Targeted Endothelial Nitric Oxide Synthase Gene Delivery with Lipopolyplexes for the Treatment of Coronary Restenosis

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by

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<tr>
<td>DES</td>
<td>Drug eluting stent</td>
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<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
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<tr>
<td>DOPE</td>
<td>dioleoyl phosphatidylethanolamine</td>
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<tr>
<td>DOTAP</td>
<td>1,2-dioleoyl-3-trimethylammonium-propane</td>
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<td>EC</td>
<td>Endothelial cell</td>
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<tr>
<td>eGFP-N1</td>
<td>Enhanced green fluorescence protein</td>
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<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>HAOEC</td>
<td>Human Aortic Endothelial Cells</td>
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<td>HAOSMC</td>
<td>Human Aortic Smooth Muscle Cells</td>
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<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>LPP</td>
<td>Lipopolyplex</td>
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<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>PBAE</td>
<td>poly(beta-amino ester)</td>
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<tr>
<td>PEG</td>
<td>Poly ethylene glycol</td>
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<tr>
<td>PLGA</td>
<td>poly(D,L-lactide-co-glycolide)</td>
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<tr>
<td>PTCA</td>
<td>Percutaneous transluminal coronary angioplasty</td>
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<tr>
<td>qPCR</td>
<td>Quanatative polymerase chain reaction</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartic acid tri-peptide sequence</td>
</tr>
<tr>
<td>rtPCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
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<td>TEM</td>
<td>Transmission electron microscopy</td>
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ABSTRACT

Current treatments of restenosis employ cytostatic or cytotoxic drugs. Despite having a lower incidence of restenosis after intervention, other problems such as late stent thrombosis arise since these drugs stop all cell proliferation, including endothelial cells, which have been shown to be critical in the healing of the blood vessel over time. Nitric oxide synthases are enzymes responsible for generating nitric oxide, a gas responsible for inhibition of smooth muscle cells and re-endothelialization in addition to a host of other vascular responses. The use of nitric oxide releasing polymers has been investigated with limited success. Alternative approaches using viral vectors or naked plasmid DNA coated onto stents have shown promise but are not viable in the clinic due to the problems of immunogenicity in the case of viral vectors or the unrealistic high doses used on stents coated with naked plasmid DNA.

Non-viral cationic vectors have been used to transfect the vasculature both in-vitro and in a number of different restenosis models in vivo. Preliminary work was conducted in-vitro to develop a gene delivery system that transfected endothelial and smooth muscle cells with no toxicity. A combination of a cationic lipid (DOTAP), a and a cationic polymer (PBAE) was found to transfect the most efficiently with no toxicity to the cells at the doses required to transfect. These lipopolyplexes were then coated onto stainless steel substrates using different types of gelatin and were incubated with smooth muscle cells to assess transfection.

Based on the preliminary formulation development work in-vitro a stent coating was developed and tested for ability to transfect and efficacy in a rabbit model for
restenosis. The stents were coated with lipopolyplexes that contained a plasmid DNA encoding for eNOS and type B gelatin. The studies reveal that the lipopolyplex coated stent can transfect and proves efficacious in the treatment of restenosis in a well established pre-clinical model for restenosis. The data supports the hypothesis that potent non-viral gene vector encoding for endothelial nitric oxide synthase coated onto a stent can inhibit restenosis through inhibition of smooth muscle cell growth and promotion of a healthy endothelium. Thus, a novel therapeutic strategy is presented for the treatment of restenosis that shows promise for clinical success.
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CHAPTER 1: INTRODUCTION

Introduction to Coronary Restenosis

Over 24 million people are diagnosed with some type of heart disease every year and cardiovascular diseases and complications continues to be associated with highest fatality in the United States [1]. Almost 29% of all deaths in this country are somehow linked to cardiovascular diseases. Coronary atherosclerosis is a condition where a plaque made of cholesterol, lipids, calcium, cells and other material traveling in the blood will deposit on the arterial vessel wall. As the plaque size increases, it can become unstable and rupture, this rupture causes a clotting cascade that forms thrombi within the vessel and fully occludes the blood flow. The obstruction in coronary artery leads to myocardial ischemia and infarction, and can be fatal. Removal of the plaque is commonly performed by non-invasive techniques, such as percutaneous transluminal coronary angioplasty (PTCA), atherectomy and stent implantation procedures. All of these procedures can lead to a complication called restenosis, where the vessel lumen size is reduced, due to proliferation of smooth muscle cells or neointimal hyperplasia in the lumen of the artery. The placement of metallic stents has become the treatment of choice as compared to other procedures due to lower percentage of restenosis and the prevention of vessel recoil [2].

As shown in Figure 1, there are three primary layers in a healthy coronary artery. The tunica intima or intimal layer is the innermost layer, which is in contact with the flowing blood and consists primarily of endothelial cells. Adjacent to the intimal layer is the tunica media or medial layer, consisting primarily of smooth muscle cells. This layer is responsible for the vascular tone. The outermost layer is the tunica adventitia or adventitial layer, consisting primarily of collagen. Following vascular injury and removal
Figure 1. Schematic illustration of the processes leading to restenotic lesion development. The figures show healthy blood vessel (A), formation of atherosclerotic plaque within the blood vessel showing fatty material and macrophages encapsulated within a fibrotic tissue (B), insertion of a balloon angioplasty catheter to remove the plaque (C), damage due to stripping of the endothelial cells of the vessel wall after removal of the balloon (D), platelet accumulation and activation as well as rapid growth of smooth muscle cells and fibrous extracellular matrix forming the scaffolding (E), and the late stage restenosis showing neointima protruding into the lumen causing occlusion within the vessel (F).
of endothelial layer from the initial layer, inflammatory cells such as macrophages, T-cells, and a small number of B-cells are recruited at the site of injury [3]. Initiation of the inflammatory reaction and stimulation of smooth muscle cell (SMC) proliferation from the medial layer are responsible for restenosis. Goldberg et al., (7) defined restenosis as a greater than 50% narrowing of the vessel as determined by a follow-up angiogram. This effect is typically classified into two distinct steps: (1) SMC proliferation in the arterial lumen also referred to as neointimal hyperplasia and (2) vessel remodeling [4]. Restenosis caused by balloon angioplasty is distinctly different from angioplasty caused by implantable stents and is attributed to three different factors: the elastic response that occurs after the over-stretching of the vessel, neointimal formation, and chronic remodeling [5]. It has been shown that restenosis caused by PTCA is molecularly different than restenosis caused by stents, even though they both cause a decrease in the lumen with smooth muscle cell in-growth [6].

The cascade of events following denudation of the luminal endothelial layer following PTCA or stent placement starts with activation of various molecular markers [6]. Inflammatory reactions are initiated as soon as 15 minutes after injury to the vessel where first white blood cells invade the site of injury, followed by deposition of neutrophils, monocytes, platelets and fibrinogen [3]. As time progresses macrophages are recruited within the neointima. The recruitment of fibrinogen receptors and neutrophils lead to an up-regulation of adhesion receptors on the cell surface [7]. After a week of stent placement, cytokines including monocyte chemo-attractant protein-1 (MCP-1), interleukin-6 (IL-6) and interleukin-8 (IL-8) are recruited depending on the type of injury causing an inflammatory response. A leukocyte integrin class of adhesion
molecules Mac-1 interacts with activated platelet receptors (P-selectin glycoprotein) leading to an accumulation of platelets on the intimal wall [4]. Blockade of the MCP-1 receptor was able to reduce neointimal hyperplasia in stented vessels, while having no effect on balloon injured vessels [8]. Targeting leukocyte β2-integrin β-subunit CD18 was shown to reduce neointimal hyperplasia in balloon injured models [8]. Balloon injured models have shown that there is no macrophage infiltration in the neointima, but there is significant neutrophil infiltration [9].

In addition to an inflammatory response, growth factors are also secreted to initiate SMC proliferation and migration into the lumen. There are a number of growth factors and cytokines that play a role in restenosis. Fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) A and B, insulin like growth factors (IGF), and transforming growth factor β (TGF-β) are produced by SMC’s and are responsible for SMC proliferation [6]. Additionally PDGF A and B, released from SMC’s, platelets, and endothelial cells, are responsible for SMC migration into the arterial lumen. Vascular endothelial growth factor (VEGF) derived from endothelial cells is responsible for endothelialization and angiogenesis. The cytokines involved in restenosis are MCP-1, IL-8, and IL-6 [6]. These cytokines, which are present during early stages, are responsible for monocyte and neutrophil recruitment and are derived from a number of cells such as macrophages, SMCs, endothelial cells, fibroblasts, T-cells and polymorphonuclear leukocytes. The cytokine signals are key indicators of an inflammatory response at the site of damage. Another molecular signal associated with inflammation and restenosis is the β-2 integrin molecule Mac-1 (CD11b/CD18), which is responsible for monocyte recruitment [5]. In addition to the inflammatory and growth
factors mentioned, other proteins and enzymes are effected by restenosis, including p27, p70, p16, TGF-β, and matrix metalloproteinases [4].

