times 500\ \text{nm} \times 250\ \text{nm}$
42	35	Convex lens (Square array)	$10\ \mu\text{m} \times 20\ \mu\text{m} \times 200\ \text{nm}$
43	36	Convex lens (Square array)	$10\ \mu\text{m} \times 20\ \mu\text{m} \times 2\ \mu\text{m}$
44	37	Convex lens (Square array)	$2\ \mu\text{m} \times 4\ \mu\text{m} \times 400\ \text{nm}$
45	38	Convex lens (Square array)	$2\ \mu\text{m} \times 4\ \mu\text{m} \times 800\ \text{nm}$
46	39	Convex hexagon lens (Square array)	$10\ \mu\text{m} \times 20\ \mu\text{m} \times 800\ \text{nm}$
47	40	Convex hexagon lens (Square array)	$10\ \mu\text{m} \times 20\ \mu\text{m} \times 2\ \mu\text{m}$
48	UP	Unpattern	-
49	41	Hexagonal donut	$50\ \mu\text{m}$ (outer), $10\ \mu\text{m}$ (inner) $\times \mu\text{m} \times 800\ \text{nm}$

Table 3. List of patterns on MARC-C. The first column shows the position of the pattern on the MARC chip whereas the second column indicates the ID number of pattern.

3.2.8 Cell Seeding & Staining

The general experimental workflow with the MARC-C is shown in **Figure 7**. Prior to seeding, the MARC chamber is washed with 100% ethanol and then dried in the 60°C oven for 30 minutes. The chamber is then plasma treated by pumping for 5 minutes and treating for 1 minute. Subsequently, the seeding area on the chamber is immersed in 70% ethanol (1 mL) and then UV sterilized for 1 hour. To facilitate cell attachment, a collagen coating is necessary. A collagen coating solution is prepared in a 20mM acetic acid (diluted in sterile DI H₂O and sterilized using a 0.2 μm filter) to achieve a final coating density of 2.5 $\mu\text{g}/\text{cm}^2$. For the MARC-C and UP-C, a 20 $\mu\text{g}/\text{mL}$ bovine collagen solution in filtered 20mM acetic acid is prepared and warmed to 37°C for approximately 15 minutes. Following UV sterilization, the 70% EtOH is aspirated, and the sample is washed thrice with 1mL of sterile, 1X

PBS for 1 minute. 500 μ L of the collagen solution is added to the MARC-C and UP-C and then incubated for at 37°C and 5% CO₂ for a minimum of 1 hour. While the samples incubate with the ECM solution, a cell suspension is prepared by washing, trypsinizing, and resuspending COS-7 or HUVECs. In all cases, cells are seeded at a seeding density of 25,000 cells/cm² (100,000 cells per chamber). The cells are incubated on the chambers for 24 hours prior to transfection.

3.2.9 Single-Pattern Studies

4 $\times$ 4cm² TCPS or PDMS molds of the pattern(s) selected for further study are silanized and washed with 0.01% Triton-X 100 as described in Section 3.2.7. The TCPS molds are prepared with hot embossing. Briefly, a 4 $\times$ 4cm² piece of TCPS is cut from the bottom of a polystyrene petri dish, cleaned with 100% EtOH and then dried with air. A glass coverslip is placed on top of several layers of aluminum foil on a hotplate, which is warmed to 250°C. The piece of TCPS is placed on top of the coverslip and heated for ~20s. A PDMS (5:1 ratio) mold of the pattern is then placed on top of the TCPS and a 5lb weight is quickly placed on top of the mold and held for 90s. After 90s, the aluminum foil is used to transfer the coverslip, TCPS, mold, and weight to the benchtop, where it is maintained for 30 seconds. The weight is then removed, and the mold is gently peeled from the TPCS. PDMS replicates of the pattern are prepared and then cut with scalpel blade to fit securely into the bottom of a 12-well plate. Unpatterned controls are prepared by pouring the PDMS mixture into a polystyrene petri dish and using the unpatterned surface as a mold.

3.2.10 Flow Cytometry

Samples for flow cytometry are prepared according to Section 3.2.9. Thin PDMS films of the mold are washed in 100% EtOH and securely fit into a 12-well plate. The size of the samples is selected to mimic the MARC-C. The plate is baked at 60°C for 30min to ensure the samples are completely dry. The samples are then plasma treated by pumping for 5min and treating for 1min. The samples are quickly immersed in 70% ethanol (1 mL) and then UV sterilized for 30min – 1hr. A collagen coating solution is prepared as described in Section 3.2.8 to achieve a final density of 2.5 μ g/cm². COS-7 are seeded on the PDMS samples at a density of 25,000 cells/cm² (following the seeding conditions of the MARC-C). 24hrs after seeding, the cells are transfected with a dose of 4 μ g of pDNA. For flow cytometry, an unpatterned, non-transfected control is prepared. Additionally, an unpatterned positive control is prepared with Lipofectamine™ 3000 according to the manufacturer's recommendations for pDNA transfection in a 12-well plate. Media is changed 24hrs after transfection. 48hrs after transfection, cells

are washed with prewarmed 1X PBS, trypsinized, and pelleted with centrifugation for 5 minutes at 1.3 rpm. Cells are resuspended in maintenance media, counted, and then transferred into a microcentrifuge tube. The cell suspension is centrifuged for 5min at 1.5rpm and then resuspended by pipetting in 500μL sterile, ice cold 1X PBS. 500μL of ice cold 2% PFA is added to achieve a final concentration of 1% PFA. While 2% PFA is added, the cell suspension is vigorously mixed by pipetting to avoid cell clumping. Cells are fixed on ice for 20 minutes, centrifuged for 5min at 1.5rpm, and resuspended in 500μL of ice cold 1X PBS. A minimum concentration of 200,000 cells/mL is used. Cells are analysed using the Becton Dickinson FACS Calibur flow cytometer (Mountain View, CA, USA). 10,000 events are captured per sample. Gating is based on the forward and side scattering profile of the unpatterned, non-transfected control. The mean fluorescence intensity (MFI) is determined from the mean or median of the transfected cells. Data is analyzed using the Floreada.io online software.

3.2.11 Microscopy & Image Analysis

Microscope analysis is conducted using the Axio Observer Z Microscope. Image analysis is conducted using ImageJ. To determine the transfection efficiency (%), the total number of EGFP positive (EGFP+) cells and the total number of cells is counted. Transfection % is then determined with the following equation:

$$\text{Transfection \%} = \frac{\text{total number of EGFP positive cells}}{\text{total number of cells}} \times 100\%$$

For MARC-C analysis, only the cells are on the pattern are counted; the region of interest (ROI) is defined prior to counting to exclude cells located in the space/groove between patterns.

3.2.12 Statistics

All statistical analysis was performed in GraphPad Prism 10. The values of all data are shown as the mean $\pm$ standard error of the mean (SEM). The number of biological replicas (*n*) varied across experiments and is indicated in the methods and/or the figure captions. One-way with Tukey's post hoc test or two-way ANOVA with Sidak's post hoc test were used to determine statistical significance and is indicated in the figure captions in Section 3.3. Statistically significant data is indicated in the figures using *, **, and *** for $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$ respectively, unless otherwise specified. Principal component analysis is performed in R using the FactoMineR and factoextra packages.

3.3 Results

3.3.1 CS-PEI/pEGFP N/P Ratio Screening

The N/P ratio is the ratio of positively charged amine (N) groups present on the cationic polymer to the negative charged phosphate (P) groups on the nucleic acid. It is a key physiochemical parameter when optimizing polyplexes for non-viral gene delivery, as it dictates nucleic acid condensation and complexation. Likewise, the N/P ratio determines the capacity of the polymer to protect the nucleic acid from degradation via nucleases. Moreover, the N/P ratio governs the surface charge of the polyplex and thus its capacity to interact with the plasma membrane and escape the endosomal system via the proton sponge effect. Therefore, prior to use on the MARC-C, the optimal N/P ratio for transfection in COS-7 and hUVECs had to be determined. Four N/P ratios, 10, 15, 20 and 40 were selected for evaluation.

3.3.1.1 Zeta Potential & Particle Size Measurements

The physicochemical characterization of CS-PEI/pEGFP NPs was conducted by evaluating the zeta potential, particle size, and polydispersity index (PDI) (**Figure 8**). Across all N/P ratios, the surface charge was positive, suggesting full complexation with the nucleic acid had taken place. The hydrodynamic diameter of the complexes was determined using the Z-average (**Figure 8B**). An N/P ratio of 40 yielded the largest average diameter (~170nm), which was significantly larger than all other N/P ratios. Interestingly, the N/P ratio of 20 resulted in the smallest particle size (~130nm). Importantly, all polyplexes are within the size range for CME. All formulations exhibited low polydispersity (**Figure 8C**), suggesting the samples are relatively monodispersed.

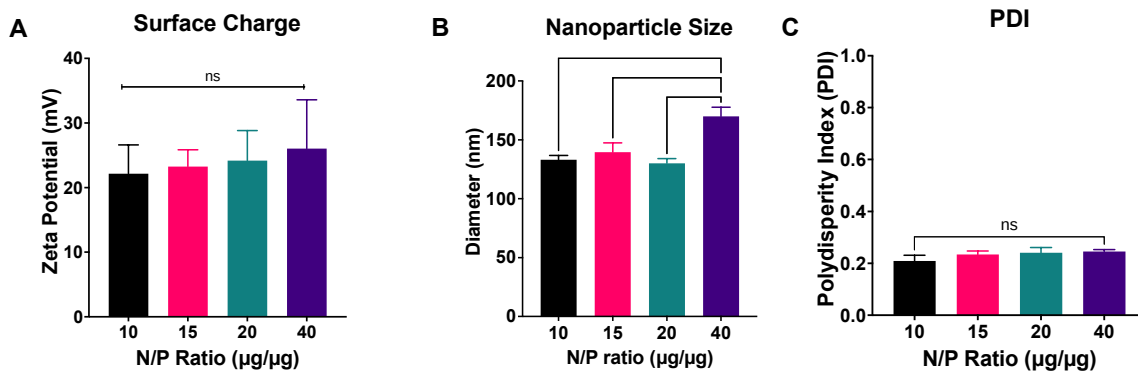


Figure 8. Physicochemical characterization of the CS-PEI/pEGFP polyplexes across N/P ratios. (A) Zeta potential measurements. (B) The diameter of the polyplexes as determined from the Z-average

size. (C) The dimensionless polydispersity index of polyplexes, indicating the size distribution of the complexes. Statistically significant data is indicated in the figures using *, **, and *** for $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$ respectively. Error bars represent the SEM. $n = 4$.

3.3.1.2 Cytotoxicity

The second step in screening for the optimal N/P ratio is examining cell viability (**Figure 9**), which was achieved using the alamarBlue viability assay. Cells were seeded at 1,000 cells/well in 96-well plate 24 hours prior to transfection. For sample controls, CS-PEI and naked pEGFP were utilized. A low dose of CS-PEI (2 μ g) was utilized and the effects on viability are minimal. Although no significant difference was observed across the N/P ratios, it is evident that an N/P ratio of 40 is the most detrimental to cell viability. As shown in **Figure 9**, the cell viability remains mostly unaffected after 24 hours and experiences a marked decrease 48 hours post-transfection. This is likely due to the second stage of PEI-induced cell death, which arises days after exposure. The initial stage of cell death, which is generally recognized as being caused by perturbations of the cell membrane by free PEI, appears to be minimal in this instance as it is known to arise shortly after exposure. As described in Casper et al., the later stage of cytotoxicity is caused by mitochondrial dysfunction and by the release of lysosomal components into the cytoplasm – a product of endolysosomal escape. The restoration of cell viability 72hrs post-transfection is likely due to the proliferation of non-transfected cells or those that have exocytosed and/or degraded the polyplexes, as well as those that were successfully transfection and achieved transgene expression.

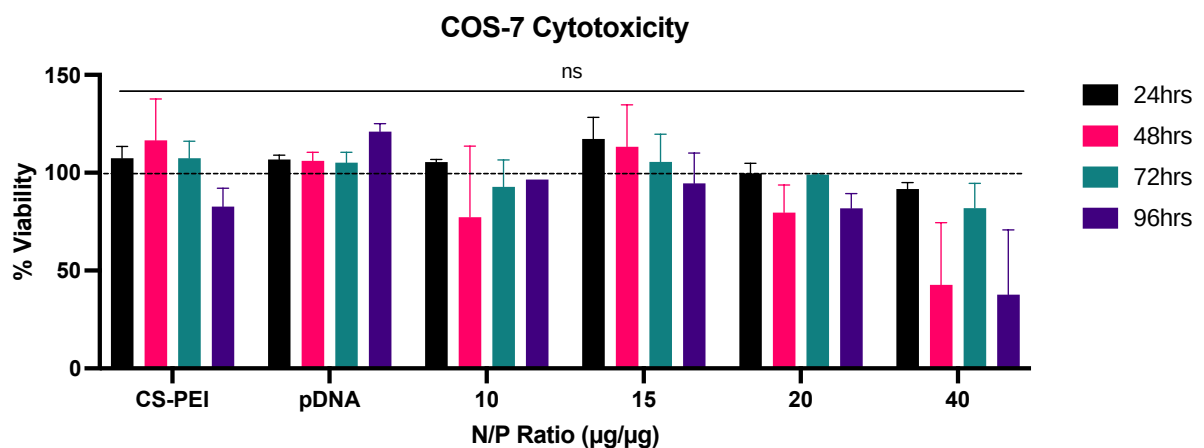


Figure 9. The effects of the N/P ratio on the viability of COS-7 over 96hrs. The grey dashed line indicates the untreated sample to which all values were normalized. Statistically significant data is

indicated in the figures using *, **, and *** for $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$ respectively. Error bars represent the SEM. $n = 3$.

3.3.1.3 Transfection Efficiency

To further screen for the optimal N/P ratio in COS-7, the transfection efficiency (the percentage of transfected cells) was evaluated across different mass formulations (**Figure 10**). As shown in Figure X, the N/P ratio that yielded the highest transfection efficiency was 40, which was significantly higher than 10, 15 and 20. Lipofectamine3000 resulted in significantly higher ($p \leq 0.0001$) transfection compared to all N/P ratios (denoted with an asterisk). However, as shown in Figure 9, this N/P also resulted in the greatest cytotoxicity across all time points. Therefore, an N/P ratio of 20 offered a balance between moderately high transfection efficiency and cytotoxicity and was selected for subsequent applications.

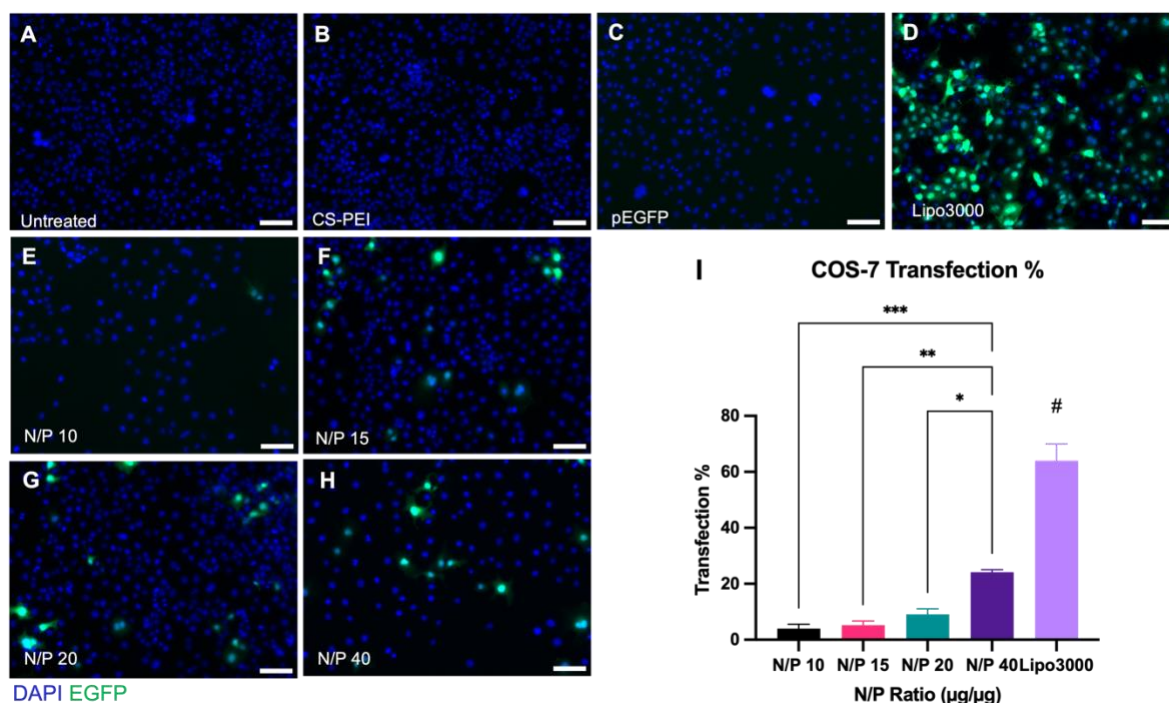


Figure 10. The transfection efficiency of varying N/P ratios. (A)-(H) Representative figures for the CS-PEI transfection efficiency 24hrs post-transfection. (A)-(C) show controls for untreated, CS-PEI alone, and pEGFP alone. (D) Shows transfection for the positive control, Lipofectmine3000. (E)-(H) display the varying CS-PEI/pEGFP ratios. (I) Shows the average transfection efficiency and SD. Scale bars = 100μm. Statistically significant data is indicated in the figures using *, **, and *** for $p \leq 0.05$,

$p \leq 0.01$, and $p \leq 0.0001$ respectively. # indicates $p \leq 0.0001$ relative to all other samples. Error bars represent the SEM. $n = 4$.

3.3.2 Preliminary Screening of CS-PEI/pEGFP Transfection on Topographical Features using the MultiARChitecture Chip

The effects of topography on CS-PEI/pEGFP transfection were then evaluated in COS-7 using an N/P ratio of 20 and a dose of 4 μ g, which is consistent with our previous report [69].

3.3.2.1 Transfection Efficiency

The transfection efficiency of the CS-PEI/pEGFP polyplexes on the topographical patterns in shown in **Figure 11**. Overall, the average transfection efficiency on the unpatterned sample is low (1.114%). Despite this, several patterns did increase the transfection efficiency of patterns relative to the unpatterned control (indicated by the dashed line in **Figure 11**). These include pattern ID #4, #19, #25, #26, #29, and #39. Laser scanning microscope images of these patterns are shown in **Figure 12**. Representative images for the transfection efficiency on these patterns is shown in **Figure 13**. The geometry and dimensions of these patterns is outlined in **Table 4**.