**Therapeutic Strategies for Restenosis**

There are a variety of ways to treat restenosis. Mechanical stents that keep the vessel open have been coated with drugs to prevent the cells from growing into the vessel lumen [10]. A variety of drug classes have been used to treat restenosis, including anti-cancer and anti-inflammatory agents [4]. Modulation of genes with plasmid DNA and RNA interference have also been tried in preclinical models to create an increase or decrease in the local concentrations of specific signaling molecules used to inhibit the growth of certain cells, while promoting the growth of others [11]. The key point for each of these therapies is to inhibit the growth of SMC, while promoting re-endothelialization of the vessel, such as to restore the injured vessel back into a healthy one.

**Bare Metal and Biodegradable Stents:** Originally developed and used by Dr. Charles Stent, a dentist, in the year 1856 for facial reconstruction, this device has radically changed non-invasive cardiac intervention since the early 1990’s [12]. Stent usage has continuously grown and is now a widely accepted treatment for occluded arteries. The use of balloon angioplasty to remove a clot is associated with a greater than 70% restenosis rate, with greater percent restenosis in larger vessels. The advent of the bare metal stent reduced the incidence of restenosis to 20-53% depending on the type of stent used. An important design feature of stents is the thickness of the stent strut, with the thicker struts causing greater incidence of restenosis [4].
Alternatives to bare stent designs have been tested, including the use of biodegradable polymer-based stents to inhibit restenosis after initial implantation and then having them degrade in situ after the vessel has stabilized. Poly (L-lactic acid)-based biodegradable stent has been used in humans with favorable results, showing complete degradation after 6 months. The researchers did not evaluate the length of time required for the stent to degrade or if the acidic bi-products of the degraded stent had unfavorable interactions with the vessel wall. A limitation of polymer-based stents is that they may not be mechanically as strong as the metal-based stent, especially as the polymer begins to degrade [13]. An alternative approach to using polymeric materials is the use of a magnesium-based stent that will erode in the body leaving no metal behind after a set time [14]. A follow up clinical study showed the magnesium stent was absorbed within 3 weeks of implantation. More studies are necessary to show whether the residence time of the device is appropriate as vessel remodeling is likely to occur for at least a month after clearing of the vessel.

**Drug-Eluting Stents (DES):** The introduction of DES has brought the use of drugs and genes to treat restenosis to the forefront of cardiovascular research. There have been a variety of different drug classes that have been evaluated to modulate SMC growth and proliferation. Antineoplastic agents, such as cytarabine, doxorubicin, and vincristine have been tested to determine their effects on SMC growth with some success [4, 15]. Voisard et al., [16] have also tested doxorubicin in addition to four other drugs - dalteparin sodium, cyclosporine A, colchicines, and etoposide - on whether the SMC proliferation was inhibited. Each of these agents showed some degree of SMC growth inhibition. DES further reduced the percentage of restenosis to less than 10% in initial
clinical trials. These results have led to the current use of DES in greater than 85% of all coronary interventions. The use of bare metal stents has fallen sharply since the introduction of DES, though that seems to be changing with the current studies showing that DES can lead to possible life threatening in-stent thrombosis conditions. In-stent thrombosis, or the formation of blood clots, occurs more frequently in DES as compared to bare metal stents since it is shown to occur prior to cell healing. In general, DES prolong the healing process and, as such, are more prone to thrombotic events [17, 18]. Despite the high usage of DES in the interventional cardiology field, there is still substantial opportunity for improvement. The polymer coating on stent surfaces is thought to invoke an inflammatory response at the site of injury creating a potential for restenosis [13, 19].

Currently there four approved DES in the market: the Cypher® stent, Taxus® stent, XIENCE V stent, and the Endeavor® stent. Each stent has a different drug and coating on it. The Cypher® stent has the drug sirolimus, which is also known as rapamycin, coated on the surface with poly(ethylene-co-vinyl acetate) and poly(n-butyl methacrylate) blend of non-erodable and non-thrombogenic polymers [20]. Sirolimus is a cytostatic agent, which arrests the cell in the G1 phase of cell division [21]. The Taxus® stent has the anticancer drug, paclitaxel, embedded in a poly(styrene-b-isobutylene-b-styrene) triblock polymer [22]. Similar to sirolimus, paclitaxel is a cytostatic agent, arresting the cell in the G1/M phase [22]. The XIENCE V stent has the cytostatic drug everolimus coated on the stent with a poly n-butyl methacrylate (PBMA) that acts as an adhesion layer for the second coating of a vinylidene fluoride and hexafluoropropylene monomers (PVDF-HFP) that contains the drug [23]. The Endeavor
stent has the drug zotarolamus coated on the surface with a phosphorylcholine polymer coating [24]. For reviews on the clinical performance of the Cypher® and Taxus® stents, please refer to the following publications [20, 25].

The other approaches to DES design include removal of the polymer-drug reservoirs and replacing them with non-polymeric nano-porous reservoirs. The use of carbon-coated stents has been shown to have comparable results to current metal-based DES. However, additional studies will have to be performed under the United States Food and Drug Administration guidelines to confirm these preliminary findings [26]. Nanoporous aluminum oxide-coated stents have also been loaded with potent immunosuppressant tacrolimus and shown to inhibit neointimal growth. Subsequent studies seem to contradict these findings, concluding that particle debris from these stents could negate any positive inhibition of restenosis [27, 28]. Additional issues such as thrombosis and inflammatory response to these implantable devices need to be well understood before they are considered clinically viable alternatives.

**Nanoparticulate Drug Delivery Systems:** Nanoparticulate delivery systems have been used to treat a variety of systemic disease, including cancer [29]. In restenosis treatment, there is a need for an alternative strategy to stents, since there are limitations to current therapies, such as in-stent restenosis including the development for DES-related thrombosis and the high rate of restenosis with the use of bare metal stents and balloon angioplasty. Both conventional drug- and gene-based medicine have been used together with advances in nanoparticle delivery to achieve desirable therapeutic results. A nanoparticle delivery system is well suited for the treatment of restenosis since local or targeted delivery can be achieved, lowering systemic toxicity, while reaching specific cell
types in sufficient concentrations for the necessary period of time, especially after intracellular uptake and localization. Biocompatible lipids and polymers used in fabrication of nano-carriers do not create an inflammatory response. The high shear pressure in the arterial blood supply leads to a very short residence time for the therapeutic agent at the target cells in the arterial wall. The use of nanoparticles allows for the rapid incorporation of the drug and gene into the cell, thus reducing the shear effects of the arterial pressure. Studies using fluorescently-labeled nanoparticles have shown a size dependency in arterial wall gene transfection administered \textit{in vivo} with a SCIMED\textsuperscript{®} REMEDY porous balloon catheter [30]. Three particle sizes were tested and the data showed that the smallest size particles (~110 nm in diameter) had the greatest fluorescence intensity within the cell. Larger particles showed very little fluorescence due to short residence time on the arterial wall surface. Other studies have shown an increased in inflammatory response with particles of 5-10 μm in diameter and no therapeutic response, probably due to uptake by the macrophages and other immune cells rather than SMC or endothelial cells [31]. Cells can incorporate particles varying from 50 nm – 300 nm in diameter based upon a variety of different internalization pathways including non-specific or receptor-mediated endocytosis [32-36]. Additionally, nanoparticles larger than 300 nm in diameter were found to accumulate in the liver, spleen and lung, rendering them unavailable for arterial delivery upon systemic administration [37]. Other known factors for cellular uptake of nanoparticles include volume, concentration, infusion pressure, and the type of infusion balloon. Increased delivery pressure and large volumes can cause an increase in the intimal thickening, while increased particle concentration leads to effective delivery [30, 38]. In addition, the
use of cell specific surface modification strategies, such as attachment of arginine-lysine-aspartic acid (RGD) tri-peptide sequence, can increase the residence time of the nanoparticles at the desired site by selective binding with cell surface integrins [39].

Significant work has been ongoing and completed in the field of polymer-based nanoparticle drug delivery for the treatment of restenosis. The bisphosphonate agent, alendronate, was encapsulated in a poly(D,L-lactide-co-glycolide) (PLGA) based nanoparticle system with an average size of 223 nm [40]. The presence of calcium ions was found to be critical for the successful entrapment of the hydrophilic drug compound in PLGA matrices. Alendronate in this formulation was delivered subcutaneously and by intravenous administration. The results showed reduction in neointimal growth in a balloon injured rabbit model using both routes of administration. Interestingly, unlike the liposomal formulation, the subcutaneous PLGA nanoparticle formulation of alendronate showed a greater reduction in neointimal growth as compared to the intravenous formulation in a rabbit model.

Paclitaxel has been used in a number of nanoparticle formulations for restenosis therapy. An alternative to paclitaxel-containing stent coating was developed by Bhargava et al.,[26] A cobalt-chromium stent loaded with carbon-carbon nanoparticles was used in a stented injury swine model. In this study, three doses of paclitaxel in nanoparticle formulation showed inhibition of neointimal growth with less fibrosis and inflammation when compared to the Cordis Cypher® stent. A comparison to the Taxus® DES would have been more relevant to see if the nanoparticle based therapy is better than the current therapy on the market. Albumin-based nanoparticles containing paclitaxel have also been used to prevent in-stent restenosis [41]. The paclitaxel concentration was lower than
typically used for chemotherapy in a denuded stented rabbit model. After 28 days, neointimal growth was inhibited with a single dose locally administered to the iliac bifurcation, while a second systemically administered dose was needed to see the effects prolonged out to 90 days. Doxorubicin, an anthracycline anticancer agent, and paclitaxel were used in a tissue factor targeted lipid/perflorocarbon nanoemulsion to target vascular smooth muscle cell proliferation *in vitro* using porcine SMC [42]. Targeted nanoemulsion had greater efficacy in preventing smooth muscle cell growth in these studies. The formulation containing doxorubicin and paclitaxel had a greater effect than single drug probably due to synergistic pro-apoptotic activity of these chemotherapeutic agents.

Doxorubicin has also been used in a polymeric micelle consisting of NK911, a self assembling block co-polymer, to treat restenosis in a balloon-injured rat carotid artery model. This study showed that doxorubicin encapsulated within NK911 permeated the arterial wall and prevented neointimal growth. The authors claim that the formulation was able to target the damaged endothelium due to a local enhanced permeability and retention effect in the arterial wall. This effect has not been confirmed in any other reports. The small size (40 nm) of the delivery system could lead to an increased uptake in a damaged arterial wall after systemic administration [15]. Another anti-proliferative agent formulated in a PLGA based nanoparticle system was U-86, a 2-aminochromone, which is an experimental anti-proliferative compound [43]. A number of different surface modification strategies to alter the charge of the nanoparticle system and to promote adhesion to the cells were tested. The results of this study showed that surface modification with a cationic surfactant, didodecyldimethylammonium bromide, was most
beneficial in producing a 7-10 fold increase in arterial drug levels due to an increase in
the residence time of the nanoparticles on the endothelial surface.

**Gene Therapy for Coronary Restenosis**

Restenosis gene therapy can be categorized by the mode of gene product action
and the cellular targets [44, 45]. The reduction of intimal hyperplasia has been the most
investigated treatment option. Intimal hyperplasia can be reduced through three distinct
modes of action. First, by transfecting cells with genes that encode for proteins known to
destroy the cells as they enter the S-phase of cell division cycle, such as thimidine kinase,
cytosine deaminase, and Fas ligand [46-51]. Alternatively, the cell cycle can be arrested
by certain gene products. These gene products take part in cellular regulation and,
therefore, modulation of these genes would stop cell division [52-54]. Genes that encode
for kinases such as CDK2, CDC3, and cyclin B as well as CDK inhibitors such as p21,
p27, a fusion of p16 and p27, and p53 are all modulators of the cell cycle [55-60]. hRAD
50 gene delivery affects p21 levels, subsequently inhibiting the cell cycle [61].
Alternatively, targeting of E2F a protein involved in the G1/S transition in cells has been
approached through a variety of different pathways. For example, with the use of non-
phosphorylatable retinoblastoma gene, a protein that inhibits E2F, has been used to
inhibit intimal hyperplasia [62]. An E2F decoy and a E2F-Rb chimera were used
separately to successfully inhibit E2F levels [63-65]. Another pathway targeted for
restenosis gene therapy is the protein kinase G (PKG) pathway. Truncated PKG gene has
been used to increase PKG levels, a known inhibitor of neointimal formation [66].
Similarly proliferating cell nuclear antigen (PCNA) ribozymes were shown to inhibit
neointimal growth by inhibiting PCNA [67]. Other pathways targeted successfully
include early growth response factor (Egr-1), dominant-negative H-ras, Gax homeobox, GATA homeobox and interferon (INF)-β [68-73]. Modulating local CO levels through iron metabolism with heme oxygenase-1 inhibited intimal hyperplasia despite the exact understanding of the mechanism being unknown [74, 75].

The second approach for reducing restenosis via the reduction of intimal hyperplasia would be to prevent the SMCs from migrating and forming a lesion in the artery. Without SMC movement, neointimal growth and occlusion would not be possible. It should be noted that these genes do not promote re-endothelialization, which is important in the long term health of the vessel. Soluble platelet-derived growth factor receptor β, a scavenger of PDGF, which is known to be involved in cell signaling and migration, has been shown to inhibit neointima formation [40]. Alternative approaches have included the use of genes that encode for extracellular matrix modifying proteases, such as tissue inhibitor of metalloproteinases and plasminogen activator inhibitor [76-81].

Lastly, the use of anti-thrombotic gene therapy has been investigated to reduce restenosis. For instance, local transfection with a gene encoding thrombin inhibitor (hirudin) showed inhibition of restenosis [82]. Other successful approaches include transfecting a gene encoding TFPI, the target of the anti-thrombotic drug heparin to inhibit restenosis [83, 84]. Prostacyclin synthase encoding gene has been used directly and indirectly by modulating cyclooxygenase-1 (COX-1) production to inhibit restenosis [85-88]. COX-1 has also been shown to open the arterial wall, reducing pressure, and ultimately reducing degree of restenosis, though it did not directly affect intimal hyperplasia growth. In combination with other genes, this could be a promising target [87, 89].
eNOS Gene Therapy for Restenosis

Nitric oxide (NO) is a small gaseous molecule at standard pressure and room temperature. NO plays important biological functions and is produced locally by a series of enzymes called nitric oxide synthases (NOS). There are 3 isoforms of NOS, neuronal NOS (nNOS, NOS-1), endothelial NOS (ENOS, NOS-2), and inducible NOS (iNOS, NOS-3). NO is produced in the body through a reaction of L-arginine and oxygen that requires calmodulin and pteridine tetrahydrobiopterin (BH4) as cofactors; resulting in the production of NO and citrulline through a 5 step oxidation reaction. As shown in Figure 2, NO is a critical molecule in the vessel wall and is responsible for a host of different mechanisms including inhibition of platelets adhesion and aggregation on the vessel wall, inhibition of leukocyte chemotaxis, inhibition of SMC growth and migration, vasodilatation and re-endothelialization [90]. NO acts on the vasculature through a variety of different molecular mechanisms, primarily through guanyl cyclase and cyclic GMP dependent and independent pathways [91].

Endothelial nitric oxide synthase (eNOS) is an enzyme responsible for local endothelial NO production in the cardiovascular system. eNOS is a 135 kDa heme-containing enzyme, and is expressed in a variety of different cells including endocardial cells, endothelial cells of arteries, veins and capillaries, kidney epithelial cells, hippocampal pyramidal neurons, skeletal monocytes, and cardiac sinoatrial cells [92]. eNOS transcription is upregulated in response to shear stress, exercise, estradiol, lysophosphatidylcholine, and oxidized LDL [92]. Once transcribed the eNOS enzyme undergoes a series of post-translational modifications. Depending on the modifications, the enzyme can either be found in the membrane or in the cytosolic fraction [92]. The
role of eNOS post-translational modifications is critical to its function on the cell surface or within the cell [93].

Due to the pleiotropic effect of NO in the artery, the use of eNOS encoding plasmid DNA for gene therapy is extremely attractive for coronary restenosis. Although NO releasing systems, including drugs and polymers, have been tried, the short half-life (~2-5 seconds) and potential for cardiovascular side effects distal from the restenosis site have limited their use. As such, gene eluting stents would be a much more attractive strategy for local eNOS transgene expression and local NO production at the site of injury.

Non-viral Gene Delivery in Restenosis

A recent publication by Sharif, et al., [94] has discussed local eNOS gene therapy using adenoviral vector embedded in stents for restenosis. Other groups have used an adenoviral vector coding for iNOS to inhibit restenosis [95]. However, viral vectors have a history of serious toxicity, and as such, their routine use in the clinic is questionable. Non-viral vectors, on the other hand, promise to have a high safety profile and can be made to be efficacious in gene delivery and transfection by judicious selection of the material and construct design. As such, a significant amount of work has been accomplished in the field of non-viral nanoparticle-based gene delivery for restenosis. Most of the work on non-viral transfection in the field of gene therapy for restenosis has been performed with commercially-available cationic lipid transfection reagents such as Lipofectamine® and Lipofectamine Plus®. The use of Lipofectamine® or Lipofectamine® Plus in delivery of genes is not a clinically viable option due to the known toxicity of these transfection reagents [96]. Several examples of NOS transfection have used non-
viral vectors that were not optimized for the specific application, such as Lipofectamine® [97, 98]. Armeanu et al., [96] systemically evaluated transfection of a number of different non-viral vectors in SMC and found that the cationic agent, spermine-DOCSPER, was most successful in transfecting after the plasmid DNA was pre-complexed with poly(L-lysine).