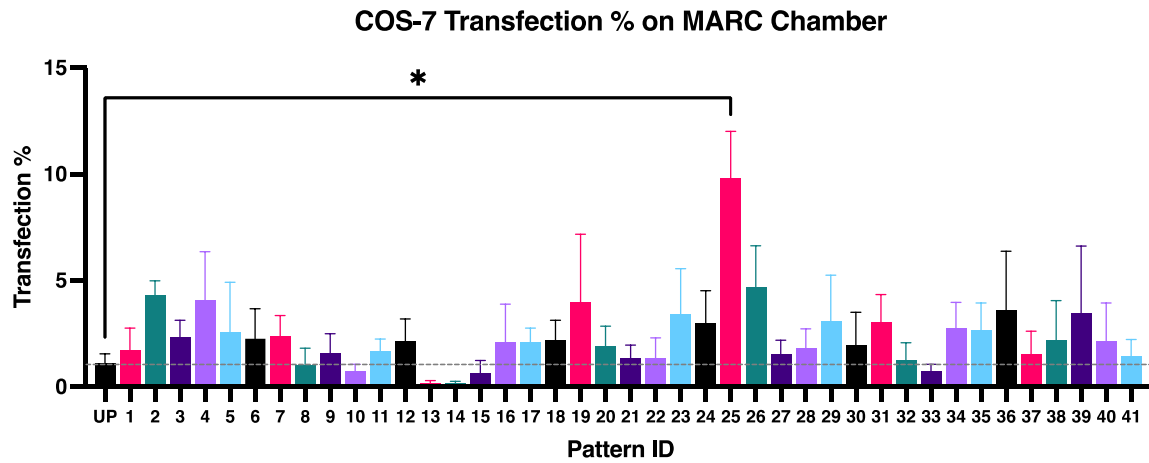


Figure 11. CS-PEI/pEGFP transfection on MARC-C organized by pattern ID. The grey dashed line signifies the transfection efficiency of the unpatterned (UP) case. Statistically significant data is indicated in the figures using *, **, and *** for $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$ respectively. Error bars represent the SEM. $n = 4$.

ID	Topography Geometry	Topography dimensions [diameter or width x pitch (center to center) x height or depth]
4	Gratings	250 nm × 500 nm × 250 nm
19	Pillars (Square array)	500 nm × 10 μm × 200 nm
25	Hierarchical micron gratings with // nano gratings	Primary topography: 2 μm × 4 μm × 2 μm Secondary topography: 250 nm × 500 nm × 150 nm
26	Hierarchical micron gratings with nano pillars	Primary topography: 2 μm × 4 μm × 2 μm Secondary topography: 250 nm × 500 nm × 250 nm
29	Inverse cones	270 nm × 300 nm × 350 nm
39	Convex hexagon lens (Square array)	10 μm × 20 μm × 800 nm

Table 4. Description of topographies that resulted in elevated transfection efficiency on the MARC-C.

A single pattern, (pattern ID #25) resulted in significantly average higher transfection efficiency (9.822%) compared to the unpatterned sample () ($p = 0.0231$). Pattern ID #25 is a hierarchal topography, with a primary topography of 2 μm × 4 μm × 2 μm microgratings and a secondary topography of 250 nm × 500 nm × 150 nm, which is parallel (//) to the microscale feature.

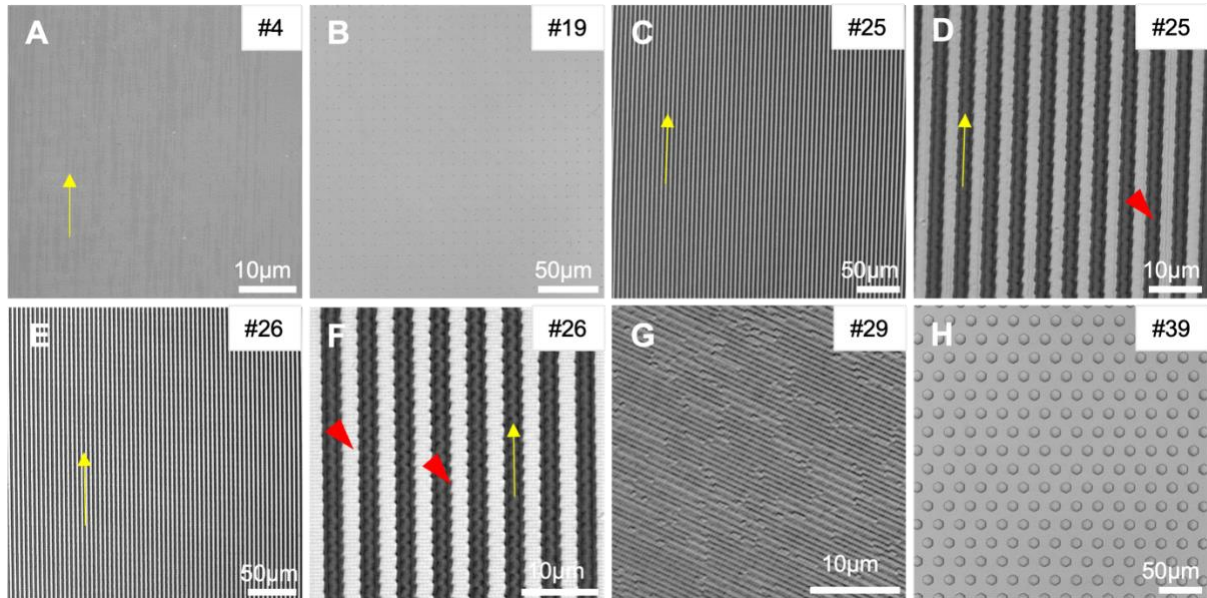


Figure 12. LEXT images of topographies that result in enhanced transfection, as shown in Figure 13. Scale bars are indicated in the figure. Yellow arrows show the direction of the anisotropic topography (e.g., microtopography). (C) and (E) are hierarchal topographies. Higher magnification images of these patterns to show the secondary topography are shown in (D) and (F) respectively. Red arrows highlight the secondary topography on hierarchal topographies.

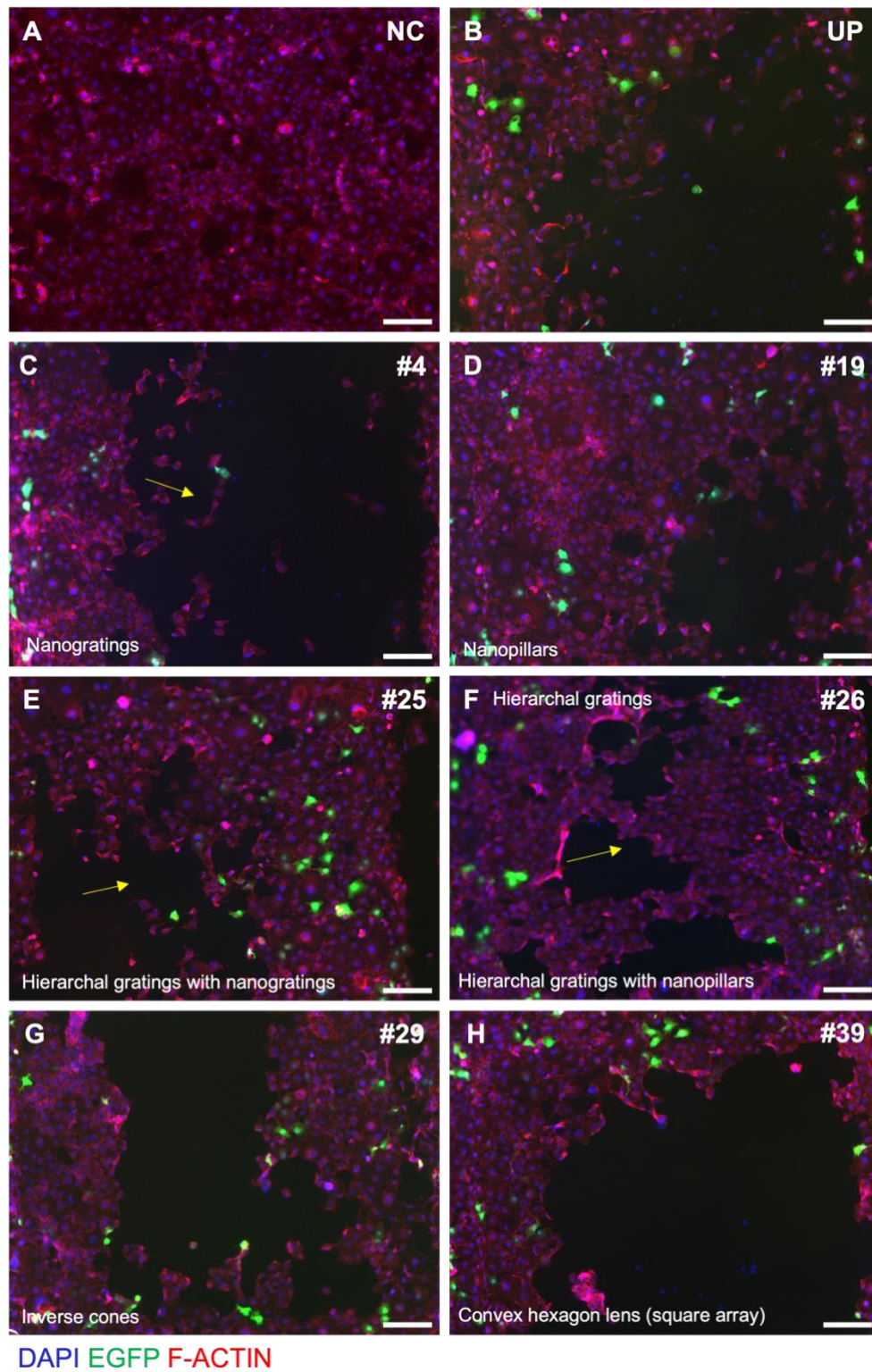


Figure 13. (previous page) Representative figures of CS-PEI/pEGFP transfection on the MARC-C. Cells are stained for the F-actin (red) and counterstained with DAPI (blue) to highlight the nuclei. (A) Represents the negative control, or non-transfected cells on unpatterned (UP) PDMS. (B) Transfection % on the UP sample on the MARC-C. (C)-(H) show the transfection efficiency on the patterns, indicated by their pattern ID #. The yellow arrow on (E) and (F) shows the direction of the $2\ \mu\text{m} \times 4\ \mu\text{m} \times 2\ \mu\text{m}$ gratings (the primary topography). Scale bar = $200\ \mu\text{m}$. $n = 4$.

3.3.2.2 Subgroup Analysis

To further evaluate which aspects of biointerface topography contribute to the variation in transfection, subgroup analysis was conducted. In **Figure 14**, the patterns are arranged based on whether they are anisotropic or isotropic topographies. The transfection efficiency on pattern ID #25 was also significantly higher than pattern ID #8 ($p = 0.0205$), #9 ($p = 0.0475$), #10 ($p = 0.0124$), #13 ($p = 0.0161$), #14 ($p = 0.0045$), #15 ($p = 0.0108$), and #27 ($p = 0.0464$).

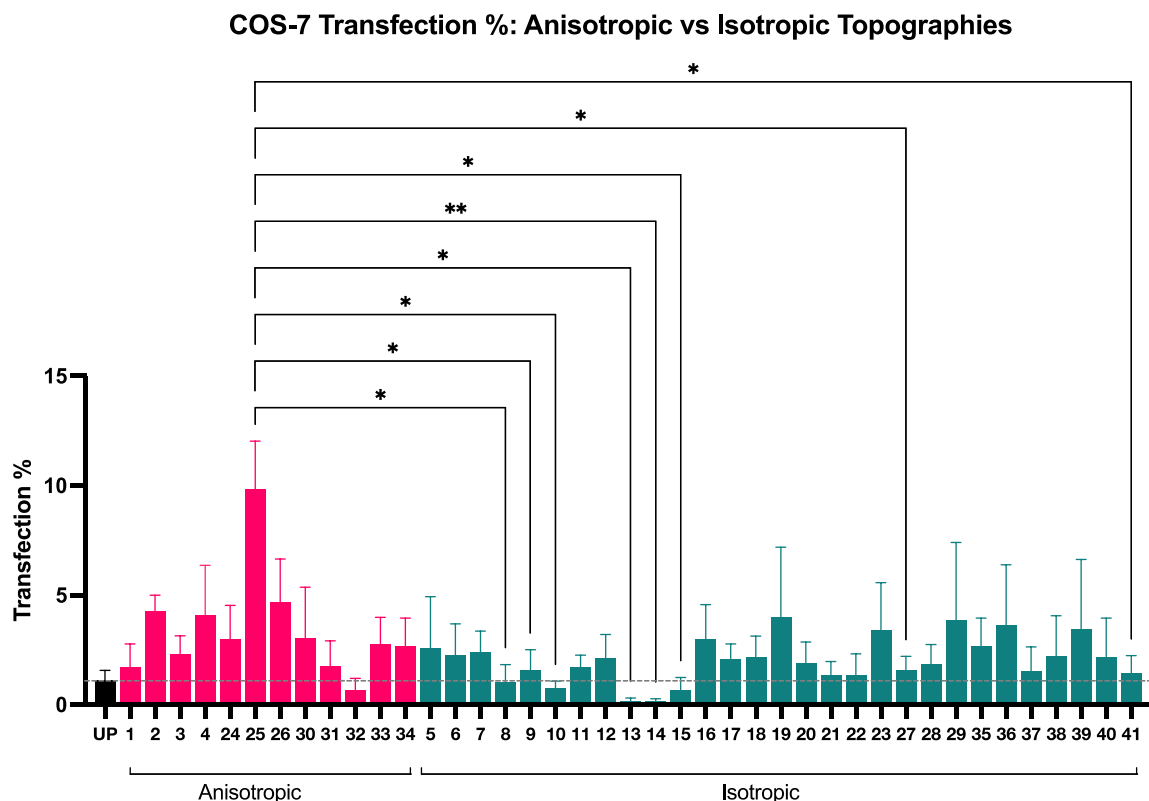


Figure 14. The effects of anisotropic and isotropic topographies on COS-7 transfection efficiency. The unpatterned sample is shown in black. Anisotropic topographies are represented in pink, whereas

isotropic topographies are represented in green. The grey dashed line indicates the transfection efficiency of the unpatterned (UP) case. Statistically significant data is indicated in the figures using *, **, and *** for $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$ respectively. Error bars represent the SEM. $n=4$.

As exhibited in **Figure 14**, pattern ID #25 (an anisotropic topography) resulted in the greatest transfection efficiency, which was significantly higher than numerous, isotropic topographies. However, there is no distinct effect of pattern directionality; although not statistically significant, pattern ID #19 and #39 yield comparatively high transfection efficiencies. Pattern ID #19 represents a square array of nanopillars with dimensions of $500 \text{ nm} \times 10 \mu\text{m} \times 200 \text{ nm}$ (diameter $\times$ spacing $\times$ height). Pattern ID #39 represents a square array of hexagonal convex lens topographies. The lowest transfection efficiencies were observed on pattern ID #13 and ID #14, which are both square arrays of micropillars. The dimensions of the former are $2 \mu\text{m} \times 12 \mu\text{m} \times 700 \text{ nm}$ and the dimensions of the latter are $2 \mu\text{m} \times 12 \mu\text{m} \times 500 \text{ nm}$ (diameter $\times$ spacing $\times$ height). As highlighted **Figure 15A**, the height of the $2 \mu\text{m}$ diameter pillars plays a significant role in determining the transfection efficiency, with the largest height, $2 \mu\text{m}$, yielding the greatest percentage of EGFP positive cells. The transfection efficiency shows a marked decrease on $2 \mu\text{m}$ (2000 nm) pillars with heights of 700 and 500 nm , and then a steady increase as the height decreases. A similar trend can be observed for the 500 nm pillars (**Figure 15B**); the transfection efficiency shows a steady increase as the height decreases, with the 200 nm height yielding the highest percentage of EGFP positive cells.

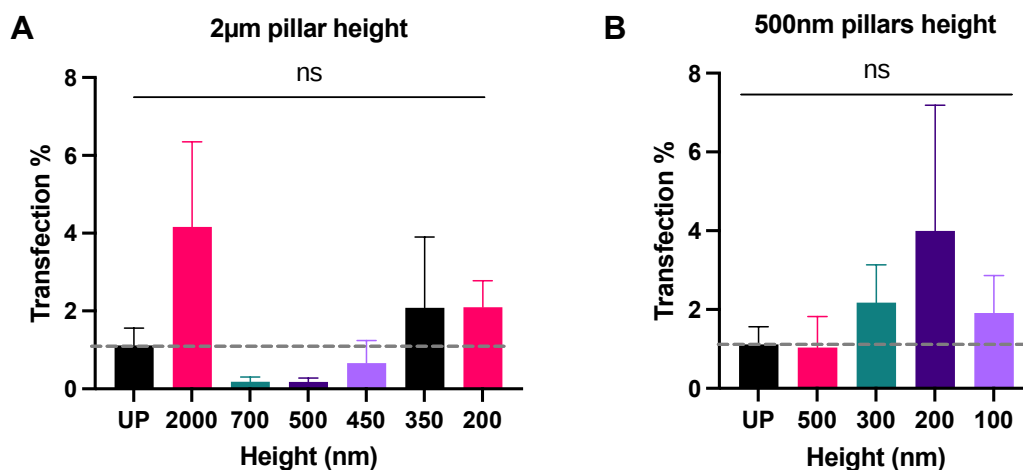


Figure 15. Pillar subgroup analysis: the effect of pillar height. (A) The effect of varying $2 \mu\text{m}$ diameter micropillar height on the transfection efficiency. (B) The effect of varying 500 nm diameter nanopillar

height on the transfection efficiency. The grey dashed line represents the average transfection efficiency of the unpatterned case. Statistically significant data is indicated in the figures using *, **, and *** for $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$ respectively. Error bars represent the SEM. $n=4$.

The effect of membrane curvature is also examined in greater depth in **Figure 16**. The topographies represented here possess both x-y and z-axis curvature. Both microscale and nanoscale V- and U-gratings are evaluated. In the case of the V-gratings, the nanoscale pattern appears to have a beneficial effect relative to microscale topography. Contrastingly, the microscale U-gratings appear to increase transfection relative to their nanoscale counterparts. Interestingly, the sharp edges (V-gratings) appear to enhance transfection compared to the soft edges (U-gratings). There appears to be little difference between the convex and concave micro lens features. However, both features result in a higher transfection efficiency compared to the unpatterned sample.

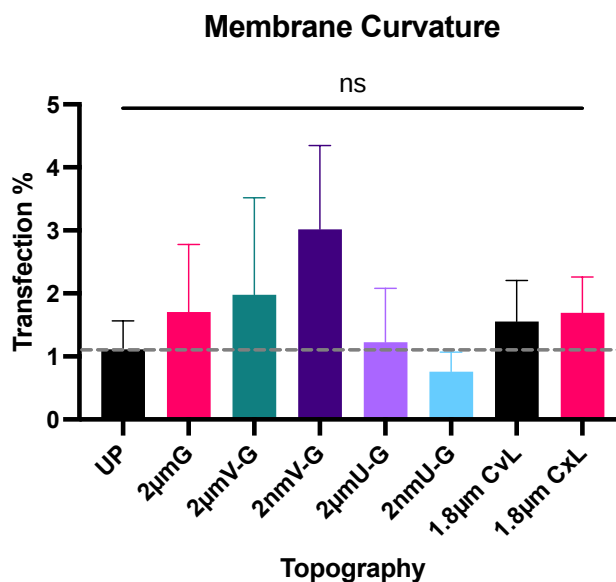


Figure 16. The effect of membrane curvature on transfection efficiency. The dashed grey line represents the average transfection efficiency of the unpatterned case. Statistically significant data is indicated in the figures using *, **, and *** for $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$ respectively. Error bars represent the SEM. $n=4$.

3.3.3 Principal Component Analysis (PCA)

Principal component analysis (PCA) was used to evaluate how different aspects of topography, such as the normalized transfection efficiency, normalized cell density, feature height/width ratio, feature spacing, feature height, and feature width. The results of the PCA are shown in **Figure 17**. A correlation plot is shown in **Figure 17A** and shows weak positive correlation between the transfection efficiency and feature height/width ratio, width, and height as well as the normalized cell density. There is no correlation between the transfection efficiency and feature spacing ($r = 0.02$). Notably, there is a weak negative correlation between the seeding density and the height/width ratio ($r = -0.23$). Contrastingly, the cell density is weakly positively correlated with the feature spacing and width. A scree plot of the eigenvalues from all variables or factors is shown in **Figure 17B**. The scree plot shows the percentage of explained variation from each principal component. The first two dimensions explain 58.7% of the variation, which provides an indication that this data can be explored using PCA. The contribution of each variable to the first five dimensions is shown in **Table 5**.

Variable	Dim. 1	Dim. 2	Dim. 3	Dim. 4	Dim. 5
Transfection	2.043231	33.8294936	41.791383	4.69936090	16.572367
Density	9.446417	8.8437492	40.675837	20.10973637	20.924007
Spacing	27.931616	0.0264823	8.337845	17.66561574	14.220116
Height	21.634457	2.5075764	1.945139	33.94972285	30.807138
Width	36.672689	0.7939948	2.865330	0.06237066	1.816886
Height/Width	2.271589	53.9987037	4.384466	23.51319349	15.659487

Table 5. The percentage contribution of each variable to the first five dimensions (Dim.) or principal components.

The bar plot shown in **Figure 17C** shows the $\cos^2$ values for each variable in the first two principal components or dimensions. As shown, feature width and the height/width ratio possesses the largest $\cos^2$ values and are well-represented within the two dimensions. This is further illustrated in the variable factor or loading plot shown in **Figure 17D**. As shown by their distance from the origin of the plot, feature width is the primary contributor to the first principal component and the height/width ratio is the primary contributor to the second principal component. The variable factor plot also shows the relationship between the cell density and the height/width ratio, also known as the aspect ratio. These variables are located on diagonally opposed quadrants, emphasizing their inverse correlation. In other words, as the aspect ratio increases, the density of cells on the topography is likely to decrease.

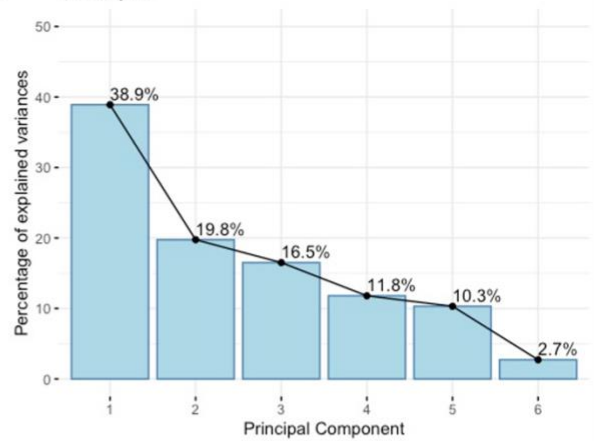
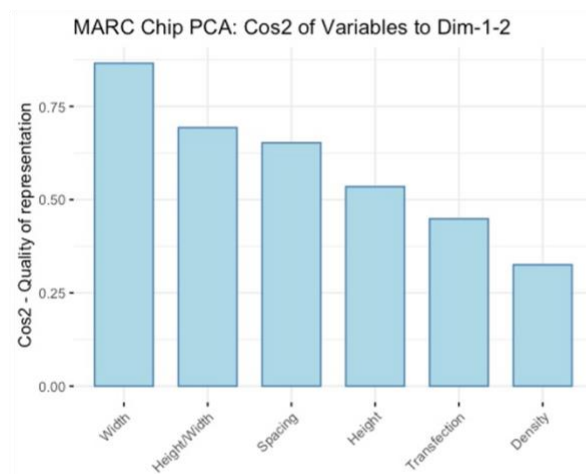
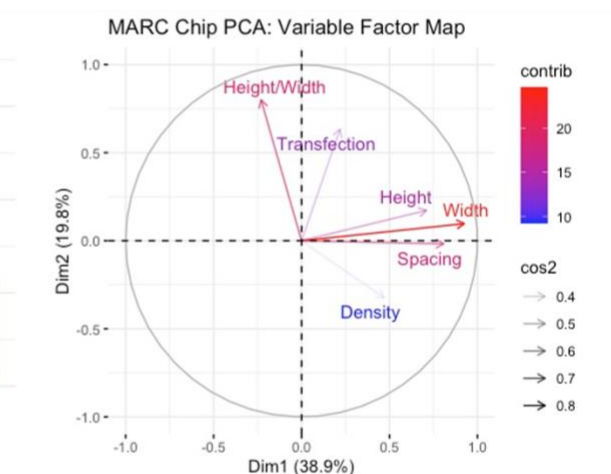
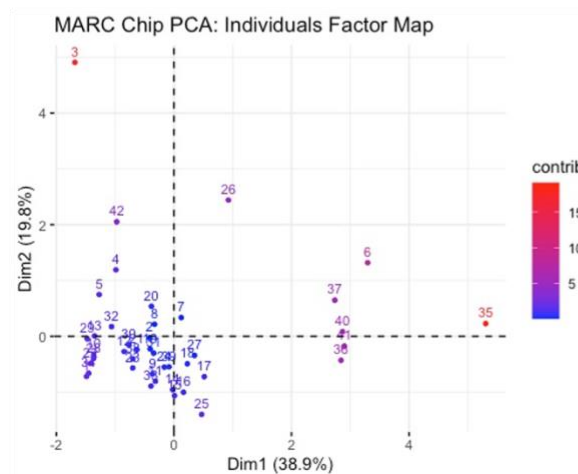
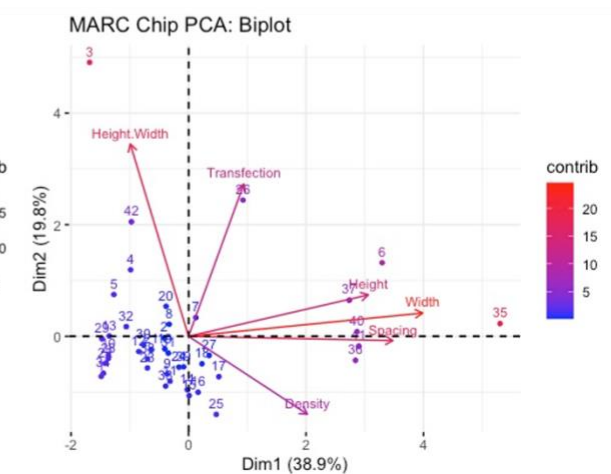
A**B** Scree plot**C****D****E****F**

Figure 17. (previous page) Results of PCA analysis of transfection efficiency, cell density, and the width, spacing, height, and height/width ratio of the topographical features. (A) A correlation plot of the variables. (B) A screen plot generated the eigenvalues, using the total percentage of contribution to the variation. (C) A bar plot of the total cos2 of variables in principal components or dimensions 1 and 2, to estimate the quality of the representation in the principal component. (D) A variable correlation plot, in which variables are colored by their contribution to the first two principal components. The intensity of the line is determined by the cos2 of the variable for the first two principal components.

However, the variable plot suggests that the feature height, width, and spacing have little correlation with the transfection efficiency. Furthermore, the near right angle between the transfection efficiency vector and the spacing vector confirms the aforementioned relationship: there appears to be no correlation between these two variables. The variable factor plot emphasizes the potential relationship between the transfection efficiency and the aspect ratio, as shown by the smaller angle between these two vectors. The individuals plot or factor map is shown in **Figure 17E**. The topographies with the lowest cos2 values are located close to the origin of the graph, indicating poor representation by the principal components. Individual number 3 and 35, or pattern ID #2 and #34, are the furthest from the origin and have the highest cos2 numbers, suggesting they are the best represented by the principal components. The majority of the samples are clustered together, suggesting they have similar values. Looking at the biplot in **Figure 17F**, one can evaluate relationship between the individual samples and the variables.

3.3.4 Secondary Screening on Individual Topographies

As shown in the MARC-C analysis, pattern ID #25 resulted in the highest transfection %, which was significantly higher than the unpatterned case. Thus, this pattern was selected for further analysis with flow cytometry. As described previously, pattern ID #25 is a hierarchal pattern. To determine which component (the primary or secondary topography) of the pattern contributes to the heightened rate of transfection, the transfection efficiency was evaluated on 2 μ m gratings, 250nm gratings, and the hierarchal topography. Flow cytometry was utilized to overcome the limitations of fluorescence image analysis in evaluating transfection efficiency (**Figure 18** and **Figure 19**).

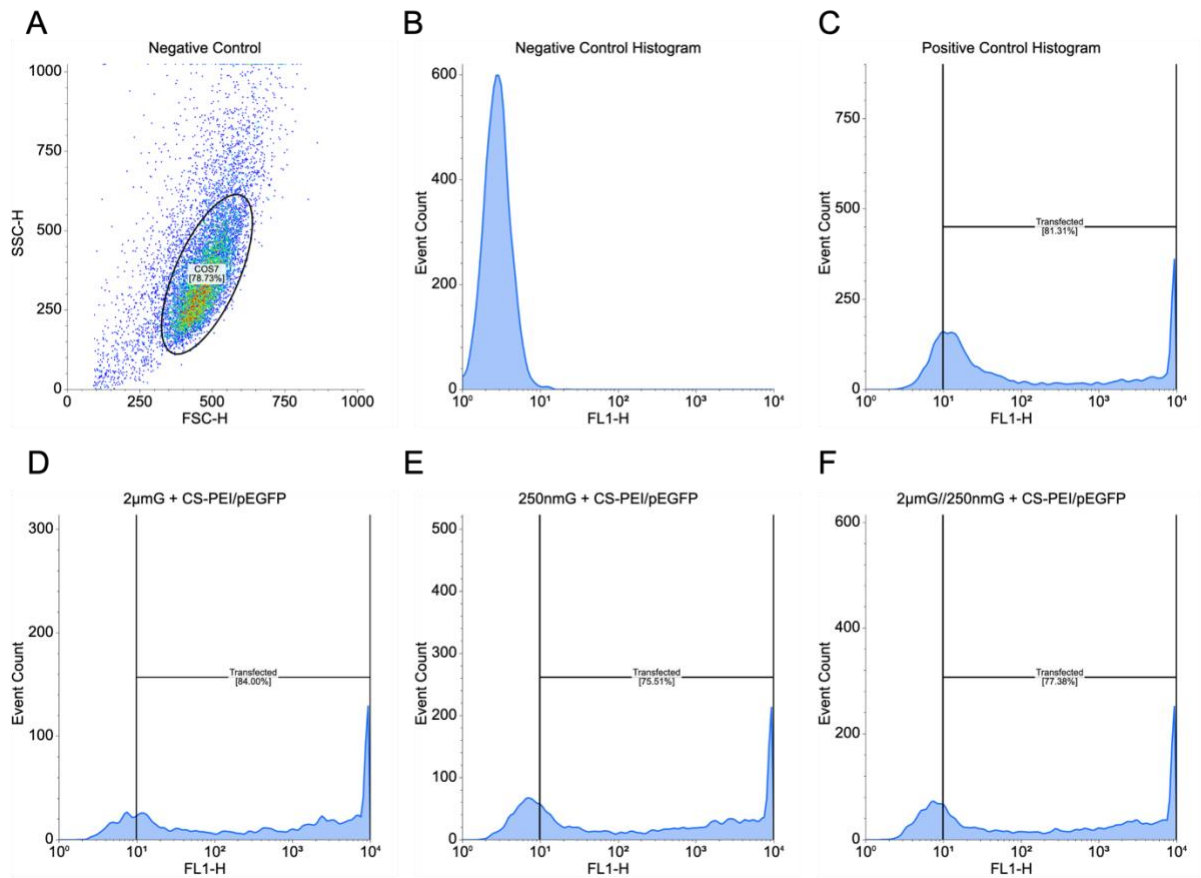


Figure 18. The percentage of EGFP positive cells on different topographies as determined by flow cytometry and with the gates determined in (A) and (C). $n = 2$.

As shown in **Figure 18** and in **Figure 19A**, there is little difference in the percentage of transfected or EGFP positive cells across the topographies. However, the percentage of transfected cells is only one parameter to quantify transfection efficiency. The mean fluorescence intensity (MFI) is also a key tool to evaluate the success of transfection. There are a number of ways to calculate the MFI, including the arithmetic mean, median, and geometric mean. The median is generally considered to be the most reliable metric. In **Figure 19**, both the median and mean are shown.

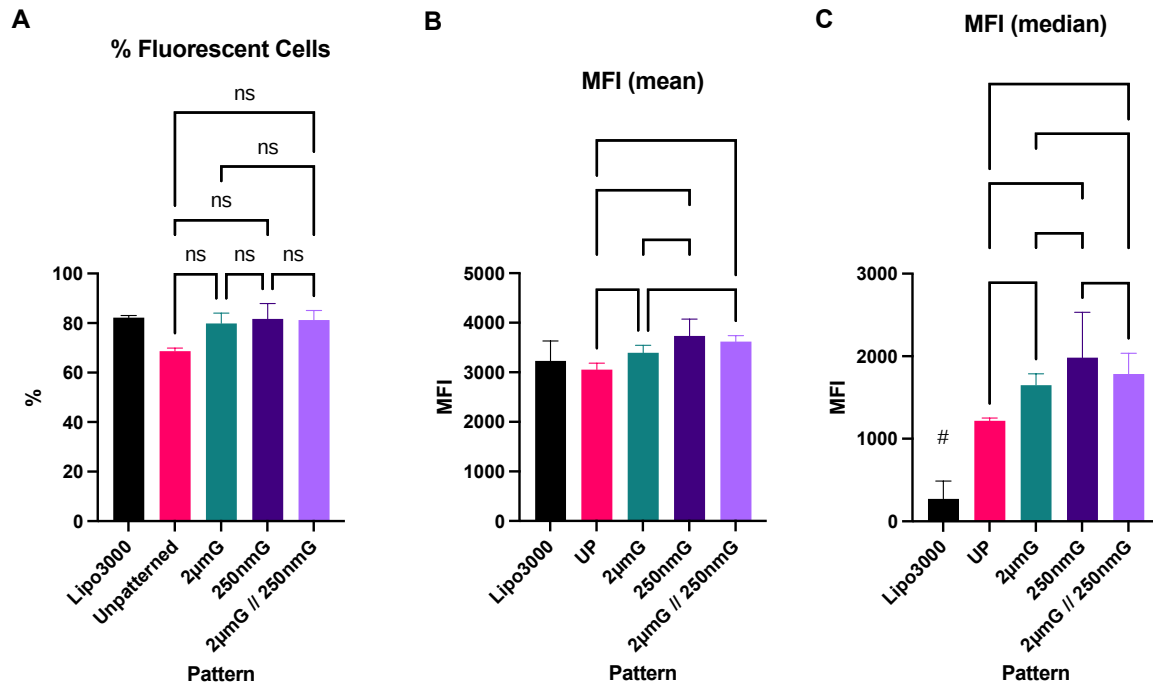


Figure. 19. The percentage of transfected cells and mean fluorescent intensity (MFI) cells transfected on 2µm microgratings, 250nm nanogratings, and the hierarchal topography (2µm gratings with parallel (/) 250nm gratings). (A) The percentage of fluorescent cells. (B) The MFI (mean) of the EGFP positive cells. (C) The MFI (median) of the EGFP positive cells.

While there are no significant differences in the percentage of fluorescent cells on the topography compared to the unpatterned case, there are differences in the MFI, when represented by the median and mean. Considering the MFI in both contexts, all topographies significantly upregulate the intensity relative to the unpatterned control. Interestingly, the hierarchal topography does not result in the highest MFI. The 250nm gratings alone result in the highest the MFI. The median intensity is significantly higher on 250nm gratings compared to both the 2µm gratings ($p < 0.0001$) and the hierarchal topography ($p = 0.0008$). The MFI (median) on the hierarchal topography is also significantly higher than the MFI on the 2µm gratings ($p = 0.0425$).

3.3.5 Preliminary Screening with hUVECs

The cytotoxicity of hUVECs at varying N/P ratios after 24hrs was evaluated to determine if an N/P ratio of 20 is suitable for this cell type and this application and is shown in **Figure 20**. In this case, the hUVECs were incubated with the polyplexes for 24hrs. As shown, the N/P ratio of 40 resulted in a clear

decrease in viability after 24hrs, resulting in less than half (~49%) of viable cells compared to the untreated control. Alternatively, N/P ratios of 10 and 15 moderately decreased viability, resulting ~85% and ~84% viability. The N/P ratio 20 reduced viability, resulting in ~67% viable cells. Although cell proliferation was reduced in the presence of CS-PEI/pEGFP polyplexes prepared at an N/P ratio of 20, it was predicted that a similar trade-off in terms of viability and transfection efficiency would be observed in hUVECs as was seen in COS-7. Therefore, preliminary studies were conducted with hUVECs on the MARC-C using a similar experimental approach. However, further experiments are needed to determine the viability of the hUVECs after 48 and 72hrs, as these time points demonstrate the late-stage cytotoxicity of the polyplexes and the potential recovery, as was observed in Figure 10. Furthermore, it is necessary to quantify the transfection efficiency at these ratios, particularly N/P ratios 10 and 15. Likewise, the percentage of fluorescent cells after 48 and 72 hours must be evaluated to explore the time of peak transfection in this cell type.

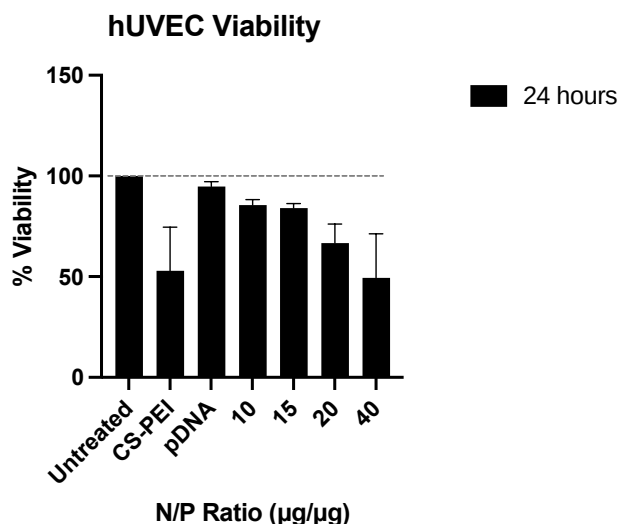


Figure 20. The effects of the N/P ratio on the viability of hUVECs after 24hrs. The grey dashed line indicates the untreated sample to which all values were normalized. Statistically significant data is indicated in the figures using *, **, and *** for $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$ respectively. Error bars represent the SEM. $n = 2$.