The use of plasmid DNA encoding for inducible and endothelial nitric oxide synthase (iNOS and eNOS, respectively) has been studied extensively in other types of cationic liposomes. In one study, the researchers examined local iNOS plasmid delivery to an injured mini pig femoral and coronary artery model [99]. Administration of the complexed plasmid was performed using an Infiltrator® infusion catheter and the animals were subsequently stented to produce the injury. The lipid-DNA complexed (lipoplex) nanoparticle formulation contained monocationic lipid 3β-(N,N’-di-methylaminoethane)-carbamoylcholesterol (DAC) at 30% (w/w) and dioleoyl phosphatidylethanolamine (DOPE) at 70% (w/w) concentrations. When administered to the injured model, despite the breakdown of excess NO in the bloodstream by hemoglobin, significant reduction of neointimal growth was observed with this DNA delivery system. Using a similar liposomal system, Pfeiffer et al., [100] used a double-balloon Infiltrator® catheter to administer iNOS expressing plasmid DNA in a foxhound dog model after an arterial graft. In this study, the intimal hyperplasia was inhibited for up to 6 months with a single local plasmid DNA administration. *In-vivo* studies of eNOS plasmid DNA formulated in cationic lipid 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM) and cholesterol in a 1:1 weight ratio, was performed [101]. Using a Stauffland rabbit model, an *in-situ* transfected carotid artery was grafted into the animal. The results
indicated that eNOS transfected in this manner, reduced neointimal formation with effects lasting up to 21 days after transfection [101]. The same formulation was also used in a heart transplanted rabbit model with similar results [102].

Polymer based pre-complexation has been used to enhance transfection efficiency. Pre-complexation is where the DNA is incubated with a polymer to reduce the size of the plasmid DNA, once the plasmid is condensed, a positively charged lipid / liposome or polymer is then added. Lampela et al., used PEI to precomplex DNA to a variety of different systems including a dendrimer DOCSPER liposomes and DOTAP liposomes, results showed that efficient transfection was seen even in the presence of serum [103, 104]. Poly lysine has also been used as a pre-complexation agent to increase transfection efficiencies within smooth muscle cells [105]. Besides increasing the stability of DNA upon intracellular vesicular transport, pre-complexation may also enhance endosomal escape leading to higher transfection efficiency. There are several proposed mechanisms for endosomal escape, including the proton sponge effect and fusion of the lipid with the endosome [106].

Poly(beta-amino ester)s (PBAE) are a class of biodegradable and biocompatible polymers synthesized by addition reaction of primary or secondary amines with diol diacrylates (Figure 3) [107]. Recently, Professor Langer’s group at MIT have developed a large library of PBAE’s based on parallel synthesis using different classes of amines and diol-diacrylates and these have been tested for gene delivery applications [107, 108]. A hydrophobic representative PBAE (MW ~ 10 kDa) was synthesized by the addition reaction of 4,4’-trimethylpiperidine with 1,4-butanediol diacrylate in
Figure 3. Schematic illustration for the synthesis of poly(beta-amino ester)s using primary or secondary diamines and diol-diacrylates.
dimethylformamide for 48 hours at 50°C and purified according to the synthesis scheme described earlier [107]. This particular PBAE has a specific pH-dependent solubility profile. The polymer is insoluble in aqueous media at pH above 6.5. This allows for a pH responsive release of the DNA from the polyplex that is formed upon cellular internalization as the pH of the endosome/lysosomes is significantly lower than extracellular pH [109]. Additionally, in vitro transfection with this PBAE in serum containing media has showed relatively good results [110]. The use of PBAE as a pre-complexation agent has not been investigated to date. Table 1 summarizes non-viral gene therapy for restenosis.

**Localized Arterial Delivery**

Functionalized nanoparticles can target specific cell types. A major limitation of this is that the area of a restenotic lesion is so small compared to the total area of the circulatory system. Very highly specific ligands must be used to effectively target restenotic cells. Even with those ligands the amount of particles needed to inhibit restenosis would likely lead to systemic toxicity since targeted particles only modify the biodistribution slightly. One way to circumvent this is by locally administering the treatment using an infusion catheter as previously described with a Dispatch®, Infiltrator®, or other cardiac infusion catheters [38, 111]. These catheters employ a porous double balloon design. The inner balloon has no pores in it, and is inflated first to remove the atherosclerotic plaque. Once fully inflated a solution is added through the second balloon, causing high local levels of the therapy. Unless there is some targeting ligand associated with the therapy it can get washed away quickly after the balloon is removed.
Table 1:
Non viral gene delivery for restenosis

<table>
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<tr>
<th>Genes</th>
<th>Nanocarrier System</th>
<th>Model and Route</th>
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<tr>
<td>eNOS (endothelial nitric oxide synthase)</td>
<td>Cationic Liposomes (Lipofectamine plus)</td>
<td>Adult male mongrel dogs, ex-vivo saphenous vein transfection, both intimal and adventitial.</td>
<td>No efficient gene transfection with animal transfusion, adventitial transfusion showed elevated mRNA levels compared to controls.</td>
<td>[98]</td>
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<td>PCNA chimeric hammerhead ribozyme</td>
<td>Cationic liposomes (Lipofectamine / Lipofectin)</td>
<td>Balloon injured porcine coronary artery</td>
<td>Inhibition of intimal hyperplasia and reduction of coronary artery restenosis.</td>
<td>[67]</td>
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<td>Prostacyclin synthase (PGIS)</td>
<td>Lipofectamine (Cationic liposomes)</td>
<td>Balloon injured and stented New Zealand White rabbits with local delivery using dispatch catheter.</td>
<td>PGIS induced PGI2 expression and inhibited VSMC growth and accelerated re-endothelialization, preventing neointimal formation.</td>
<td>[86]</td>
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<tr>
<td>iNOS</td>
<td>Non-liposomal lipid (FuGENE 6, Boehringer Mannheim)</td>
<td>Coronary stented porcine model, local delivery using Dispatch catheter</td>
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<td>VEGF</td>
<td>DAC (30% w/w) and DOPE (70% w/w)</td>
<td>Coronary and femoral artery stented mini pigs, local administration of lipoplex performed prior to stenting with Inflitrator catheter</td>
<td>Inhibition of intimal hyperplasia.</td>
<td>[99]</td>
</tr>
<tr>
<td>eNOS (endothelial nitric oxide synthase)</td>
<td>Cationic liposomes</td>
<td>Transgenic F344 rats carotid artery bypass grafts, local delivery using Inflitrator catheter</td>
<td>Single transfecion able to show inhibition of intimal growth for up to 6 months.</td>
<td>[100]</td>
</tr>
<tr>
<td>eNOS (endothelial nitric oxide synthase)</td>
<td>Cationic liposomes</td>
<td>Human umbilical vein endothelial cell (ECV304) in vitro</td>
<td>Successful transfer of eNOS in-vitro, saw increase in NO.</td>
<td>[97]</td>
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<tr>
<td>eNOS (endothelial nitric oxide synthase)</td>
<td>Liposomes / cationic liposomes</td>
<td>New Zealand white rabbits and Starfrand rabbits heart transplants, liposomes delivered ex-vivo to aortic roots of donor heart</td>
<td>Despite transfection inefficiencies with liposomes, measurable NO expression was achieved, reducing endothelial activation.</td>
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<td>Chlamydomonas acryl transisnase (CAT)</td>
<td>Cationic liposomes</td>
<td>Balloon injured Yorkshire pig iliofemoral arteries, local delivery using catheter.</td>
<td>Saw increased CAT activity after liposome transfection in artery.</td>
<td>[112]</td>
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<td>C-type natriuretic peptide (CNP)</td>
<td>Liposomes (DOCSPER, 1,3-dioleoyloxy-2-carbamoyl-spermine)-propane)</td>
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<td>[113, 114]</td>
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<tr>
<td>VEGF</td>
<td>Cationic liposomes</td>
<td>Human trial containing patients with angina and 60-99% diameter stenosis in 1-2 of major coronary arteries. Local delivery achieved using Dispatch catheter.</td>
<td>Did not affect post angioplasty restenosis, but significant improvement in myocardial perfusion was observed in viral vector treated patients.</td>
<td>[115]</td>
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<td>TIMP-1 (tissue inhibitors of metalloproteinase 1)</td>
<td>Self assembling cationic targeted vector</td>
<td>Balloon denuded rat carotid artery, delivery to adventitial carotid artery.</td>
<td>TIMP-1 effects seen up to 28 days after initial transfection when delivered to adventitial vascular cells.</td>
<td>[116]</td>
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<tr>
<td>E2F oligonucleotide decay</td>
<td>Hemaglutin virus of Japan (HJV) liposomes</td>
<td>10.5D mice and Japanese monkeys hearts were treated ex-vivo prior to transplant with viral liposomes</td>
<td>Ex vivo liposomal transfection suppressed neointimal hyperplasia after cardiac transplant surgery.</td>
<td>[64]</td>
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<tr>
<td>E2F oligonucleotide decay</td>
<td>Hemaglutin virus of Japan (HJV) liposomes</td>
<td>Balloon denuded rat carotid artery, local delivery using catheter</td>
<td>Neointima inhibition seen up to 8 weeks after single intraluminal injection.</td>
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<tr>
<td>Wildtype p53</td>
<td>Hemaglutin virus of Japan (HJV) liposomes</td>
<td>Balloon denuded Japanese white rabbits with local delivery using double-lumen catheter</td>
<td>Successful inhibition of neointimal formation without apoptotic stimuli. VSMC growth inhibited.</td>
<td>[60]</td>
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<td>Tissue factor pathway inhibitor gene</td>
<td>Hemaglutin virus of Japan (HJV) liposomes</td>
<td>High cholesterol fed-rabbit model. Blue artery, balloon angioplasty was used to open stenosis, local liposome delivery using Dispatch catheter.</td>
<td>Significant reduction of intimal hyperplasia compared to controls, confirmed through histology and angiography.</td>
<td>[83]</td>
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<tr>
<td>Prostaclyn synthase (PGIS)</td>
<td>Hemaglutin virus of Japan (HJV) liposomes</td>
<td>Balloon denuded Sprawley rat carotid artery, local delivery using infusion catheter.</td>
<td>PGIS expression in neointima, inhibiting neointimal growth.</td>
<td>[38]</td>
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<tr>
<td>PDGF receptor β antagonist</td>
<td>PLGA nanoparticles</td>
<td>Explanted rat SMC in-vitro, injected rat carotid artery in-vivo</td>
<td>Controlled release of oligonucleotide over 1 month, neointima growth inhibited in nked AS and PLGA NP, AS, PLGA NP believed to be effective at lower concentrations, due to sustained release.</td>
<td>[40]</td>
</tr>
<tr>
<td>Monocyte chemoattractant protein 1 (MCP-1)</td>
<td>PEI / PEI / PCG, PEI / melatin</td>
<td>In-vivo SMC, and EC</td>
<td>Inhibition of SMC growth, incorporation of melatin increased activity, while decreasing toxicity.</td>
<td>[117]</td>
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<tr>
<td>Early growth response Factor 1 (EGR-1)</td>
<td>DNAzyme</td>
<td>Porcine coronary stent model, local delivery with Transport catheter</td>
<td>Inhibition of SMC, reducing in-stent restenosis.</td>
<td>[68]</td>
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</tbody>
</table>
Ex vivo application of the drug or gene in the cells is also another possible way to reduce systemic effects of the administered drug. However, clinical application of the ex vivo approach will be quite limited. Local administration is also achieved through the use of drug and/or gene eluting stents such as the Cypher® and Taxus® stents [45, 112-115]. Other drug-coated stents using different designs, such as biodegradable stents, as well as other systems are being evaluated in preclinical and clinical studies [116]. Gene eluting stent studies have used a variety of methods including the plasmid DNA within the polymer coating, or including it within a viral vector that is associated a polymer coating via an antibody [45, 117]. Since the surface area of a stent is extremely small (<1cm²) a vector that is used must be very potent [45].