Preliminary screening with hUVECs was conducted on the MARC-C using the same conditions (e.g., seeding density, dose, N/P ratio) as with COS-7. However, marked toxicity was observed. Shortly after transfection, widespread cytotoxicity was observed on the MARC-C despite the high seeding density,

resulting in cell death after incubation with the polyplexes after 24 hours. Therefore, a range of incubation times were evaluated, as shown in **Figure 21**. Clearly, there is a near linear decrease in viability as the incubation time increases, resulting in less than 25% viability after incubation for 24hrs. Incubation with the polyplexes for 3hrs resulted in an average viability of ~88%. Therefore, an initial investigation into the transfection efficiency on the MARC-C after incubation for 3 hours was conducted. While the viability of hUVECs is maintained after incubation for 3 hours, the transfection efficiency was very low, with no EGFP positive cells detected. These results demonstrate the importance of optimizing the N/P ratio and the dose for evaluation with hUVECs and emphasize cell-type specific responses to gene carriers.

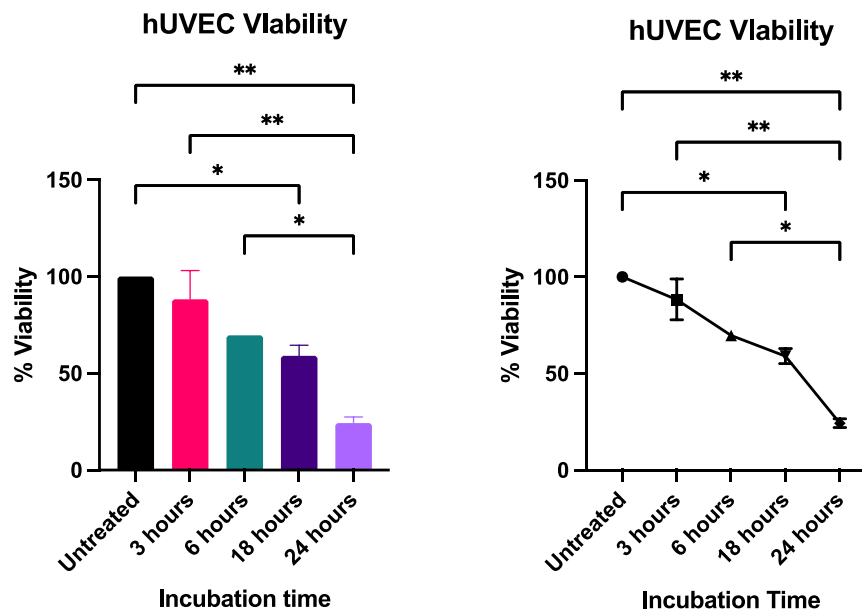


Figure 21. The effect of incubation time with the CS-PEI/pEGFP polyplexes at an N/P ratio of 20 and a dose of 4 μ g of pDNA. Statistically significant data is indicated in the figures using *, **, and *** for $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$ respectively. Error bars represent the SEM. $n = 2$.

3.4 Discussion

Substrate-mediated nonviral gene delivery has widespread applications in tissue engineering and regenerative medicine due to its ability to provide targeted transfection and localize the gene carrier in the cellular microenvironment. Moreover, the functionalization of the biomaterial surface with topographical cues could be utilized to not only manipulate cell behaviour but enhance transfection.

Our lab has previously shown that topographical cues such as micropillars and nanopillars enhance transfection by interacting with different endocytic pathways. The MultiARChitecture (MARC) chip is a microarray with 41 different patterns, each with an area of $\sim 4\text{mm}^2$ that allows for high-throughput evaluation of cellular behavior on topography under identical experimental conditions. In this chapter, the effects of topography on CS-PEI transfection efficiency are evaluated using the MARC chip to screen for potential topographies that enhance the transfection. The pattern that resulted in the highest transfection efficiency was selected for downstream analysis.

The CS-PEI copolymer was selected as nonviral gene carrier for investigation due to its potential use *in vivo*. A plasmid encoding EGFP was used as a transgene to evaluate the transfection efficiency due to its ease of detection. Prior to its evaluation with the MARC chip, it was necessary to determine the optimal N/P ratio for transfection in COS-7, a cell line often used in transfection studies. By investigating the cytotoxic effects and transfection efficiencies of varying N/P ratios, as well as the nanoparticle size and charge, an N/P ratio of 20 was selected for further use. However, it must be noted that further optimization of the N/P ratio is required for translation to primary cells or more sensitive cell types, such as hUVECs. hUVECs are highly sensitive to the CS-PEI/pEGFP polyplexes at an N/P ratio of 20 and there is a clear relationship between cytotoxicity and incubation time. COS-7 are a robust cell line, and do not experience the same detrimental effects of the CS-PEI polyplexes and maintains sufficient viability for analysis at this N/P ratio. It would also be of interest to explore lower and higher N/P ratios with more negative and positive charges, respectively, to gain further insight into the charge-dependent viability in both primary and immortalized cell types. Furthermore, determining the peak transfection could be helpful in defining the optimal time point at which to evaluate transfection efficiency. An experiment of this nature could also be utilized to refine the selection of the N/P ratio. The relationship between the pDNA dose and viability is also of interest.

The transfection efficiency of the CS-PEI polyplexes on topographical features was then evaluated 48 hours post-transfection with the MARC chip at a dose of $4\mu\text{g}$ of pDNA. It is critical to note that all transfections were performed as forward transfections, with the CS-PEI polyplexes added to adhered cells on already stably attached to the PDMS substrate. MARC chip screening revealed that pattern ID #25 resulted in the highest transfection efficiency and was selected for further analysis. The percentage of EGFP positive cells on this pattern was significantly higher compared to not only the control but an array of other patterns. The average transfection efficiency on the unpatterned sample was $\sim 1.114\%$, whereas the average transfection efficiency on pattern ID #25 was $\sim 9.822\%$. This

suggests that this pattern could be utilized to significantly increase SMD compared to an unpatterned biomaterial. Other patterns also resulted in increased transfection efficiency, albeit not significantly.

However, there are numerous limitations to the MARC chip and the experimental set up that must be acknowledged and considered when discussing this finding. Firstly, there are no defined barriers between patterns on the MARC chip, which allows for cell-cell and paracrine signalling to induce cell migration from one topography to another. This may influence the density of cells on a single pattern, thus skewing the transfection efficiency in both directions. An additional consequence of this phenomena is dependency of transfection efficiency on the location of the pattern in the chip. A pattern with few cells and low transfection efficiency may be located next to a pattern that promotes cell migration. Additionally, cells show a preference for the slight groove between the patterns as well as the outer edges of the chip, and the majority of transfected cells are within this region. To circumvent this issue and promote cell attachment on the topographies, a high seeding density was chosen. However, the high seeding density combined with a total culture time of 72hrs results in a high proportion of cells the per pattern, also skewing the transfection efficiency. Cell density, proliferation, and endocytosis are connected, and should be investigated in further depth. Furthermore, as will be discussed subsequently, the PCA highlights a potential negative correlation between cell density and transfection efficiency. Therefore, future experiments should evaluate the transfection efficiency with multiple seeding densities. Additionally, to examine the effects of cell-cell signalling on the chip, it is also recommended to explore a wider range of features with flow cytometry, such as those that result in low and intermediate levels of EGFP positive cells.

Subgroup analysis and PCA revealed the contribution of dimensions, curvature, aspect ratio, and cell density to the transfection efficiency. With regards to the former, trends regarding pillar height and membrane curvature were investigated. There is a clear height dependency for both micro and nanoscale pillars. In the case of the micropillars, decreasing pillar heights in the nanoscale range resulted in higher transfection efficiency. Interestingly, pillars with a height of 2 μ m and an aspect ratio (height/width ratio) of 1 resulted much higher transfection efficiency. The effects of topographies that induce membrane curvature appear to be dependent on the scale of the feature (micro vs nano) and whether the edges of the feature are rounded or sharp. The role of membrane curvature requires further investigation and could provide intriguing insight into the mechanism of endocytosis. The topographies that were included in the subgroup analysis do not include pillar geometries, which could introduce high membrane curvature via the high aspect ratio. The PCA showed that the aspect/ratio negatively

correlates with cell density. Furthermore, the transfection efficiency appears to be negatively correlated with cell density. PCA demonstrates key link between aspect ratio, cell density, and transfection efficiency. This begs further evaluation of the ties between proliferation, membrane curvature, actin cytoskeleton reorganization, cell area, and CS-PEI/pEGFP transfection. These parameters are intimately associated with the aspect ratio, cell density, and endocytosis, and would provide clarity regarding the key variables that drive transfection on topographical cues.

Flow cytometry analysis provided insight into the metrics for evaluating transfection efficiency as well as the driving forces behind enhanced transfection on the hierarchal topography. Firstly, flow cytometric analysis demonstrates that the percentage of transfected cells must be considered in addition to the fluorescent intensity. Transgene expression appears to be more significantly affected by topographical cues than the percentage of EGFP positive, or transfected cells. The MFI is significantly affected by not only topography but different patterns, whereas the percentage of EGFP positive cells shows no significant difference across all conditions. Moreover, the topographical cues resulted in a significantly higher MFI compared to the positive control which is promising in the context of *in vivo* applications. The flow cytometric analysis revealed that the 250nm gratings resulted in the highest MFI, followed by the hierarchal topography. This suggests that the 250nm gratings are the driving force behind the enhanced transfection and explains the high transfection efficiency on hierarchal patterns ID #25 and #26, which possess 250nm gratings as the secondary topography. The 250nm gratings alone also resulted in a relatively high percentage of transfected cells on the MARC chip (pattern ID #4), although still much lower relative to other the hierarchal topography and others, such as pattern ID #19 (500nm pillars). This may be due to the location of the pattern; pattern ID# 4 is located in the top row of the MARC chip and borders the edges of the chip, which cells have a strong preference towards. As such, it is likely the cells migrated from the topography to the chip edge, resulting in a lower cell density and lower percentage of EGFP positive cells on this pattern. This may also explain why the transfection efficiency is high on both pattern ID #25 and #26 compared to the 250nm gratings. These topographies are located more centrally and may not experience the same paracrine effects to drive migration. Furthermore, the 2 μ m primary topography may drive increased cell migration and proliferation further contributing to the results observed on the MARC chip. Flow cytometry has clearly demonstrated that MFI is a key parameter to evaluate transfection and should be utilized to further examine the candidate topographies discussed in [Section 3.3.2.1](#). However, the underlying mechanisms that drive enhanced transfection efficiency and transgene expression on 250nm gratings require further investigation.

Chapter 4 Topography-Mediated Nonviral Delivery of microRNA-145

4.1 Introduction & Background:

MicroRNAs (miRNAs) are small, non-coding RNAs (~22 nucleotides) that exhibit post-transcriptional gene silencing activity and mediate the expression more than a third of genome. A central miRNA in VSMC biology is miR-145, which is integral in the transcriptional networks that mediate phenotypic switching [27], [84]. VSMCs are a uniquely plastic cell type, and may dedifferentiate from their quiescent, contractile phenotype to a fibroblast-like, synthetic migratory state in response to vascular injury. miR-145 plays a key role in mediating this process and promotes the contractile phenotype by repressing the expression of genes that contribute to VSMC dedifferentiation, actin remodelling, and podosome formation. The genetic targets of miR-145 include *KLF4/5*, *CAMK2* and *ELK-1* [23]–[26], [30], [32], which are mediate the expression of synthetic genes by interacting with myocardin-related transcription factors (MRTFs) or acting as transcription factors themselves. Additional targets include *SRGAP1/2*, *SSH2*, and *FSCN1* (fascin) [33], which are involved in actin cytoskeleton reorganization during migration and ACE, which is critical for VSMC contraction. Importantly, miR-145 is downregulated after vascular injury and in cardiovascular diseases, such as atherosclerosis [36], [37], [85], [86]. Furthermore, lower plasma levels of miR-145 are associated with increased risks of coronary in-stent restenosis [87]. VSMC dedifferentiation and phenotypic switching underpins the pathological response observed in intimal hyperplasia (IH). As such, recent efforts have sought to develop biomaterial-mediated delivery platforms to enhance endogenous miR-145 expression and limit IH initiation and progression [41], [43], [44], [88].

The primary strategy used in these studies involves the incorporation of a nonviral gene carrier containing pDNA encoding miR-145 or synthetic miR-145 mimics into a graft material that is implantable. This approach is referred to as substrate-mediated gene delivery (SMD). These studies have demonstrated that miR-145 overexpression via a graft material can reduce the formation of neointimal lesions in the lumen of the graft and promote the contractile phenotype. However, these studies have demonstrated that miR-145 alone is insufficient to promote EC coverage of the graft surface. Thus, additional biochemical or biophysical stimuli are needed to promote endothelialization. A key biophysical parameter that has shown great promise is topography. Topographical cues can be patterned onto the luminal surface of a vascular implant to promote EC colonization of the graft material. Anisotropic features such as grooves or gratings have shown great promise both in vitro and

in vivo. In blood vessels, ECs form a monolayer atop a basement membrane, which comprises the tunica intima or the innermost layer of the vasculature. The basement membrane possesses a nanoscale, fibrillar structure that is organized into microscale undulations or wave-like features. As such, anisotropic features mimic the native topography of blood vessels and encourage vascular remodelling. Numerous studies have shown that 2 μ m gratings patterned on the luminal surface to promote EC attachment, proliferation, and migration throughout the graft lumen. Our lab has developed a vascular graft composed of poly(vinyl alcohol) (PVA) with 2 μ m gratings patterned onto the lumen [89]–[93]. Yao et al., 2023 have shown promising results with this platform, demonstrating enhanced endothelialization through the combination of topographical and biochemical cues both *in vitro* and *in vivo* [92]. To further improve this graft system and combat IH formation at the graft anastomoses, SMD of miR-145 can be utilized. In this chapter, the use of the CS-PEI copolymer as a gene carrier for miR-145 mimics will be evaluated as an initial step towards its integration into the fucoidan-modified and topographically enhanced PVA-based sSDVG.

Synthetic miRNA mimics are small double-stranded oligonucleotides that imitate the sequence of the endogenous miRNA molecule [94]. Here, a miRNA mimic of the guide strand of human miR-145 (hsa-miR-145-5p) will be delivered to hUASMCs and hUVECs with the CS-PEI copolymer. To examine the therapeutic efficacy of the CS-PEI/miR-145 polyplexes, its effects on hUASMC behavior and gene expression will be assessed. Moreover, to work towards the integration of gene carrier with the PVA-based sSDVG platform our lab has pioneered, the CS-PEI/miR-145 nanoparticles will be utilized in combination with 2 μ m microgratings. This work represents an initial investigation into the combined use of miRNA gene therapy and topographical cues, which has rarely been investigated.

4.2 Materials & Methods:

4.2.1 CS-PEI/miR-145 Polyplex Formation & Transfection

A description of the CS-PEI copolymer is provided in Section 3.2.1. The formation of CS-PEI nanoparticles is the same as outlined in Section 3.2.4. Two types of miR-145 mimics are utilized in this chapter. Firstly, a double-stranded Cy3-hsa-miR-145-5p mimic from Creative Biogene, Inc, and secondly, a double-stranded custom mimic from Integrated DNA Technologies (Coralville, IA). A scramble control (Creative Biogene, Inc) is utilized as a negative control. The scramble control possesses a random sequence that has been validated to have no targets in mammalian cells. The

sequences of the mimics and scramble control are shown in **Table 6**. As evidenced in Table 5, the 5' to 3' sequences are identical.

Synthetic RNA Name & Origin	Sequence
Cy3-hsa-miR-145-5p (Creative Biogene, Inc)	5'-Cy3-GUCCAGUUUCCCCAGGAAUCCCU-3'
RNA duplex (Integrated DNA Technologies)	5'-/5PHOS/rGrU rCrCrA rGrUrU rUrUrC rCrCrA rGrGrA rArUrC mCmCrU-3'
miRNA Control Sequence (Creative Biogene, Inc)	5'-UUGUACUACACAAAAGUACUG-3'

Table 6. The RNA sequences of miRNA mimics and scramble control. In the case of the RNA duplex obtained from Integrated DNA Technologies, the nucleotides in the sequence are labelled with “r” or “m”. The “r” indicates that the nucleotide is a ribonucleotide (RNA), and the “m” indicates that the nucleotide possesses a 2'-O-methyl modification.

The duplexed RNA is shipped lyophilized, briefly centrifuged, and resuspended in DPEC-treated ultrapure DI H₂O. The miR-145 mimics and scramble RNA duplex are stored at -80°C and maintained on ice during transfections to reduce degradation. The mass of the miRNA mimics is determined from the molecular weight and micromolar concentration. As such, the volume of miR-145 is maintained at 1μL per transfection. Lipofectamine™ 3000 serves as the positive control for all miRNA mimic transfections. As per the manufacturer's protocol, the P3000 reagent is excluded from small RNA transfections. For a Lipofectamine™ 3000 transfections in a 24-well plate, 1μL of miRNA mimics are diluted in 25μL Opti-MEM and then combined with 1.5μL of Lipofectamine™ 3000 Reagent in 25μL to achieve a final volume of 50μL. The Cy3-hsa-miR-145-5p mimic (herein referred to as Cy3-miR-145) is used in transfections to evaluate and confirm internalization. The unlabelled mimic (herein referred to as miR-145) is used in all other transfections. The doses of mimics are specified in the figure captions or results.

4.2.2 Cell Culture

The maintenance of hUVECs is described in Section 3.2.5. Briefly, hUVECs (P6-P11) maintained in Vasculife® VEGF Endothelial Media (Lifeline Cell Technologies) and passaged at 80% cell confluency. Every 48hrs, 3mL of the maintenance media is replaced and replenished. hUASMCs (P4-P8) are purchased from Lonza (VWR, catalog # CA10064-266; lot # 458z025.2) and are cultured in maintenance media, prepared in-house using a custom recipe. The composition of hUASMC

maintenance media is shown in **Table 7**. A 40% w/v D-glucose solution is prepared by mixing 40g of D-(+)-glucose or dextrose powder with DI H₂O. The solution is sterilized by autoclaving, aliquoted, and stored at -20°C. To prepare maintenance media, 10mL of DMEM/F-12 with L-Glutamine and HEPES (DMEM/F-12) is added to a 50mL conical centrifuge tube. The remaining components of the maintenance media as detailed in **Table 7** are then added. 10mL of DMEM/F-12 is added to the conical centrifuge tube and mixed well. 15mL of the media is added to a second 50mL conical centrifuge tube. 35mL of DMEM/F-12 is added to both tubes, which are mixed thoroughly and then stored at 4°C. To thaw hUASMCs, 5mL of maintenance media is added to a T-25 flask and allowed to equilibrate in the incubator at 37°C and 5% CO₂ for 15 minutes. The cap of the cryovial is briefly loosened to release pressure inside and the cells are thawed in a water bath at 37°C for ~2min or until a small piece of ice remains. The cells are then added to a 15mL conical centrifuge tube and then diluted 1:10 by the dropwise addition of prewarmed maintenance media. The cells are centrifuged for 5min at 1.0rpm, then resuspended in 5mL of the equilibrated media, and added to the T-25 flask. The maintenance media is replaced every 48hrs. hUASMCs are passaged at 90% confluency with a high splitting ratio (1:3).

Component	Stock Concentration	Volume	Supplier
DMEM/F-12 with L-Glutamine and HEPES	-	90	Wisent BioProducts
FBS	-	5 mL	Wisent BioProducts
GlutaMAX™	100X	3.75 mL	Gibco™
PenStrep	10,000 U/mL	1 mL	Gibco™
D-(+)-glucose (aqueous)	40% (w/v)	125µL	Sigma-Aldrich
Human recombinant fibroblast growth factor-basic (bFGF)	0.5 µg/µL	0.5µL	Gibco™
Human recombinant epithelial growth factor (EGF)	0.2 µg/mL	6.25µL	Gibco™

Table 7. The composition of hUASMC maintenance media.

4.2.3 N/P Ratio Screening: alamarBlue™ Cell Viability Assay

To evaluate the cell viability across N/P ratios, hUASMCs are seeded in a tissue-cultured treated polystyrene flat bottom 96-well plate at a density of 1,000 cells/cm². Cells are seeded in four replicates per treatment (four N/P ratios). Furthermore, the outer wells of the 96-well plate are filled with 100µL of 1X PBS to reduce evaporation. Polyplexes are prepared as per Section 3.2.4 yet resuspended in 367µL to achieve a final volume of 400µL. 100µL of the polyplex solution is added to each replicate

well in the 96-well plate. 24 hours after transfection, the transfection media is removed and then replaced with 90µL of the full maintenance media using a multichannel pipette. 10µL of prewarmed alamarBlue™ Cell Viability Reagent (Invitrogen™) is added to each well and then incubated for 1.5 hours protected from light at 37°C and 5% CO₂ prior to measurement with the BioTek Synergy HTX microplate reader (Agilent Technologies). The absorbance is measured at 570nm and 600nm. Every 24 hours, this procedure is repeated to evaluate changes in viability over time. 2µg of CS-PEI and 7.5nM of miR-145 are used as negative controls. Media with no cells is used to control for background absorbance. The percentage viability is calculated as described in Section 3.2.6.

4.2.4 Dose-Response Evaluation

To evaluate the minimum dose of miR-145 required to modify hUASMC phenotype, the alamarBlue™ Cell Viability assay (Invitrogen™) and the LIVE/DEAD™ Viability/Cytotoxicity kit (Invitrogen™) were employed. The former was utilized to determine the effect of varying miR-145 doses on cell metabolism, whereas the latter allowed for an evaluation of the percentage of alive and dead cells.

hUASMC were seeded on glass coverslips in a 24-well plate a seeding density of 20,000 cells/cm² in full maintenance media. 24 hours after seeding, the cells are transfected with CS-PEI/miR-145 polyplexes with varying doses of miR-145 (50nM, 1µM, 10µM, 20µM, 40µM, and 80µM) of miR-145 at an N/P ratio of 20. Lipofectamine 3000-mediated transfection of miR-145 and the miRNA scramble control are used as positive and negative controls respectively. After 24hrs, the transfection media was removed and replaced with full maintenance media. The assays are performed in succession 48hrs post-transfection. Culture media is gently removed with a pipette and replaced with 360µL of maintenance media. For the positive controls, 100% EtOH is added to kill the cells. Subsequently 40µL of the alamarBlue™ Cell Viability reagent is added to each well. Maintenance media with no cells serves as the background control. The cells are incubated for 1.5hrs at 37°C and 5% CO₂ for 1.5 hours. During the incubation period, the LIVE/DEAD™ Viability/Cytotoxicity staining solution is prepared. 0.5µL of calcein AM and 2µL of ethidium bromide (EthD-1) are added to 1mL of sterile 1X DPBS. The final concentrations of calcein AM and EthD-1 are 2µM and 4µM respectively. Two separate staining solutions are prepared with calcein AM and EthD-1 at the same concentrations for the control samples (alive and dead controls) labelled with either dye only.

To take alamarBlue™ Cell Viability reagent measurements using the microplate reader, 50µL of the reduced alamarBlue™ Cell Viability Reagent is added to a flat bottom, opaque (black) 96-well plate (8 replicates for each sample). Until measurements are taken, the plate is sealed and kept away from light. Meanwhile, the samples are gently washed with prewarmed 1X DPBS. 100µL of the staining solution is added to each well and incubated for 30 minutes at room temperature away from light. While the samples incubate with the LIVE/DEAD™ Viability/Cytotoxicity staining solution, the fluorescence of the reduced alamarBlue™ Cell Viability Reagent is measured using the BioTek Synergy HTX microplate reader (Agilent Technologies) with an excitation of 560nm and an emission of 590nm. The cell viability is determined with the following the equation:

$$\% \text{ viability} = \frac{FI \text{ 590 test well}}{FI \text{ 590 untreated control}}$$

The fluorescence of the LIVE/DEAD™ Viability/Cytotoxicity reagents is also measured using the BioTek Synergy HTX microplate reader (Agilent Technologies) with an excitation of 495nm and an emission of 515nm and 635nm for calcein AM and EthD-1 respectively. Finally, the coverslips are mounted in 10µL of DPBS on a microscope slide and examined with the fluorescent microscope.

4.2.5 hUASMC Seeding & Transfection on PDMS

hUASMCs were seeded on PDMS substrates either unpatterned (UP) or with 2µm microgratings (2µmG) at a seeding density of 20,000 cells/cm². The PDMS substrates were first cut to fit securely into the bottom of a 24-well plate and washed with 100% EtOH. The plate was baked at 60°C for 15 minutes to ensure the samples were completely dry. The plate was then plasma treated by pumping for 5 minutes and treating for 2 minutes. Quickly, the PDMS samples were immersed in 500µL of 70% EtOH and then UV sterilized for 30 minutes. A 10µg/mL collagen I solution in 20mM acetic acid was prepared and then prewarmed to 37°C in the water bath. After UV sterilized, excess EtOH is aspirated, and the samples are washed twice with 1X PBS. 500µL of the collagen solution is added to each well, resulting in a final coating density of 2.5µg/cm². The collagen coating is incubated for 1 hour at 37°C and 5% CO₂. Meanwhile, hUASMCs are washed, trypsinized, and counted with the hemocytometer to prepare a cell suspension of 80,000 cells/mL in full maintenance media. Excess collagen is aspirated and washed thrice with 1X PBS. 500µL of the cell suspension is added to each well. 24 hours after seeding, cells are transfected following the protocol described in Section 4.2.1. For RT-qPCR applications, the 5 technical replicates of each transfection/ are performed and then pooled to ensure a

sufficient amount of RNA can be collected. hUASMCs are maintained on PDMS for 72 hours post-transfection before downstream assays are performed.

4.2.6 RNA Isolation and RT-qPCR

Prior to RNA isolation, cDNA synthesis, and preparing the polymerase chain reaction (PCR), the workspace is thoroughly cleaned with 10% bleach or the RNaseZap™ RNase Decontamination Solution. Pipettes and pipette tips used for RNA and DNA work are kept isolated and decontaminated. All steps (unless otherwise noted) are performed on ice.

4.2.6.1 Total RNA isolation

RNA isolation is conducted using the TRIzol™ LS reagent. To extract a sufficient amount of RNA, total RNA is pooled from five wells in a 24-well plate. The maintenance media is gently removed with a pipette. 200µL of ice cold TRIzol™ LS reagent is added to each well. Ice cold TRIzol™ LS reagent is used to limit the effects of RNases. The lysate is repeatably pipetted up and down in the well to homogenise the cells. Quickly, the TRIzol™ LS reagent from each of the five wells is added into a 1.5 mL microcentrifuge tube. To allow for complete dissociation of the nucleoprotein complexes, the samples are incubated for 5min at room temperature. 200µL of chloroform is added to the microcentrifuge tube, quickly inverted 6-8 times, and then incubated for 3min at room temperature. Samples are centrifuged for 15min at $12,000 \times g$ at room temperature. The clear aqueous phase is transferred to a new 1.5mL microcentrifuge tube. 500µL of 100% isopropanol is added to the supernatant, gently inverted 4-6 times, and incubated overnight at -80°C . The RNA samples are thawed on ice and then centrifuged for 10min at $12,000 \times g$. The supernatant is carefully removed from the RNA pellet with a pipette, avoiding the sides of the microcentrifuge tube. The RNA pellet is washed with ice cold 75% EtOH (prepared in DPEC-treated ultrapure DI H₂O) and briefly vortexed. The sample is centrifuged for 5min at $7,500 \times g$. The supernatant is carefully removed from the RNA pellet with a pipette, leaving a small volume of EtOH remaining to ensure total solubilization. The pellet is air dried on ice for 10 minutes) and then resuspended in 20µL DPEC-treated ultrapure DI H₂O by pipetting up and down numerous times. The RNA pellet is added to a heat block and incubated for 10min at 60°C . RNA samples are placed on ice and immediately quantified using the NanoDrop ND-1000 spectrophotometer. Two measurements are made for each sample, and the 260/230 and 260/280 ratios

are recorded. For downstream applications, a 2ng/ μ L working solution is prepared in ice cold DPEC-treated ultrapure DI H₂O. All RNA samples are stored at -80°C.

4.2.6.2 TaqMan™ Real-Time Quantitative Polymerase Chain Reaction

miR-145 expression is evaluated using the TaqMan™ MicroRNA Assay (Applied Biosystems™). The RNU44 TaqMan™ MicroRNA Assay is used as the endogenous control; miR-145 expression is normalized to RNU44 expression. The reverse-transcriptase reaction is performed with the TaqMan™ Reverse Transcription Kit (Applied Biosystems™) according to the manufacturer's protocol. Briefly, the reverse transcription (RT) master mix is prepared by combining the kit components (with 10% overage) according to **Table 8**, vortexed, centrifuged, and then kept on ice. 5 μ L of the total RNA working stock (2ng/ μ L) is combined with 3 μ L of 5X RT primer in a 0.2mL PCR tube. Therefore, 10ng of total RNA is used for each reverse transcription reaction. The sample and primer are incubated at 85°C for 5min and then at 60°C for 5min. This step is required for duplexed miRNA mimics. Subsequently, 7 μ L of the RT master mix is added to each tube, which is placed on a thermocycler (Veriti™ 96-Well Fast Thermal Cycler, Applied Biosystems™) and incubated according to the manufacturer's specifications. The RT product is either used immediately for real-time quantitative polymerase chain reaction (RT-qPCR) or stored at -20°C overnight. RT-qPCR is run according to the TaqMan™ MicroRNA Assay (Applied Biosystems™) protocol with the TaqMan™ Universal PCR Master Mix (Applied Biosystems™). Importantly, the RT product is not diluted; 1.37 μ L of the RT product is added to the 18.67 μ L of PCR master mix in each well. Four technical replicates of each reaction are performed. The RT-qPCR is performed in the StepOnePlus™ Real-Time PCR System (Applied Biosystems™) in a 96-well PCR plate with the fast-cycling mode. Relative gene expression was determined via the $2^{-\Delta\Delta C_t}$ (Livak-Schmittgen) method, normalizing to RNU44 expression on the untreated control.

Component	Volume (1× rxn)	Volume (6× rxns)	Volume (6× rxns + 10%)
100mM dNTPs (with dTTP)	0.15 μ L	0.9 μ L	1.035 μ L
MultiScribe™ Reverse Transcriptase (50 U/ μ L)	1.00 μ L	6 μ L	6.9 μ L
RNase Inhibitor (20 U/ μ L)	1.50 μ L	9 μ L	10.35 μ L
10× Reverse Transcription Buffer	0.19 μ L	1.14 μ L	1.311 μ L
Nuclease-free Water	4.16 μ L	24.96 μ L	28.704 μ L

Table 8. The components of the RT master mix, for one reaction (rxn) and six reactions (rxns), without and without 10% overage.

4.2.6.3 SYBR® Green RT-qPCR

SYBR® Green RT-qPCR was utilized to evaluate mRNA expression of hUASMCs genes. The PowerTrack™ SYBR Green Master Mix for qPCR (Applied Biosystems™) was used in combination with the PrimeTime™ qPCR Primer Assays from Integrated DNA Technologies. The sequence of each primer is shown in **Table 9**. Primers are diluted in TE buffer to obtain a 20× working stock concentration. Prior to RT-qPCR, the complementary DNA (cDNA) is synthesized using the iScript™ cDNA Synthesis Kit. 10ng of total RNA is used in each 20μL cDNA synthesis reaction. cDNA synthesis is performed with the Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems™) in a 0.2mL PCR tube. The cDNA product is diluted 1:3 in TE buffer and either used immediately or stored overnight at -20°C. 500ng of cDNA is used in each reaction. The PCR reaction mixture was prepared according to the PowerTrack™ SYBR Green Master Mix for qPCR (Applied Biosystems™) manual. Importantly, the concentration for both forward and reverse primers is 500nM. The RT-qPCR reaction is performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems™) in a 96-well PCR plate with the standard-cycling mode. Relative gene expression was determined via the $2^{-\Delta\Delta CT}$ (Livak-Schmittgen) method, normalizing to *HPRT1* expression (the endogenous control) on the untreated sample.

Gene Name	Primer Assay	Sequence
<i>HPRT1</i>	Hs.PT.58v.45621572	GCG ATG TCA ATA GGA CTC CAG
		TTG TTG TAG GAT ATG CCC TTG A
<i>MYH11</i>	Hs.PT.58.2909933	GAA GTC ACG TTG TCA TTC AGC
		TCA AGG ACA AGA CTG AGT TCT C
<i>ACTA2</i>	Hs.PT.56a.2542642	CTG TTG TAG GTG GTT TCA TGG A
		AGA GTT ACG AGT TGC CTG ATG
<i>CNN1</i>	Hs.PT.58.38799164	CAT GAA GTT GTT GCC GAT GC
		TCA GCC GAG GTT AAG AAC AAG
<i>KLF5</i>	Hs.PT.56a.40282397	CCA GGT ACA CTT GTA TGG CT
		GAG AAA CGA CGC ATC CAC TA
<i>TAGLN</i>	Hs.PT.58.41066034	CAG CTC CTC GTC ATA CTT CTT C
		ACA CAA GTC TTC ACT CCT TCC

Table 9. Primer sequences.

4.2.7 EdU Assay

72 hours post-transfection, the EdU cell proliferation assay was performed. Cell media is carefully removed with a pipette and then replenished with 500 μ L of fresh maintenance media. 0.5 μ L of the stock EdU solution (10mM) is added to each well to achieve a final concentration of 10 μ M. Cells are incubated with EdU at 37°C and 5% CO₂ for 4 hours. Once the incubation period has ended, culture media is carefully removed with a pipette and the hUASMCs are washed thrice with sterile, pre-warmed 1X PBS for 30s each. Cells are then fixed with pre-warmed 4% PFA for 15 minutes. Meanwhile, the EdU blocking solution is prepared. The EdU blocking solution consists of 10% goat serum and 0.3% Triton X-1000 in 1X PBS. Once fixation is complete, the samples are washed thrice with 1X PBS for 30 seconds each. 500 μ L of the blocking solution is added to the plate and incubated at room temperature for 30 minutes. During the blocking period, the EdU staining cocktail is prepared. The composition of the EdU staining cocktail is shown in **Table 9**. The volume of each component is scaled up to ensure sufficient staining solution for all samples. Importantly, the ascorbic acid is prepared in DI H₂O and protected from light just before preparing the staining cocktail. Furthermore, the ascorbic acid is added last to the cocktail, which is then vortexed well. The blocking solution is aspirated, and the cells are washed with 1X PBS thrice for 30s each. 300 μ L of the staining cocktail is added to each well and incubated for 30 minutes at room temperature away from light. The cells are washed thrice and then counterstained for DAPI (1:900) for 20 minutes. Samples are mounted on microscope slides with Fluoromount-GTM Mounting Media and sealed with clear nail polish. Slides are allowed to dry for ~15 minutes at room temperature away from light prior to microscope analysis.

Component	Stock Concentration	Final Concentration	Volume
1X PBS	-	-	676.4 μ L
Tris	0.92M	100mM	108.6 μ L
CuSO ₄	100mM	1mM	10 μ L
Alexa Fluor Azide 488	-	10 μ M	5 μ L
Ascorbic acid	0.5M	100mM	200 μ L
Total volume:			1 mL

Table 9. Components of the EdU staining cocktail.

4.2.8 Microscopy & Image Analysis

Microscope analysis is conducted using the Axio Observer Z Microscope (Zeiss). Image analysis is conducted using ImageJ. For the EdU assay, 6 images are taken at random across the sample to determine the average number of EdU positive cells per biological replicate.

4.2.9 Statistics

All statistical analysis was performed in GraphPad Prism 10. The values of all data are shown as the mean $\pm$ standard deviation (SD). The number of biological replicates (n) varied across experiments and is indicated in the methods and/or the figure captions. One-way with Tukey's post hoc test or two-way ANOVA with Sidak's post hoc test were used to determine statistical significance. For data in which less than 3 biological replicates have been collected, mixed-effects analysis is used to evaluate statistical significance. If mixed-effects analysis is employed, it is indicated in the figure caption. Statistically significant data is indicated in the figures using *, **, and *** for $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$ respectively, unless otherwise specified.

4.3 Results

4.3.1.1 Cytotoxicity

As described in [Section 3.3.1](#), the N/P ratio of the CS-PEI/nucleic polyplexes is a critical parameter that must be evaluated. Therefore, to determine which N/P ratio is optimal for transfections in hUASMCs, the alamarBlue™ Cell Viability assay was used to determine which formulations have the least effect on cell viability (**Figure 22**). Surprisingly, there was minimal reduction in cell viability across N/P ratios. As primary cells are considered to be more sensitive to the positively charged polyplexes, as was observed in the previous chapter. Contradictorily, hUASMCs appear to be quite tolerant to the increasing zeta potential, which suggests miR-145 is conferring a protective effect and reducing the toxicity induced by the CS-PEI. Consistent with the results shown in **Figure 8**, there is a sharp decrease in viability 48hrs after transfection, which is regained after 72hrs. Of the N/P ratios, this effect was only significant with the N/P ratio of 10. Likewise, viability with N/P 10 is also significantly reduced after 72 hrs relative to the untreated control. Taken into context with results of **Figure 9**, it is likely that the transfection efficiency of miR-145 is insufficient to ensure an adequate quantity of miR-145 is internalized and provide a beneficial effect and counteract the cytotoxic effects of CS-PEI. Similar to the results in Figure 9, an N/P ratio of 40 results in the lowest viability.