The first study employing a gene eluting stent used an emulsion of PLGA and plasmid DNA that was subsequently coated onto a stent, a total of 1mg of DNA was loaded onto the stent [118]. Encouraged by the PGLA results Perlstein et al., used denatured collagen as a stent coating [119]. These results showed much higher levels of transfection with half of the dose compared to the PLGA coated stent. The researchers chose to use collagen since there have been results indicating that integrins may promote gene transfection using naked and adenoviral vectors [120-122]. These studies both employed no vector, only naked DNA embedded within a polymer matrix. Stents have been coated with crosslinked gelatin containing adenoviral vector containing β-gal [123]. Results showed transfection at the stented vessel, though some of methods employed in the study were not clear. Other strategies to use stents as a gene delivery vehicle include viral vectors tethered onto collagen coated stents and polyurethane coated stents [124, 125]. Recently there have been reports of layer by layer (LBL) loading of DNA onto a
stent using cationic lipids and polymers[126-129]. Studies have used cationic polymers such as phosphorylcholine and poly beta amino esters on stent surfaces to complex with the antisense RNA or DNA respectively [126, 127]. Using gold wire and lipid complexed with DNA Yamauchi et. al. showed that a LBL assembly can be used in medical device applications with good levels of reporter plasmid transfection [128, 129]. Limitations of these types of systems are likely to be the amount of DNA loaded onto the stent and the total release of DNA from the stent. Jewell et al., showed a stent with 8 layers on with a PBAE polymer where only 7 μg of DNA was released. In-vitro results did show good levels of transfection using eGFP-N1. Compared to previous gene eluting stent studies it is not clear if 7 μg of DNA will be enough to transfect vascular cells. The use of antibodies to tether DNA onto the surface of the stent has been evaluated with positive results [125]. Viral vectors have been evaluated on a variety of coating platforms for stents. The use of viral vectors tethered onto stents has been studied extensively [95, 117, 124, 130]. Inhibition of restenosis was achieved using a viral vector encoding for iNOS tethered to a stent in rats [130]. The use of a viral vector encoding eNOS dip coated onto a BiodivYsio HI matrix PC-coated stent showed inhibition of restenosis and showed an improvement in endothelialization by day 14 [94].

The use of liposomes coated onto stents has been investigated by a few groups [131-134]. A brief review of this topic is presented here [131]. All of these studies employed liposomes as agents to deliver drugs, not genes. These studies also did not use any polymer coating; the liposomes were directly deposited onto the surface of the stent. The need for an effective gene delivery system that can deliver enough DNA to produce therapeutic efficacy is very high. Problems associated with viral vectors have hindered
the progress in gene delivery. Developing a potent gene delivery system that can transfec
t cells with very low levels of DNA and promote the re-endothelialization of the vessel wall after injury is a great challenge. The use of collagen loaded sponges with lipoplexes and polypexes has been evaluated for wound healing and tissue engineering [135-142].

**Rationale for LPP-Based Formulations in Localized Arterial Delivery**

Gene-eluting stent studies have used a variety of methods including the plasmid DNA within the polymer coating, or including it within a viral vector that is associated a polymer coating via an antibody [45, 117]. Since the surface area of a stent is extremely small (<1cm²) a vector that is used must be very potent [45]. The initial studies employing a gene eluting stent used a PLGA emulsion and denatured collagen and plasmid DNA (1 mg) that was subsequently coated onto a stent [118]. The results with denatured collagen showed much higher levels of local transfection with half of the dose as compared to the PLGA-coated stent. The researchers chose to use denatured collagen, since there have been results indicating that integrins may promote gene transfection using naked and adenoviral vectors [120-122]. Additionally, stents that have been coated with crosslinked gelatin and coated with adenoviral vector expression beta-galactosidase have also been examined [123]. Other strategies to use stents as a gene delivery vehicle include viral vectors tethered onto collagen coated stents and polyurethane coated stents [124, 125]. Recently there have been reports of layer-by-layer film formation on metallic stents and DNA loading using alternative cationic lipids and polymers [126-129].

In each of the above examples, the main challenges in local arterial gene therapy in restenosis is the small surface area available at the lesion site, residence time of non-
viral gene delivery system, cellular uptake and intracellular stability of nucleic acid construct, and efficient transcription and translation of encoded protein. Based on these challenges, we have identified the following design criteria in development of safe and effective plasmid DNA delivery system for local transfection in coronary restenosis. First, the formulation should concentrate plasmid DNA in order to accommodate an appropriate dose in the small surface area of stents. Second, the use of a stent-based gene delivery and arterial implantation during PTCA procedure will be the most effective treatment strategy. Although systemic administration of nanoparticles have been tried, their localization at the lesion site in the coronary artery is highly inefficient. Additionally, the use of double balloon porous catheters, such as Infiltrator®, can also afford local delivery. However, these catheters are not routinely used in interventional cardiology anymore.

The need for an effective gene delivery system that can deliver enough DNA to produce therapeutic efficacy is very high. Problems associated with viral vectors have hindered the progress in gene delivery. Developing a potent gene delivery system that can transfecet cells with very low levels of DNA and promote the re-endothelialization of the vessel wall after injury is a great challenge. To date there have been no studies evaluating the use of denatured collagen (gelatin) with a non-viral vector embedded in it. This dissertation outlines the development of a potent non-viral gene delivery system for primary cells, in vitro characterization, the development of a gelatin based stent coating, and in vivo evaluation with a therapeutic plasmid encoding eNOS.
CHAPTER 2: Objective and Specific Aims

Main Objective

The primary objective of this doctoral dissertation research was to develop and evaluate a gene therapy strategy using safe and effective non-viral vectors for local production of endothelial nitric oxide synthase (eNOS) on arterial wall and therapeutic response in restenosis.

Specific Aims

The specific aims of the thesis project are:

Aim 1: Development of positively-charged plasmid DNA-polymer-lipid complexes or lipopolyplex, characterization, and optimization for cellular uptake and transfection.

Aim 2: In vitro trafficking and transfection studies with lipopolyplexes in primary human aortic smooth muscle and endothelial cells.

Aim 3: Fabrication and optimization of gelatin-coated mesh and stent systems for local in vivo delivery of lipopolyplexes.

Aim 4: In vivo transfection of reporter (GFP-encoding) and therapeutic (eNOS-encoding) genes in lipopolyplex formulations in denuded rabbit iliac artery restenosis model after local administration with a stent.