Interestingly, the addition of the miR-145 mimic alone is able to reduce hUASMC proliferation, suggesting the miR-145 mimic alone may be internalized. Overall, while all N/P ratios appear to reduce viability, there is no clear formulation that stands out as optimal. As such, it is predicted that an N/P ratio of 20 would similarly provide a balance between cytotoxicity and transfection efficiency as was observed with COS-7 and was employed for further analysis.

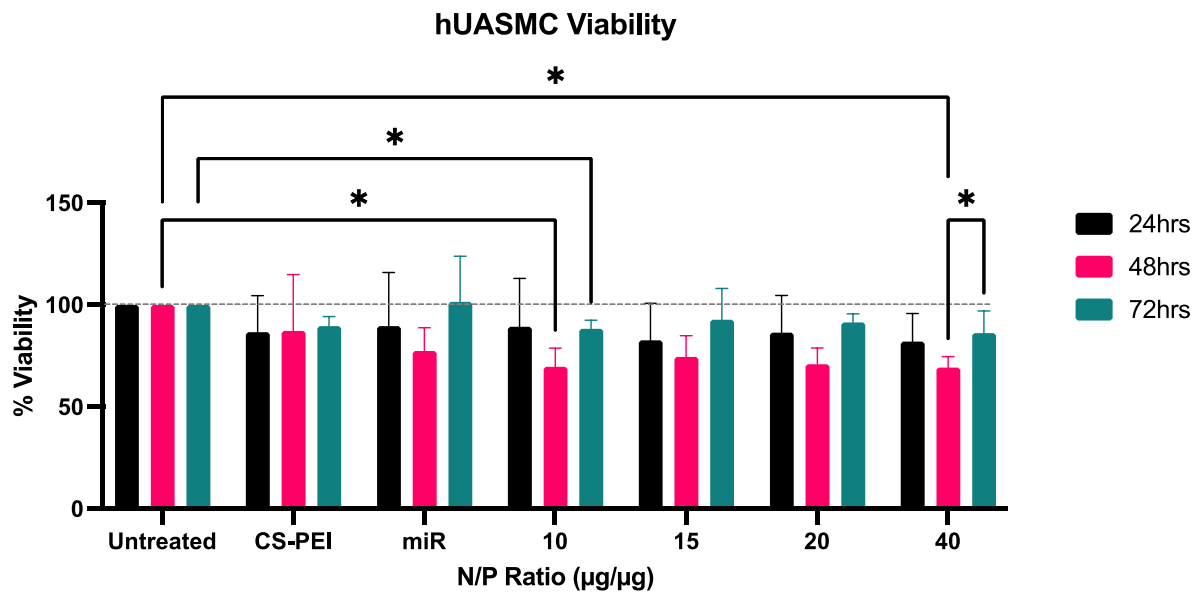


Figure 22. The effects of the N/P ratio on hUASMC viability over 72hrs. The grey dashed line indicates the untreated sample to which all values were normalized. Statistically significant data is indicated in the figures using *, **, and *** for $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$ respectively. Error bars represent the SEM. $n = 3$.

4.3.1.2 Confirmation of miR-145 Transfection

To further evaluate the CS-PEI/miR-145 polyplexes with the N/P ratio of 20, hUASMCs and hUVECs were transfected with Cy3-labelled miR-145 mimics (Cy3-miR-145), shown in **Figure 23** and **Figure 24** respectively. The positive control, Lipofectamine 3000, is shown in **Figure 23D** and **Figure 24D**, and provides insight into the internalization and uptake of the CS-PEI/Cy3-miR-145 polyplexes 24 hours post-transfection. Figure 24D shows limited uptake in hUVECS, yet Figure 23D shows clear internalization of the CS-PEI/Cy3-miR-145 polyplexes. As evidenced by Figure 23E and 24E, both hUASMCs and hUVECs, respectively, were capable of internalizing the CS-PEI/Cy3-miR-145 polyplexes. In fact, the CS-PEI/Cy3-miR transfection efficacy appears to be much higher in hUVECs

compared to the positive control. Here, one can see pockets of fluorescence located tightly around the nucleus, yet the not colocalized with the nucleus. The cytotoxicity of Lipofectamine 3000 in hUVECs is evidenced by changes in cell morphology, as shown in Figure 24C and 24D. Similar effects are observed with free CS-PEI as shown in Figure 24B. It is likely that miR-145 is capable of inducing a protective effect in hUVECs as well as hUASMCs, and the dose delivered by Lipofectamine 3000 is insufficient to mitigate the toxicity. This would explain why the morphology of CS-PEI/miR-145 transfected hUVECs appears the most similar to the untreated negative control. The Cy3 fluorescence appears to be largely concentrated the cytoplasm in the hUASMCs. A higher magnification of the Cy3-miR-145 transfections is shown in **Figure 25** and **Figure 26** providing further insight into the potential intracellular destinations of the Cy3-miR-145.

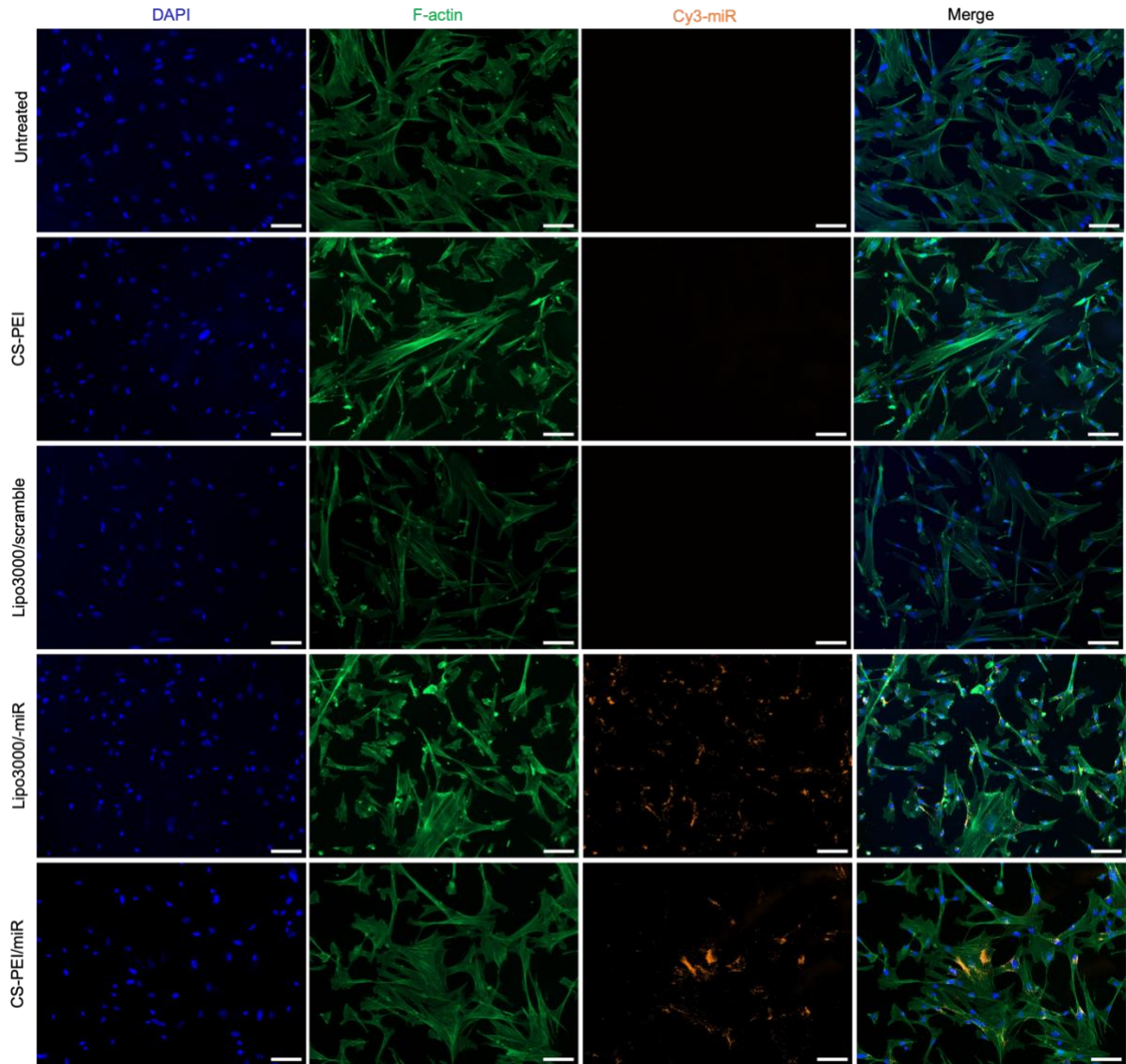


Figure 23. Confirmation of Cy3-miR-145 uptake in hUASMCs on glass coverslips 24-hours post transfection. The first panel shows the nuclei, counterstained with DAPI. The second panel shows the F-actin cytoskeleton, stained with Alexa Fluor® Phalloidin 488. The third panel shows the Cy3-miR-145 and the third panel shows the first three panels merged. Scale bar = 100µm. n = 3

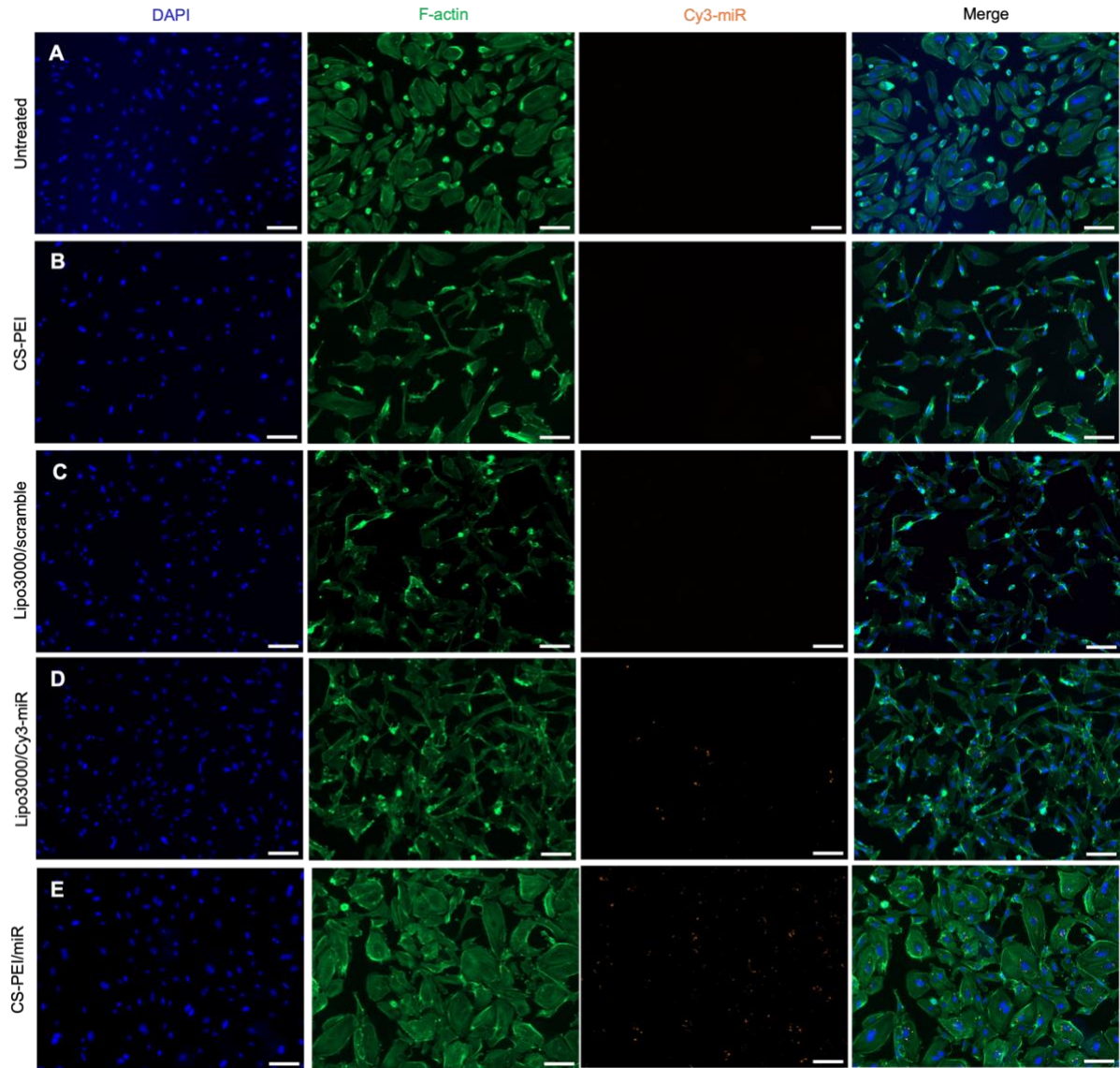


Figure 24. Confirmation of Cy3-miR-145 uptake in hUVECs on glass coverslips 24 hours post-transfection. The first panel shows the nuclei, counterstained with DAPI. The second panel shows the F-actin cytoskeleton, stained with Alexa Fluor® Phalloidin 488. The third panel shows the Cy3-miR-145 and the third panel shows the first three panels merged. Scale bar = 100μm. n = 2

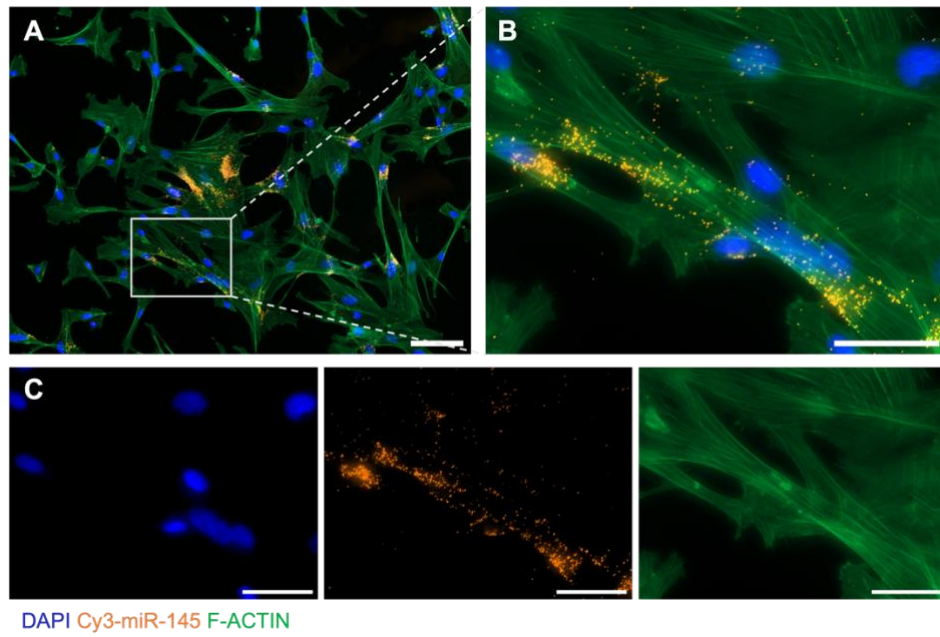


Figure 25. Confirmation of Cy3-miR-145 uptake in hUASMCs. Scale bar = 100 μ m in (A). Scale bar = 40 μ m in (B) and (C).

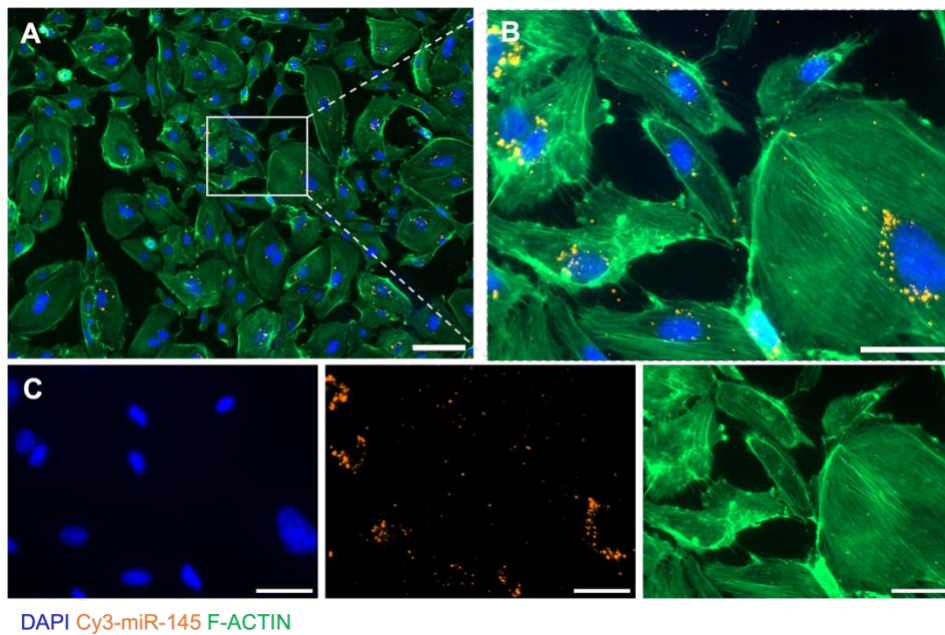


Figure 26. Confirmation of Cy3-miR-145 uptake in hUVECs. Scale bar = 100 μ m in (A). Scale bar = 40 μ m in (B) and (C).

4.3.2 The Effects of CS-PEI/miR-145 NPs on HUASMC Proliferation: Dose-Response

Once the N/P ratio was decided, it was critical to determine the minimal dose of the miR-145 mimic that was required to modulate hUASMC phenotype without affecting cell viability. Few studies have systematically evaluated the optimal dose for miRNA gain-of-function and overexpression studies. Thus, it was imperative that a dose-response relationship be evaluated. As such, two assays were performed in succession to gain a holistic understanding of the effect of the respective dose on hUASMC proliferation: the alamarBlue Cell Viability assay was performed followed by the LIVE/DEAD Viability/Cytotoxicity Assay. The active agent of the former is resazurin, which is a fluorometric redox indicator that is reduced to resorufin in the presence of a reducing environment, such as that of metabolically active cells. miR-145 is predicted to reduce hUASMC proliferation and metabolic activity, thus, a decrease in resazurin would suggest heightened miR-145 activity. However, the alamarBlue Cell Viability Reagent does not allow one to discern between a genuine effect on cellular metabolism and increased cell death. Thus, the LIVE LIVE/DEAD Viability/Cytotoxicity Assay was used to confirm if the observed responses are due to activity of miR-145 or increased cytotoxicity. Six miR-145 doses were evaluated: 50nM, 1 μ M, 10 μ M, 20 μ M, 40 μ M, and 80 μ M. Importantly, measurements were taken 48hrs post-transfection. This time point was selected based on the results of the previous section, which showed that the greatest dip in cell metabolism was found 48 hours post-transfection.

4.3.2.1 alamarBlue Cell Viability Assay

The results of the alamarBlue Cell Viability assay are shown in **Figure 27** and demonstrate that doses of 50nM, 40 μ M, and 80 μ M significantly reduce hUASMC proliferation. While the effect is not significant, doses of 1 μ M and 10 μ M also reduce proliferation relative to the control. The deleterious effect of a high dose (15 μ g) of free CS-PEI is also shown in Figure 27.

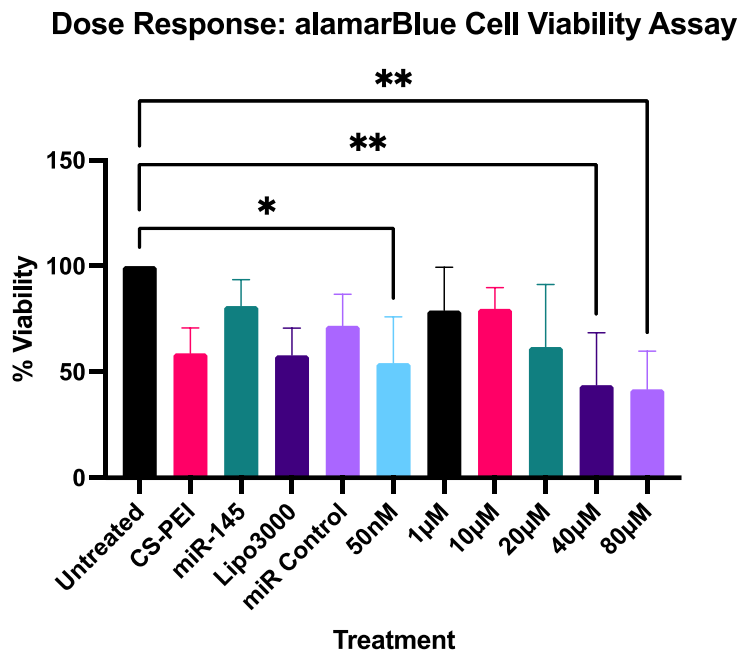


Figure 27. The effects of varying miR-145 doses on hUASMC viability, as determined by the alamarBlue™ Cell Viability reagent. Statistically significant data is indicated in the figures using *, **, and *** for $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$ respectively. Error bars represent the SEM. $n = 3$.

4.3.2.2 LIVE/DEAD Viability Assay

As described, to validate the results of the alamarBlue Cell Viability assay, the LIVE/DEAD Viability/Cytotoxicity Assay was performed. Representative figures for each dose are indicated in **Figure 27**. Clearly, doses of 40µM and 80µM are toxic to the hUASMCs. This is likely due to the high CS-PEI concentration required to adjust to the high nucleic acid masses found at these doses. This suggestion is supported by **Figure 27C**, which shows the effects of CS-PEI on cell viability and presents a similar morphology to what is observed on **Figure 27J** and **27K**. The discrepancies between Figure 26 and Figure 27 are likely owed to the metabolism of the hUASMCs at the edge of the coverslip. These cells may contribute significantly to the reduction of resazurin and thus fluorescence yet are not represented on the coverslip itself. As a dose of 1µM appeared to sufficiently lower cellular metabolism without increasing cell death (**Figure 27G**), it was selected for use in further study. The results of the microplate reader are not shown due to extreme variability. There is a requirement to optimize the volume of LIVE/DEAD Viability/Cytotoxicity staining solution needed for accurate, reproducible

results with the microplate reader. The recommended volume for microscope analysis was utilized, which appears insufficient for microplate readings.

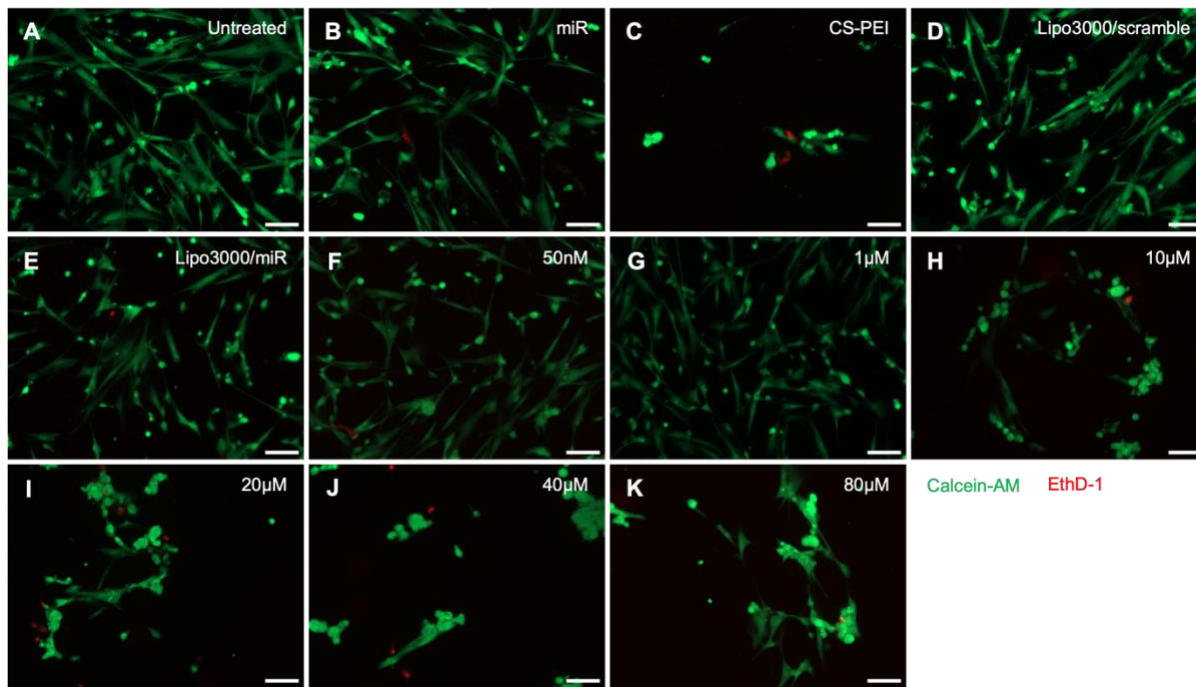


Figure 27. Representative figures from the LIVE/DEAD Viability/Cytotoxicity Assay. Green indicates cells that interact with calcein-AM, or the live cells. The red cells are those that have been stained with EthD-1, or the dead cells. Positive (dead controls are not shown) Scale bar = 100 μ m. n = 3.

4.3.3 CS-PEI Mediated Overexpression of miR-145 in Vascular Cells

With a dose of 1 μ M at an N/P ratio of 20, CS-PEI-mediated miR-145 overexpression was evaluated. As shown in **Figure 28A**, the expression of miR-145 is increased approximately 6-fold in the presence of CS-PEI/miR-145 polyplexes after 24 hours. Surprisingly, the transfection efficiency of CS-PEI/miR-145 polyplexes is nearly 4 times higher than the positive control (Lipofectamine 3000). The expression of miR-145 in the case of both CS-PEI and Lipofectamine 3000 transfection drops after 72hrs, which is consistent with the known transient nature of miRNA. However, while not significant, it is important to note that expression of miR-145 appears to be maintained to a greater extent after 72hrs after CS-PEI mediated transfection, relative to the positive control. It is important to note that expression is normalized to the untreated control on PDMS.

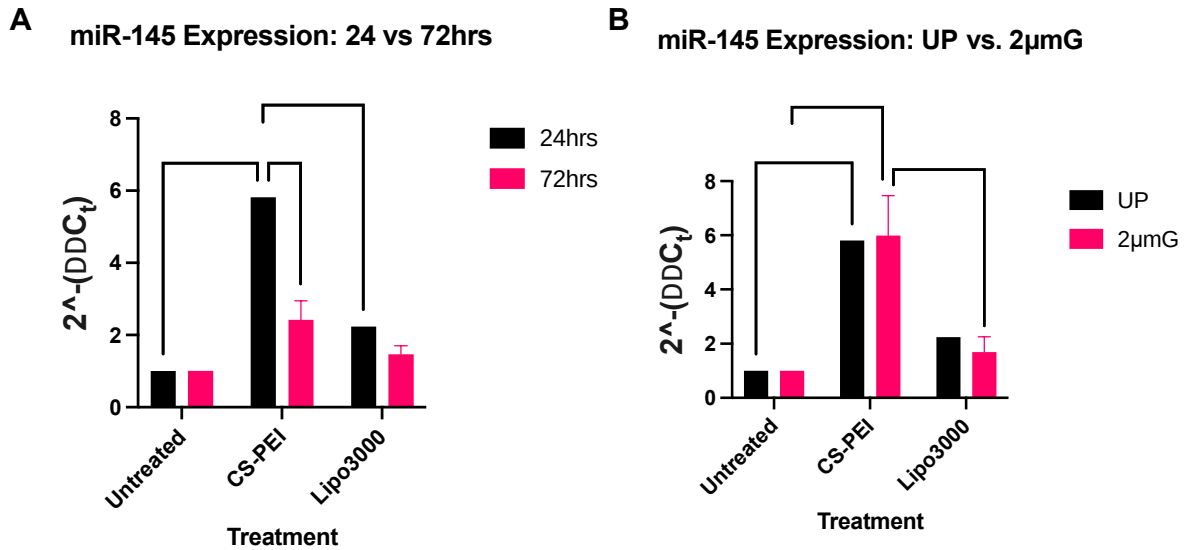


Figure 28. Transfection-mediated overexpression of miR-145 on PDMS samples. (A) The expression of miR-145 after 24hrs and 72hrs on unpatterned PDMS substrate. (B) The expression of miR-145 after 24hrs on unpatterned (UP) and 2µm microgratings (2µmG) on PDMS substrate. Mixed-effects analysis is used to evaluate statistical significance. RNU44 is the endogenous control. Statistically significant data is indicated in the figures using *, **, and *** for $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$ respectively. Error bars represent the SEM For miR-145 expression on UP PDMS after 24hrs, $n=1$. For miR-145 expression on 2µmG, $n = 2$. For miR-145 expression after 72hrs, $n = 2$.

The expression of miR-145 on 2µm microgratings on a PDMS substrate was also explored to evaluate if the topography modulates transfection efficiency (**Figure 28B**). As discussed in Section 4.1, the topography for analysis in this chapter is 2µm grooves or gratings. Based on the results of [Section 3.3.3](#), and [Section 3.3.5](#), it was hypothesized that the 2µm microgratings will enhance CS-PEI/miR-145 transfection. However, as shown in Figure 28B, there appears to be little difference between the unpatterned and patterned samples. Similar to the unpatterned case, the CS-PEI/miR-145 transfection results in significantly higher miR-145 expression (~6 times higher) compared to the untreated hUASMCs. The minimal difference between the unpatterned and patterned samples is likely due to the overall high transfection efficiency, as was observed in the flow cytometric data in Section 3.3.5.

4.3.4 The Effects of CS-PEI/miR-145 on HUASMC Gene Expression

To determine the efficacy of CS-PEI/miR-145 polyplexes, the expression of contractile markers (*ACTA2*, *CNN1*, *TAGLN*) as well as a target of miR-145 (*KLF5*) was evaluated 72 hours after transfection (**Figure 29**). *MYH11* was also evaluated yet was undetectable across all samples. RNA was collected from hUASMCs on PDMS substrates; **Figure 29** shows gene expression on unpatterned PDMS relative to the untreated control. The overexpression of miR-145 via CS-PEI or Lipofectamine 3000 resulted in higher expression of the contractile genes *ACTA2*, *CNN1*, and *TAGLN*. The effect is most pronounced on *CNN1*, with the CS-PEI/miR-145 polyplexes resulting in the highest expression of *CNN1* (although not statistically significant). The expression of *KLF5* is unexpected and contradictory. *KLF5* is a known target of miR-145, thus it is surprising that its expression is significantly higher in the presence of CS-PEI/miR-145 polyplexes and Lipofectamine 3000/miR-145 lipoplexes. Intriguingly, the expression of *KLF5* is significantly higher after Lipofectamine 3000 transfection compared to the CS-PEI/miR-145 polyplexes. This may be due to the difference in miR-145 overexpression achieved by the respective transfection reagents.

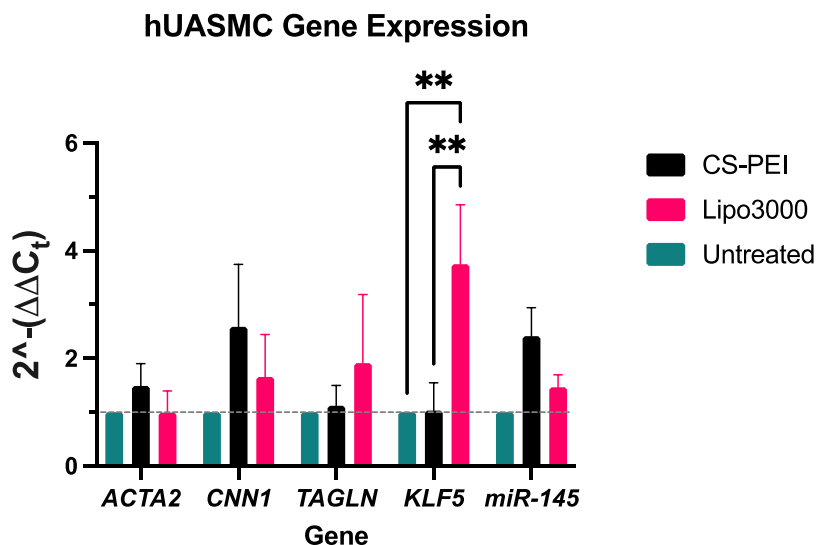


Figure 29. The expression of hUASMC contractile genes, miR-145 target, and miR-145 on unpatterned PDMS with and without transfection. Total RNA is collected 72 hours after transfection. The grey dashed line represents the untreated control to which sample expression is normalized. HPRT is the endogenous control. Statistically significant data is indicated in the figures using *, **, and *** for $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$ respectively. Error bars represent the SEM. $n = 3$.

4.3.5 The Effects of CS-PEI/miR-145 on HUASMC Behavior

To evaluate the effects of CS-PEI/miR-145 on hUASMC behaviour in combination with topography, an EdU assay was performed on unpatterned and 2µm micrograting PDMS substrates. The results of the EdU assay are shown in **Figure 30**. The representative figures for each substrate condition are shown in **Figure 31** and **Figure 32**.

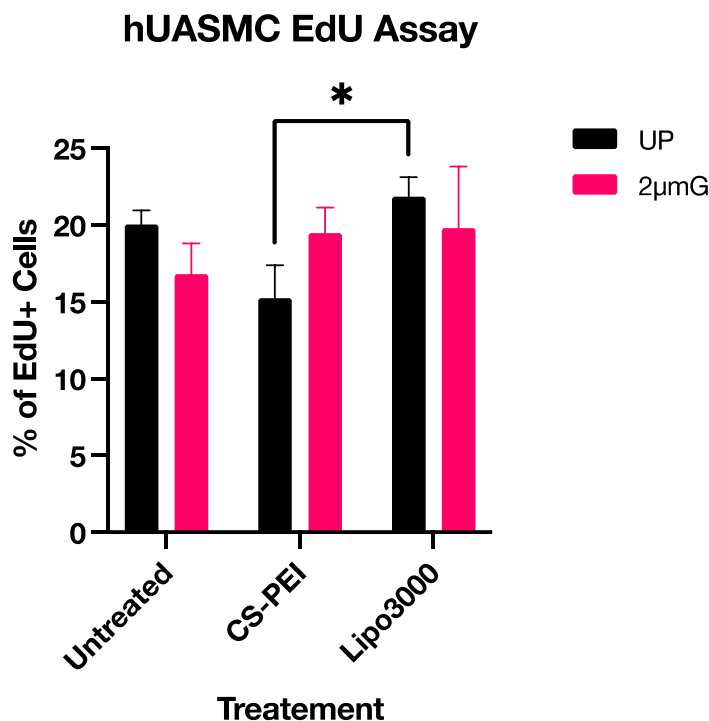


Figure 30. The percentage of EdU positive (EdU+) cells on unpatterned or patterned substrates after transfection with CS-PEI/miR-145 polyplexes or Lipofectamine 3000. Statistically significant data is indicated in the figures using *, **, and *** for $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.0001$ respectively. Error bars represent the SEM. $n = 2$.

As shown in Figure 30, the 2µm gratings reduce the percentage of EdU+ cells compared to the untreated control. However, when treated with CS-PEI/miR-145 polyplexes, the percentage of EdU+ cells increase on the 2µm gratings. Unexpectedly, this effect is greater in the presence of Lipofectamine 3000/miR-145 lipoplexes. Treatment with CS-PEI/miR-145 reduces the number of EdU+ cells compared to both the untreated and positive controls on the unpatterned substrate, yet this effect is only significant in the case of the Lipofectamine 3000 transfection.

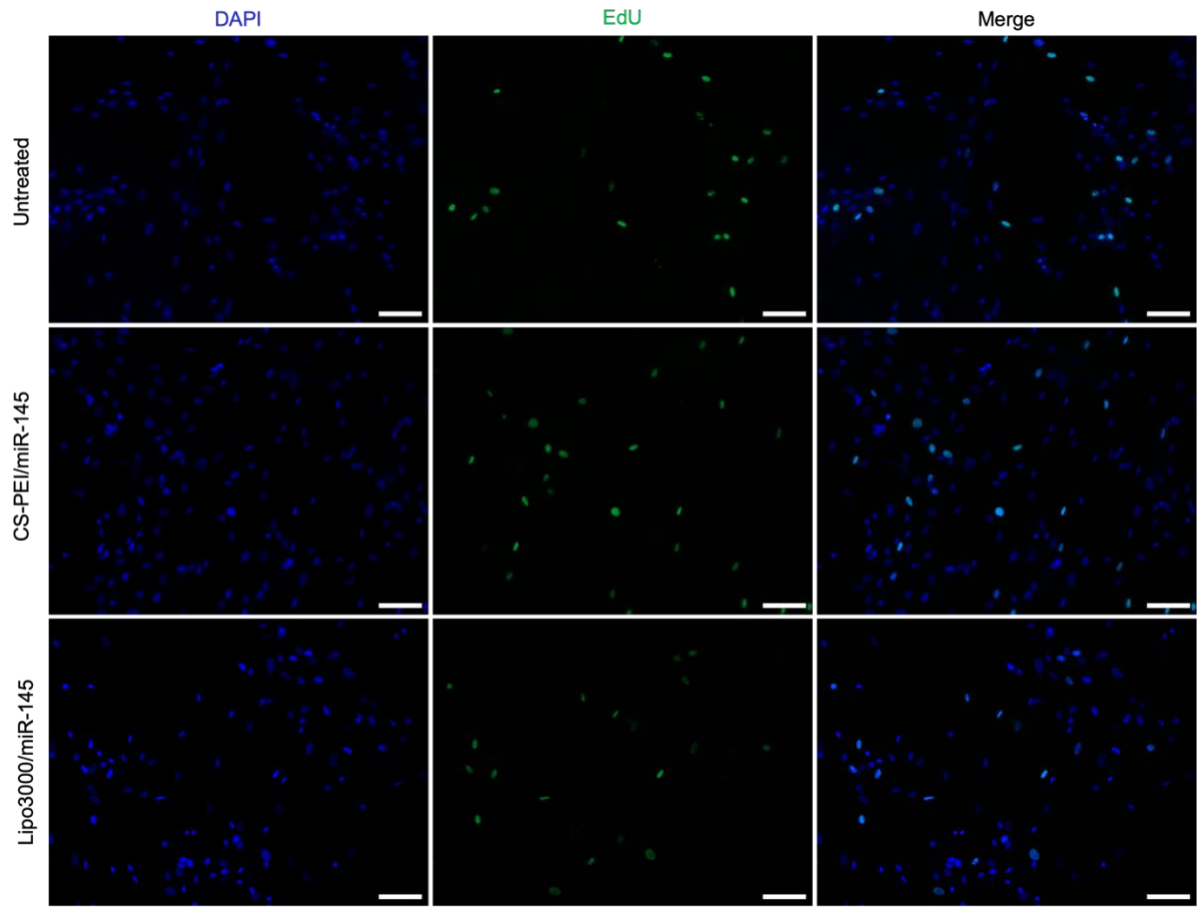


Figure 31. Representative figures for the number of EdU positive (EdU+) cells on unpatterned PDMS. The first panel shows the nuclei, counterstained with DAPI (blue) and the second panel shows the cells stained with EdU (green). The last panel shows the two panels merged. Scale bar = 100 μ m. n = 2.