Aim 5: In vivo therapeutic efficacy of local eNOS production in rabbit iliac artery model.
CHAPTER 3: PRELIMINARY EVALUATION OF NON-VIRAL GENE DELIVERY SYSTEMS

Introduction

Based on our previous studies which showed highly efficient gene delivery and transfection using type B gelatin nanoparticles [143-152], we initially evaluated these non-condensing DNA delivery systems for transfection in human aortic smooth muscle cells (SMC) and endothelial cells (EC). Based on these results, we decided to formulate lipopolyplexes made by pre-condensation of plasmid DNA with poly(beta-amino ester) (PBAE) followed by encapsulation of the pre-condensed plasmid with cationic liposomes.

Materials and Methods

Cationic phospholipid, 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), cholesterol, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine – poly(ethylene glycol) 5000 molecular weight (DSPE-PEG 5kDa) were purchased from Avanti Polar Lipids (Alabaster, AL) and were dissolved in HPLC grade chloroform (Fisher Scientific, Milwaukee, WI). Plasmid DNA expressing GFP (i.e., EGFP-N1, 4.7 Kb) was amplified and purified by Elim Biopharmaceuticals (Hayward, CA) [153]. Phosphate buffered saline (PBS, pH 7.4), Tween® 80, Type B gelatin (100 bloom strength), glyoxal, denatured ethanol, bovine serum albumin and 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Sigma-Aldrich (St. Louis, MO). Cysteine and glycine residues end-modified arginine-glycine-aspartic acid (RGD) tri-peptide sequence [RGD peptide was custom synthesized
at Tufts University Peptide Synthesis Core Facility (Boston, MA)). 2-iminothiolane (Traut’s Reagent) was purchased from Pierce (Rockford, IL). Human aortic SMC and EC were purchased from Cell Applications, Inc. (San Diego, CA) and grown in culture at 37°C and 5% CO₂ cells were grown in smooth muscle cell growth media. All aqueous solutions and reagents were prepared in deionized distilled water using Barnstead/Thermolyne Nanopure II (Dubuque, IA) system.

**Preparation of Nanoparticles Formulations**

*Synthesis of Thiol-Modified Gelatin and Preparation of Nanoparticles*: A solution of gelatin was incubated with 2-iminothiolane as previously described [147]. Briefly, 1 gram of 100 bloom strength gelatin was dissolved in 100 ml water. The solution was adjusted to pH 8.0 and 2.5 mg of 2-iminothiolane was added to the solution. The resulting mixture was left to incubate at room temperature for 15 hours. The solution was dialyzed against 5 mM and 1 mM HCl for 24 hours, respectively. Following dialysis, the thiol-modified gelatin was rapidly frozen and lyophilized.

Thiolated gelatin nanoparticles were generated by the solvent replacement technique. Using 200 mg of thiolated gelatin dissolved in 10 ml of de-ionized water. The resulting solution was adjusted to pH 7.0 with 0.1 M NaOH. For formulations that contained DNA, plasmid DNA was added at this. Under constant high speed stirring 40ml of 9:1 EtOH : deionized distilled water (volume / volume) was slowly added via a peristaltic pump at a flow rate of 20 ml/min. The resulting solution was crosslinked by slowly adding 1.5 ml of 4% glyoxal solution. The addition of 5 ml of 0.2 M glycine was added to quench any free aldehydes on the nanoparticles. The suspension was
centrifuged at 32,000 rpm for 45 minutes. The pellet was reconstituted and lyophilized for subsequent analysis.

**Preparation of RGD Modified Thiolated Gelatin Nanoparticles:** Thiolated nanoparticles were synthesized as above. After addition of EtOH, the nanoparticles were centrifuged at 32,000 rpm for 45 min. The resulting pellet was reconstituted in 10 ml of deionized distilled water and mixed at high speed. The particles were crosslinked by slowly adding 1.5 ml of 4% glyoxal. After crosslinking of the nanoparticles, 5 mg of the RGD peptide was added to the mixture and allowed to incubate for 60 minutes. Additional 0.2 M glycine was added in excess to quench any remaining aldehydes. The suspension was centrifuged at 32,000 rpm for 45 minutes. The pellet was reconstituted and lyophilized for subsequent analysis.

**Preparation of Albumin Nanoparticles:** Bovine serum albumin nanoparticles were made using the same technique as the gelatin nanoparticles. Briefly, 200 mg of albumin was dissolved in 10 ml of deionized distilled water. The solution was adjusted to pH 8.0 with 0.2 N NaOH. Plasmid DNA was added at this point for formulations that contained DNA. Under constant high speed stirring 40 ml of ethanol was slowly added via a peristaltic pump at a flow rate of 20 ml/min. The resulting solution was crosslinked by slowly adding 1.5 ml of 4% glyoxal solution. The addition of 5 ml of 0.2 M glycine was added to quench any free aldehydes on the nanoparticles. Particle size of the solution was taken at this point. The suspension was centrifuged at 32,000 RPM for 45 minutes. The pellet was reconstituted and lyophilized for subsequent analysis. Surface modification of these particles was performed in the same manner as outlined previously.
Preparation of Lipid Coated Nanoparticles: Cationic lipid films were prepared by evaporating a solution of DOTAP in chloroform at 1 mg/mL using a rotary evaporator (Buchi model number R200) at a pressure of 300 milliTorr, for 30 minutes with a water bath temperature of 50°C in a 50ml round bottom flask. Samples were then placed on a lyophilizer (Labconco Freezone® 6 plus) overnight to remove any residual solvent. The lipid film was re-hydrated by adding 5 ml of the centrifuged and re-suspended nanoparticles and vortexed extensively. Films that did not hydrate readily were incubated at 50°C for 10 minutes to allow the lipids to hydrate.

Preparation of DNA-Cationic Lipid Lipoplexes: Cationic liposomes were prepared by evaporating in chloroform at 10 mg/mL using a rotary evaporator (Buchi model number R200) at a pressure of 300 milliTorr, for 30 minutes with a water bath temperature of 50°C. Lipid compositions were varied to assess the effect of charge, and the role of additional lipids in transfection. Samples were then placed on a lyophilizer (Labconco Freezone 6 plus) overnight to remove any residual solvent. The lipid film was re-hydrated by adding 1.0 mL of filtered deionized distilled water and vortexed for 30 seconds. After the vortexing step, hydrated lipid sample was placed in a 50°C water bath for 10 minutes. The lipid was then vortexed for 30 seconds and placed on ice for 10 minutes, this was repeated a total of 3 times. The liposomes were then sonicated on ice with a Sonics Vibracell® (Newtown, CT) probe sonicator for 10 minutes at a power rating of 30% with a 1 second pulse interval. After initial formation of the liposomes, DNA was incubated in a diluted solution of liposomes for 30 minutes prior to the start of transfection.
Preparation of DNA-Poly(Beta-Amino Ester) Polyplexes: (PBAE) was dissolved in 1 N HCl and diluted in MES buffer at pH 6.0 as previously described [107, 108]. The buffer was used to keep the pH of the system fixed at 6.0 so the polymer would be positively charged and remain soluble. DNA and PBAE were mixed in different ratios to assess the effect of polymer concentration on transfection. The samples were incubated for 30 minutes prior to the start of transfection.

Preparation of DNA-PBAE-Cationic Lipid Lipopolyplexes: Polyplexes were generated as discussed above. The polyplexes were then added to diluted liposomes for 30 minutes. After 30 minutes the resulting particles were added to cells to assess their ability to transfect.

In Vitro Transfection Studies with EGFP-N1 Plasmid DNA

Quantitative Transfection by FACS: To assess the ability of the lipopolyplexes to transf ect human arterial cells, FACS analysis was carried out. Untreated cells were used as a control to set up the gating and to adjust the forward and side scatter. The cellular debris was gated out of the sample. A total of 10,000 events within the gated region was collected for each sample. Cells were plated in 6-well plates and allowed to grow to 70% confluency to minimize the effect of cell growth and division on transfection. On day of treatment, the SMC were incubated with each treatment for 6 hours. The cells were then washed with sterile PBS thrice to remove any particles that were not endocytosed and the samples were then placed in a cell culture incubator at 37°C with 5% CO₂. Twenty four hours after the start of transfection the cells were trypsinized and centrifuged. Media and trypsin were removed and the cell pellet was re-suspended in 4% paraformaldehyde /
sterile PBS (Fischer Scientific, Waltham, MA). The cell samples were placed in sheath fluid and analyzed for transfection on a FACScaliber flow cytometer (BD Biosciences, San Jose, CA). GFP expression was detected using the FL-1 channel (530/30 emission). Data was analyzed using the Cell Quest Pro® software.