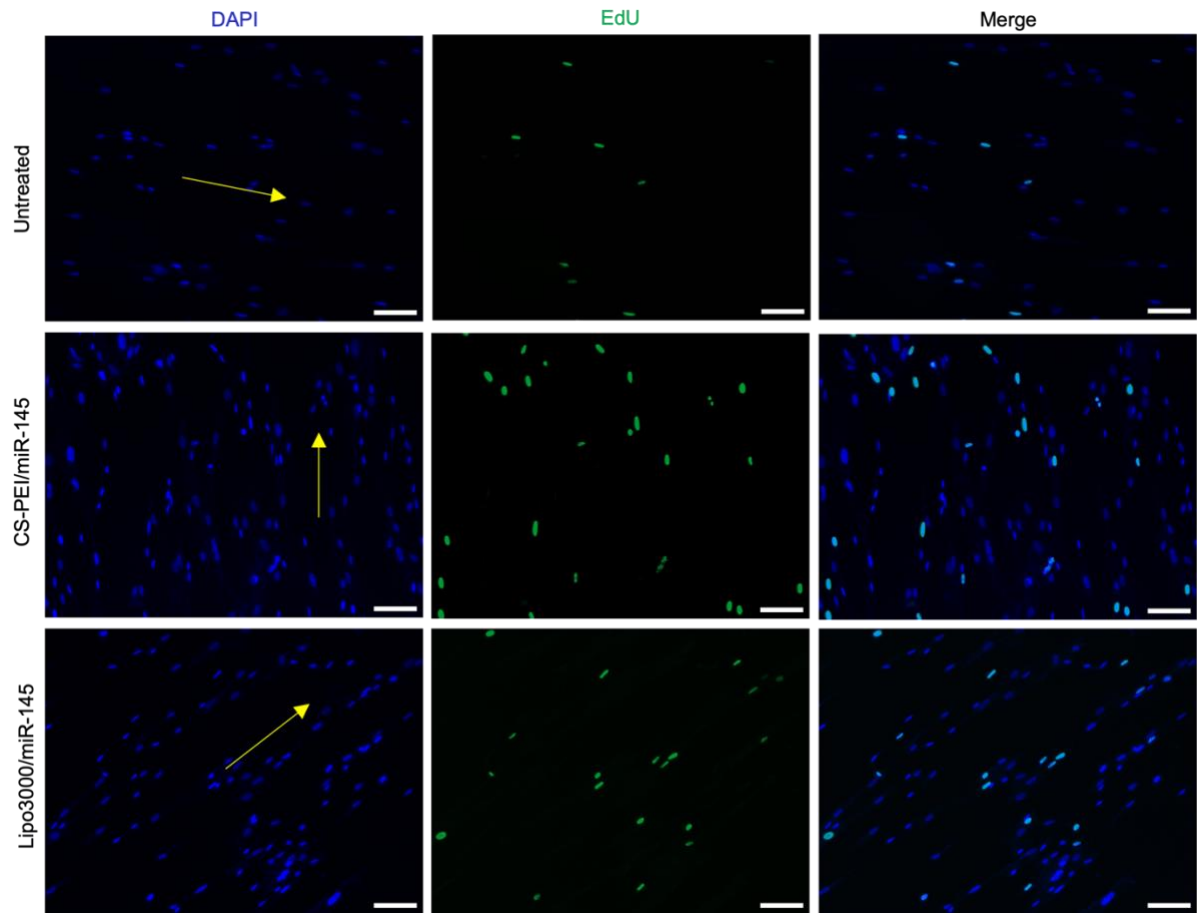


Figure 32. Representative figures for the number of EdU positive (EdU+) cells on 2 μ m micrograting PDMS. The first panel shows the nuclei, counterstained with DAPI (blue) and the second panel shows the cells stained with EdU (green). The last panel shows the two panels merged. The yellow arrow in the first panel indicates the direction of the gratings. Scale bar = 100 μ m. n = 2.

4.4 Discussion

MiRNAs exert profound influence on the transcriptional networks that govern tissue development and regeneration. Recent efforts have strived to integrate miRNA-based therapeutics and nanomedicines traditional tissue engineering approaches. Tissue engineering is contingent upon harnessing endogenous differentiation and transdifferentiation mechanisms. The combination of miRNAs with a 3D functionalized biomaterial could provide a biomimetic microenvironment that supports endogenous regeneration processes. MiR-145 has arisen as a prime candidate for vascular tissue engineering applications due to its central role in the differentiation and dedifferentiation of

VSMCS. Numerous efforts have sought to develop nonviral gene delivery platforms for miR-145 delivery from a sSDVG to reduce IH and improve long-term graft patency. In line with these works, this chapter examines the CS-PEI copolymer as a nonviral gene delivery platform for miR-145 in hUVECs and hUASMCs. Furthermore, this chapter investigates the effects of CS-PEI/miR-145 transfection on hUASMCs in combination with biointerface topography.

To address the central aim of this chapter, it was first necessary to determine the optimal N/P ratio and dose for transfections in hUASMCs, the target cell type. Similar to what was observed in the Chapter 3, the range of N/P ratios screened for did not reveal a single optimal formulation. As such, juxtaposing the results from Chapter 3 and the cytotoxicity data from Section 4.3.1, an N/P ratio of 20 appeared a strong candidate. The transfection potential of this N/P ratio is evident and is shown to result in significant overexpression of miR-145 with the selected dose. Furthermore, it is clear that this N/P ratio results in significant Cy3-miR internalization in hUVECs with minimal cytotoxicity, a promising result as hUVECs are a notoriously difficult and sensitive cell type to transfect. However, it must be noted that extrapolating CS-PEI performance with miRNA mimics from its efficiency with pDNA is limited. Firstly, the structure of miRNA and pDNA are vastly different, and may yield differing complexation efficiencies. Secondly, their target site in the cell differs significantly, and is influenced by the complexation. Therefore, while the screened for N/P ratio provides effective transfection, it is highly probable that lower N/P ratios may be able to achieve a similar effect, and present lower cytotoxicity when applied in a scaled-up setting when a higher dose is required. It would be of interest to examine the effects of a low N/P ratio such as 10 and a higher dose of miR-145. Dose-response analysis was conducted using similar cell viability assays and put forth a 1 μ M dose as sufficient to reduce cell proliferation whilst limiting cytotoxic effects. Building on from the previous discussion, the limited range of doses evaluated leaves room for alternative doses, specifically between 50nM and 1 μ M that may yield improved results. Jin et al. warn that high doses of miRNA mimics could induce the formation of high molecular weight RNA species and off-target effects [95]. Thus, it is recommended that further doses be evaluated, as well as the dose-dependent effects on miR-145 markers. Nonetheless, the screened for dose and N/P ratio was shown to have minimal effects on cell viability in a 24-well plate and outperformed the commercial reagent, Lipofectamine 3000, in terms of miR-145 expression.

To evaluate the effects of CS-PEI/miR-145 polyplexes on hUASMC phenotype, the expression of four contractile markers and one target of miR-145 was evaluated. Despite the high levels of miR-145 overexpression achieved with the CS-PEI/miR-145 polyplexes, the effects on hUASMC gene

expression appear minimal. Although the contractile markers are upregulated, the extent of upregulation is unexpectedly low. This could be owed to a variety of factors. Firstly, the hUASMCs are cultured on PDMS for gene expression analysis rather than TCPS or a glass coverslip, the most common substrate for *in vitro* analysis. The stiffness of PDMS fabricated with an elastomer to crosslinker ratio of 10:1 has an estimated stiffness of 580 kPa. Contrastingly, TCPS and glass coverslips have estimated Young's moduli of 2.28-3.28 GPa and 60-64 GPa respectively. The stiffness of the VSMC microenvironment (the medial layer of the blood vessel) is estimated to be between 30 and 196 kPa [96], [97]. As such, PDMS presents a softer, more biomimetic substrate for VSMCs. VSMC phenotypic switching is influenced by the substrate stiffness, with softer substrates promoting the contractile phenotype [98], [99]. It is predicted that the expression of the contractile markers on the PDMS substrate is elevated prior the transfection with the CS-PEI/miR-145 polyplexes. In this experimental set up, the gene expression was normalized to the unpatterned, non-transfected PDMS control. A TCPS or glass coverslip control must be introduced to isolate the effects of the substrate stiffness and the CS-PEI/miR-145 polyplexes. In fact, an evaluation of both the transfection efficiency and the expression of the contractile markers on TCPS would be beneficial to make a comparison between the two substrates. Moreover, it is predicted that the effect of CS-PEI/miR-145 polyplexes on hUASMC gene expression will be more pronounced in the presence of PDGF, which is known to stimulate the synthetic phenotype and is often used in *in vitro* assays to evaluate the efficacy of miR-145 mimics. Normalization to a TCPS or glass coverslip control could also provide insight into the effect of microtopography on CS-PEI/miR-145 transfection. Furthermore, this could reveal a potential connection between topographical cues and endogenous miR-145 expression. As found in Chang et al., 2014, microgrooves alone can upregulate miR-145 expression [100]. These modifications to the experimental set up could provide further insight into the effects of CS-PEI/miR-145 on hUASMC transcriptome.

Evaluating protein levels after miR-145 expression could provide a more holistic understanding of the combined effect of CS-PEI/miR-145 and topography on hUASMC phenotype. As discussed in Jin et al., several reports have shown that in *in vivo* gain-of-function or loss-of-function studies result in the little to no effects on mRNA target levels but rather alter protein expression [95]. This notion is echoed in fundamental studies regarding miR-145 function in vascular cells have shown that mRNA levels may not be altered by gain-of-function or loss-of-function experiments, but the protein output of the transcript is modified. For example, in Cordes et al. miR-145 knockout had no effect on *KLF4*

mRNA levels but significantly increased protein expression [23]. In this line of discussion, it is also recommended to evaluate more than one target of miR-145, such as *KLF4* or CamKII- δ , as miRNAs are known to modulate cell behaviour through multiple mRNA targets. Finally, the time point at which protein expression is evaluated is critical. In this work, RNA levels are evaluated 72hrs after transfection. The reasons for this are two-fold. Firstly, as shown in the cytotoxicity analysis, transfected cells generally experience a dip in viability 48hrs post-transfection, which is regained within 72hrs. To circumvent the effects of CS-PEI induced cytotoxicity, RNA levels were evaluated after 72hrs. Secondly, topography-induced alterations in gene expression are not often apparent until 3-4 days of culture. However, as shown in Cheng et al., miR-145 levels and contractile protein expression are time-dependent [22]. As such, the 72hr time point may be unsuitable for evaluating mRNA expression, especially considering the loss of miR-145 overexpression at this time. In summary, evaluating protein and RNA expression at a range of time points could provide clarity regarding the activity of the miR-145 mimic.

The combined effects of miR-145 and topography on hUASMC behaviour were also analyzed in the context of the synthetic phenotype. The EdU assay labels newly synthesized DNA and provides insight into the proportion of cells that are in the S-phase of the cell cycle. Previous reports have shown that nanogrooves or nanogratings can reduce the number of EdU positive VSMCs and significantly reduce proliferation [101], [102]. Here, it was shown that non-transfected hUASMCs exhibit reduced proliferation on 2 μ m gratings compared to the unpatterned sample, reflecting the literature. However, treatment with miR-145 either via CS-PEI or Lipofectamine 3000 appears to increase hUASMC proliferation on the gratings. This requires further investigation, as it is an unexpected consequence of the miR-145 overexpression. A possible explanation for the increase in the number of S-phase cells is that there is crosstalk between miR-145 targets and the intracellular signalling pathways activated by the topographical cues. As outlined in Section 1.3.3 and in Section 1.5.2, both topographical cues and miR-145 mediate actin cytoskeleton reorganization and the cell proliferation through Rho GTPases, and the synergy of the two may serve to increase proliferation in an unexpected manner. It is also possible that increase in proliferation is reflecting the trend observed in the cytotoxicity screening; between 48hrs and 72hrs post-transfection, the cells recuperate from the initial cytotoxicity effects. The hUASMCs may undergo replication to recover from the toxicity, which is aided by beneficial effects of topographical cues in this particular context. Similar to protein expression, it is also critical to evaluate behaviour at numerous time points following CS-PEI/miR-145 expression.

Overall, the results presented in this chapter present novel insight into the combination of biophysical cues and miRNA gene therapy, which remains an unexplored area of research. This chapter presents a step towards the eventual integration of the CS-PEI/miR-145 polyplexes with the PVA-based sSDVG that has been developed in our lab. Future work will evaluate the integration of the positively charged polyplexes onto the graft surface or within the graft layers. It is proposed that the immobilization of the polyplexes could be achieved by their electrostatic interactions with the anionic fucoidan molecules conjugated on the luminal surface of the PVA conduit [91], [92].

Chapter 5

Conclusion & Future Outlook

The central focus of this thesis is to investigate the utility of biointerface topography in priming cells for transfection. This thesis serves a foundational step towards substrate-mediated gene delivery systems with enhanced efficacy. In this work, the use of CS-PEI as a gene carrier for substrate-mediated gene delivery has been explored in two contexts. Firstly, the application of topographical cues to prime cells for transfection was explored. Here, it was shown that a hierarchical topography that consists of a primary topography of 2 μm gratings and a secondary topography of 250 nm gratings resulted in the highest percentage of transfected cells. Further analysis using flow cytometry revealed that the secondary topography, the nanogratings, was the driving force behind enhanced transfection and significantly higher transgene expression. This implicates this topography for further analysis and suggests that it may be useful for both *in vitro* and *in vivo* substrate-mediated gene delivery applications. For example, this topography has been utilized to drive the differentiation of hMSCs towards the neural and myogenic lineage *in vitro* in the absence of biochemical cues. The use of this topography in combination with CS-PEI-mediated delivery of growth factors could be used to augment the differentiation of hMSCs towards a desired phenotype. Furthermore, this thesis explored which aspects of various topographical cues are critical for enhanced transfection efficiency. The second context includes the use of CS-PEI for miR-145 delivery to vascular cells. This chapter emphasized the use of CS-PEI as a gene carrier for miR-145 to VSMCs, the target cell type. Furthermore, it provided novel insight into combination of topographical cues, specifically microgrooves, and miR-145 on hUASMC phenotype. The synergy of topography and miRNAs has, to the best of the author's knowledge, only been explored in two studies. Further investigation will bridge the gap between the *in vitro* platform and its incorporation into the PVA SDVG.

Despite the conclusions of this thesis, several questions remain unanswered. Firstly, the links between endocytic pathways and substrate topography remain elusive. The results of this study suggest crosstalk between topography-induced mechanotransduction and the machinery that is required to drive membrane remodelling during endocytosis, particularly curvature sensing proteins. Foremost, the endocytic pathways promoted by various topographical cues must be determined. This may be achieved by endocytosis inhibitor studies, which are able to pharmacologically or genetically knockdown the endocytic pathways described in Section 1.5 [103]. To provide further mechanistic insight, the key

players in these pathways must be investigated in terms of localization, expression, and dynamics on various topographies in the presence and absence of a gene carrier. The approach utilized in Zhao et al. provides a model experimental setup for an experiment of this nature [81]. Furthermore, while forward transfections are useful for predominantly *in vitro* applications, reverse transfections are the more relevant for substrate-mediated gene delivery applications *in vivo* [1], [4]. As such it is of critical importance to evaluate the immobilization of CS-PEI/nucleic acid polyplexes onto a substrate and how this effects cell attachment, viability, proliferation, and ultimately endocytosis. In this line of discussion, to make firm progress towards the development of a SDVG-mediated gene delivery platform it is also necessary to explore further aspects of VSMC behavior in response to topographical and miR-145 cues. As described in Section 4.4, there are several questions that remain unanswered and uncertainty in the conclusions of Chapter 4 due to crosstalk between different mechanical cues, namely stiffness and topography. Furthermore, the resulting VSMC behaviors and their correlation to sSDVG outcomes were not studied and remain unknown. To address these remaining questions and limitations, the following studies are proposed for to further expand this thesis:

Investigate effects of endocytosis inhibitors on CS-PEI/pEGFP transfection efficiency across topographies: systematic screening of the effects of endocytosis inhibitors that target each of the pathways outlined in Section 1.6. This, alongside membrane trafficking studies, can be utilized to identify which endocytic pathways preferentially interact with various topographical features.

Investigate the effects of topography on the reverse transfection of CS-PEI/pEGFP polyplexes: evaluate the transfection efficiency of the CS-PEI/pEGFP polyplexes when they are immobilized on the surface of the MARC chip. Develop a method to immobilize the nanoparticles on the PDMS surface. Topographies found to upregulate transfection in this experimental set up should be selected for further evaluation with flow cytometry and endocytosis inhibitor studies. A comparison between forward and reverse transfection efficiencies on various topographies could reveal insight into the mechanism of endocytosis.

Investigate contractile protein expression and migration velocity of hUASMCs in response to 2 μ m microgratings and CS-PEI: using Western blots and wound healing assays, determine the expression of both synthetic and contractile proteins as well as the migration velocity of hUASMCs on PDMS or TCPS and PVA.

Evaluate the effects of CS-PEI/miR-145 on endothelial monolayer formation: using immunofluorescent imaging, Western blots, RT-qPCR, and wound healing assays, determine if the CS-PEI/miR-145 polyplexes have any detrimental effects on hUVEC monolayer formation and integrity. Co-culture models could also be of interest to provide insight into how the CS-PEI/miR-145 polyplexes and topography would influence both VSMC and EC behaviour, thereby providing a more clinically relevant model.

Investigate the biodegradation and intracellular fate of the CS-PEI polymer after transfection: understanding the intracellular fate of a polymeric nanoparticle is critical in both evaluating the mechanism of transfection and providing insight into the global effects of the gene carrier. Deleterious effects may arise if the PEI accumulates inside the cell. Therefore, it is important to understand how the degradation of this polymer may be achieved *in vitro* and *in vivo* via endogenous and exogenous mechanisms.

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