**Quantitative Transfection by ELISA:** ELISA was used to determine the amount of GFP being produced in the cells for a subset of formulations. Cells were plated and grown in 6-well plates until they reached approximately 70% confluence. Cells were treated as previously described. One six well plate was used per sample to ensure adequate protein detection. The samples were lysed with 600 μl of lysis buffer with protease inhibitors on ice, and stored at -80°C until use. Using React-Bind® anti-GFP antibody coated plates (Pierce, Rockford, IL) and adding a GFP secondary antibody reactive to alkaline phosphatase, very low levels of GFP can be detected. Briefly, cell supernatants were incubated with the anti-GFP coated plates for 24 hour at 4°C, after washing with PBS - 0.5% Tween®-80, GFP secondary antibody was added to each plate and was allowed to incubate for 2 hours. The plates were washed with PBS-Tween-80® again and alkaline phosphatase was added and allowed to react for 30 minutes, 0.5 N NaOH was added to stop the reaction after 30 minutes. The plates were read on a BioTek® Synergy HT plate reader with KC4 software. The total cellular protein was determined by lysing the cells with Triton®-X100, centrifugation to remove cell membrane, and assaying the cytosolic protein levels with NanoOrange® kit (Invitrogen, Carlsbad, Ca), according to the manufacturers instructions.
Results

Quantitative Transfection Efficiency with FACS

*Gelatin-Based Nanoparticle Systems:* Thiolated gelatin nanoparticles were dosed at 20 μg/200,000 smooth muscle cells and analyzed on FACS for their ability to transfect. Thiolated gelatin nanoparticles without plasmid showed an 8.3% transfection. Alternatively when plasmid was introduced the transfection was only 1.59%. A similar trend was observed with RGD modified nanoparticles, samples with no plasmid showed a transfection of 3.56% compared to 2.22% with plasmid. The addition a cationic lipid shell on the surface of the nanoparticle yielded a 17.5% and 13.2% transfection with and without plasmid respectively.

*Albumin-Based Nanoparticle Systems:* Albumin nanoparticles were dose and analyzed similarly to the gelatin nanoparticles. Albumin particles displayed a similar trend to the gelatin based nanoparticles, samples with no plasmid yielded a transfection of 5.8%. When plasmid was encapsulated within the particles the transfection fell to 4.05%. The addition of a cationic coating to the surface of these particles resulted in a 15% transfection with plasmid and 10.3% transfection without.

*Cationic Lipid- and Polymer-Based Nanoparticle Systems:* DOTAP liposomes were complexed with EGFP-N1 plasmid DNA at 1:1, 5:1, 10:1, 20:1 ratios and were subsequently incubated with smooth muscle cells. Transfection was seen with a 1:1 ratio of DOTAP to DNA with a 10 μg dose. All other ratios resulted in cell death at that dose. The FACS data was not interpretable due to the low number of counted cells.
DOPE was added to the DOTAP liposomes in a 1:1 molar ratio. Transfection was only interpreted for liposomes incubated with a 1:1 DNA : DOTAP ratio since the other formulations (1:5 and 1:10, DNA : DOTAP) resulted in high cell death at a 10μg dose. This formulation resulted in 8.34% transfection. The addition of cholesterol and DOPC to the DOTAP liposomes in a 1:1 : 0.5 cholesterol:DOTAP:DOPC (molar ratio) produced transfection when the cationic : DNA ratio was 1:1 when dosed with 10 μg DNA. Other formulations resulted in cytotoxicity resulting in too low of a cell count to be analyzed. DOTAP:DOPE:DSPE PEG 5k, 5 4:1 (molar ratio) resulted in a transfection of 3.65%.

PBAE based formulations resulted in transfection rates between 1.84% and 9.92% when dosed with 10 μg DNA. PBAE : DNA (1 : 1 w/w) resulted in a 9.92% transfection. When DOTAP based liposomes were added to the PBAE : DNA polyplex the percent of cells transfected was 3.26% (1 : 1 : 1, PBAE : DNA : DOTAP w/w), 4.58% (1 : 1 : 1 : , PBAE : DNA : DOTAP : DOPE w/w), and 2.54% (1 : 1, (PBAE : DNA, 1 : 1) : (DOTAP : DOPE : DSPE PEG 5k, 5 : 4 : 1 molar ratio) w/w). When the amount of cationic lipid was reduced the resulting transfection percentages were 2.80% (1 : 1 : 0.1, PBAE : DNA : DOTAP w/w), 1.66% (1:1:0.1:0.1, PBAE:DNA:DOTAP:DOPE w/w), and 1.84% (1:0.1, PBAE:DNA, 1 : 1)(DOTAP : DOPE : DSPE PEG 5k, 5 : 4 : 1 molar ratio) w/w).

Alternative doses were examined as well to see if problems with cytotoxicity could be avoided without a loss in transfection. DOTAP liposomes combined with DNA in a 1 : 1 ratio, resulted in a 7.32% transfection when 5μg of DNA was added. 1 : 1 (molar ratio) DOTAP : DOPE liposomes complexed with DNA at a 1 : 1 w/w ratio resulted in a transfection of 6.78% with a 5μg dose.
Other formulations tested at 5 μg included DOTAP : DOPE : DSPE PEG 5k, 5 : 4 : 1 molar ratio, 3 : 1 (2 : 1, PBAE : DNA) : DOTAP liposomes, and 3 : 2 (2 : 1, PBAE : DNA) : DOTAP liposomes, which resulted in % transfection of 4.62%, 2.30%, and 2.88%.

Four formulations were tested at a 2 μg DNA dose. These four formulations 5 : 1, PBAE : DNA, 1 : 5 : 1 PBAE : DOTAP : DNA, 0.1 : 5 : 1 PBAE : DOTAP : DNA, and 0.5 : 5 : 1, PBAE : DOTAP : DNA resulted in % transfection of 26.86%, 17.99@ 18.75% and 28.74% respectively. All of the aforementioned transfection results are tabulated in Table 2.

An ELISA was performed to assess the amount of eGFP-N1 being produced by the SMCs. Four formulations were tested by ELISA, 0.05:5:1 PBAE : DOTAP : DNA, 0.1:5:1 PBAE : DOTAP : DNA, 1:5:1 PBAE : DOTAP : DNA, 5:1 PBAE : DNA. The formulations were all compared to Lipofectin®, a commercially available transfection reagent that has been evaluated in a number of the gene therapy based studies performed to treat restenosis [67, 86, 98]. The amount of protein produced by the cells was the highest with the 1:5:1 PBAE : DOTAP : DNA (4.5ng eGFP-N1/ mg protein) followed by, 0.05:5:1 PBAE : DOTAP : DNA (1.1 ng eGFP-N1/ mg protein), and 0.1:5:1 PBAE : DOTAP : DNA (0.9 ng eGFP-N1/ mg protein). Lipofectin and 5:1 PBAE : DNA produced a negligible amount of eGFP (less than 0.1 ng eGFP-N1/ mg protein).

Figure 4 summarizes the ELISA results.

Discussion

Based on the previous experience in our lab, we initiated the work to develop a non-viral vector for the treatment of restenosis using type B gelatin-based nanoparticles
Table 2: Formulation optimization table

<table>
<thead>
<tr>
<th>Cationic lipid : DNA ratio</th>
<th>% Transfection</th>
<th>Dose DNA (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-gel NP</td>
<td>-</td>
<td>8.36%</td>
</tr>
<tr>
<td>pGFP T-gel NP</td>
<td>-</td>
<td>1.59%</td>
</tr>
<tr>
<td>RGD T-gel NP</td>
<td>-</td>
<td>3.56%</td>
</tr>
<tr>
<td>pGFP RGD T-gel NP</td>
<td>-</td>
<td>2.22%</td>
</tr>
<tr>
<td>Cationic Lipid Thiolated gelatin NP</td>
<td>-</td>
<td>13.22%</td>
</tr>
<tr>
<td>pGFP Cationic Lipid Thiolated gelatin NP</td>
<td>-</td>
<td>17.55%</td>
</tr>
<tr>
<td>Albumin NP</td>
<td>-</td>
<td>5.83%</td>
</tr>
<tr>
<td>pGFP Albumin NP</td>
<td>-</td>
<td>4.05%</td>
</tr>
<tr>
<td>Cationic Lipid albumin NP</td>
<td>-</td>
<td>10.36%</td>
</tr>
<tr>
<td>pGFP Cationic Lipid albumin NP</td>
<td>-</td>
<td>15.80%</td>
</tr>
<tr>
<td>DOTAP</td>
<td>1:1 (w/w)</td>
<td>11.20%</td>
</tr>
<tr>
<td>DOTAP</td>
<td>5:1 (w/w)</td>
<td>*</td>
</tr>
<tr>
<td>DOTAP</td>
<td>10:1 (w/w)</td>
<td>*</td>
</tr>
<tr>
<td>DOTAP</td>
<td>20:1 (w/w)</td>
<td>*</td>
</tr>
<tr>
<td>DOPE : Dotap 1:1 (molar ratio)</td>
<td>1:1 (w/w)</td>
<td>8.34%</td>
</tr>
<tr>
<td>DOPE : Dotap 1:1 (molar ratio)</td>
<td>5:1 (w/w)</td>
<td>*</td>
</tr>
<tr>
<td>DOPE : Dotap 1:1 (molar ratio)</td>
<td>10:1 (w/w)</td>
<td>*</td>
</tr>
<tr>
<td>Cholesterol : Dotap : DOPC 1:1:0.5 (molar ratio)</td>
<td>1:1 (w/w)</td>
<td>7.24%</td>
</tr>
<tr>
<td>Cholesterol : Dotap : DOPC 1:1:0.5 (molar ratio)</td>
<td>5:1 (w/w)</td>
<td>*</td>
</tr>
<tr>
<td>Cholesterol : Dotap : DOPC 1:1:0.5 (molar ratio)</td>
<td>10:1 (w/w)</td>
<td>*</td>
</tr>
<tr>
<td>1:1 PBAE : DNA</td>
<td>-</td>
<td>9.92%</td>
</tr>
<tr>
<td>Dotap : DOPE : DSPE PEG 5k 5:4:1 (molar ratio)</td>
<td>1:1 (w/w)</td>
<td>3.65%</td>
</tr>
<tr>
<td>(1:1 PBAE : DNA) : DOTAP</td>
<td>1:1 (w/w)</td>
<td>3.26%</td>
</tr>
<tr>
<td>(1:1 PBAE : DNA) : (1:1 DOTAP : DOPE)</td>
<td>1:1 (w/w)</td>
<td>4.58%</td>
</tr>
<tr>
<td>(1:1 PBAE : DNA) : (Dotap : DOPE : DSPE PEG 5k 5:4:1)</td>
<td>1:1 (w/w)</td>
<td>2.54%</td>
</tr>
<tr>
<td>(1:1 PBAE : DNA) : DOTAP</td>
<td>0.1:1 (w/w)</td>
<td>2.80%</td>
</tr>
<tr>
<td>(1:1 PBAE : DNA) : (1:1 DOTAP : DOPE)</td>
<td>0.1:1 (w/w)</td>
<td>1.66%</td>
</tr>
<tr>
<td>(1:1 PBAE : DNA) : (Dotap : DOPE : DSPE PEG 5k 5:4:1)</td>
<td>0.1:1 (w/w)</td>
<td>1.84%</td>
</tr>
<tr>
<td>DOTAP</td>
<td>1:1 (w/w)</td>
<td>7.32%</td>
</tr>
<tr>
<td>DOTAP</td>
<td>1:1 (w/m)</td>
<td>6.78%</td>
</tr>
<tr>
<td>Dotap : DOPE : DSPE PEG 5k 5:4:1 (molar ratio)</td>
<td>1:1 (w/w)</td>
<td>4.62%</td>
</tr>
<tr>
<td>3:1 (2:1 PBAE : DNA) : DOTAP</td>
<td>1:1 (w/w)</td>
<td>2.30%</td>
</tr>
<tr>
<td>3:2 (2:1 PBAE : DNA) : (1:1 DOTAP : DOPE)</td>
<td>1:1 (w/w)</td>
<td>2.88%</td>
</tr>
<tr>
<td>PBAE:DNA 5:1</td>
<td>-</td>
<td>27.86%</td>
</tr>
<tr>
<td>PBAE:DNA 1:1 (5:1 DOTAP : DNA)</td>
<td>5:1 (w/w)</td>
<td>17.99%</td>
</tr>
<tr>
<td>PBAE:DNA 0.1:1 (5:1 DOTAP : DNA)</td>
<td>5:1 (w/w)</td>
<td>18.75%</td>
</tr>
<tr>
<td>PBAE:DNA 0.5:1 (5:1 DOTAP : DNA)</td>
<td>5:1 (w/w)</td>
<td>28.74%</td>
</tr>
</tbody>
</table>
**Figure 4:** GFP protein levels (ng of GFP / mg of total protein) assessed by ELISA for a variety of formulations, lipofectin, 0.05:5:1 PBAE : DOTAP : DNA, 0.1:5:1 PBAE : DOTAP : DNA, 1:5:1 PBAE : DOTAP : DNA, 5:1 PBAE : DNA, * - statistical difference between no treatment \((P = 0.01)\), ** - Statistical difference between all groups \((p = 0.01)\) by ANOVA.
as a potentially safe and effective gene delivery vehicle. Initial transfection studies were preformed with type B gelatin based nanoparticles made by solvent displacement due to previous success in our laboratory [143-152]. The surface of gelatin nanoparticles were modified with an RGD peptide covalently bound to the nanoparticle. Surface modification with RGD peptide is known to enhance endothelial and smooth muscle cell uptake, which would allow for an increased concentration of nanoparticles at the target site [154]. FACS studies performed in human aortic smooth muscle cells (SMC) using EGFP-N1 plasmid DNA as a reporter gene showed that small levels of transfection were observed. Nanoparticles containing no plasmid DNA also showed an equally high level of background signal. This was believed to be due to adhesion of the gelatin to the smooth muscle cells. There is a well established affinity between collagen and various vascular cell types [155]. The adhered gelatin then increased the florescence activity of the cells due to the inherent florescence activity of gelatin, leading to a false positive result by FACS. Uptake studies (data not included) concluded that the gelatin nanoparticles were indeed not entering the cells, but rather adhering to the surface. Other protein based nanoparticles were then made and tested for transfection efficiency. Serum albumin nanoparticles made by solvent displacement were tested and the results were similar to those obtained with the gelatin nanoparticles. SMC have a uniform layer of glycocalyx, an anionic surface protein making the endocytosis of these types of particles difficult [156, 157]. Lipid coating on the surface of the nanoparticles with cationic lipids, led to an increase in transfection with EGFP-N1 plasmid loaded particles compared to formulations that did not contain plasmid DNA. Despite the limited transfection, there was still a problem of nanoparticle adhesion on the surface of the cells for both albumin
and gelatin based nanoparticles. The lack of cellular uptake was considered to be a major drawback for both of these protein-based nanoparticle systems.

Initial attempts at encapsulating the plasmid DNA within a nanoparticle did not produce the levels of transfection necessary for the inhibition of restenosis that we would need in an animal model. Cellular uptake efficiency by complexing DNA with cationic lipids increased the amount of transfection dramatically. For these experiments DOTAP was used at varying DNA:DOTAP weight ratios. A number of non-cationic lipids “helper lipids” were tried at different ratio’s, these included cholesterol, L-alpha-dioleoyl phosphatidylethanolamine (DOPE), 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) and DSPE-PEG (Mol. wt. 5kDa) to increase the uptake of the lipoplexes since historically, transfection is enhanced when non-cationic lipids are introduced to the lipopolyplex [106]. Results from these studies showed that DOTAP alone had the highest transfection efficiency at 11.20% compared to other formulations at the same dose. Only one ratio of DOTAP : DNA (1:1) was able to be analyzed due to the toxicity of the other formulations at the dose tested. Since this formulation is to be placed on a stent it needs to be very potent. The doses were then reduced and a polymer was added as a pre-complexation agent. Other studies have used poly(L-lysine) and poly(ethylimine) (PEI) as a pre-complexation agent with success [103-105]. The results from these studies showed that polymer pre-complexation increases transfection efficiency within the cells. Using this rationale, we introduced the cationic polymer poly beta amino ester (PBAE), this polymer was chosen because it has been used in gene delivery applications and has a unique pH responsive nature [107, 108]. The polymer is non water-soluble above pH 6, once the pH is lower than 6 the polymer dissolves. This is
advantageous in our system because as the endosome progresses into the cell the pH inside the endosome falls. The hypothesis is that the structure of the lipopolyplex would break apart within the endosome facilitating endosomal escape and accumulation of the plasmid DNA within the nucleus leading to higher protein levels. Results from the FACS studies performed with the lipopolyplexes did indeed show higher levels of transfection with this system. One limitation of the FACS results is that it does not indicate whether the protein levels are higher. To determine the protein levels post transfection, ELISA was run on the samples 24 hours after dosing with 2.0 μg DNA. The ELISA showed that, even though higher levels of transfection were observed with different formulations, a ratio of 1 : 5 : 1 PBAE : DOTAP : DNA showed the highest amount of protein production.

**Conclusions**

Development of a non-viral non-condensing gene delivery system produced disappointing results. The addition of a cationic charge to the formulation was critical in promoting endocytosis and gene transcription. Lipid-based systems showed promise, with the addition of a cationic polymer transfection efficiency was increased. Based on the results from the formulation optimization the formulation of 1 : 5 : 1 PBAE : DOTAP : DNA was chosen for the preliminary studies.
CHAPTER 4: CELLULAR UPTAKE AND TRANSFECTION STUDIES WITH LIPOPOLYPLEXES IN HUMAN AORTIC SMOOTH MUSCLE AND ENDOTHELIAL CELLS

Introduction

To examine the potential of non-viral gene delivery in human aortic smooth muscle cells (SMC) and endothelial cells (EC), in this study, we have developed poly(β-amino ester) (PBAE) pre-complexed DNA, which was further modified with DOTAP to form lipopolyplexes. The rationale for lipopolyplex formation was to enhance cellular uptake through charge-mediated interactions between negatively-charged cell membrane and cationic lipids and enhanced intracellular stability and endosomal release using PBAE pre-complexation. The formed lipopolyplexes were analyzed for size, charge, morphology, and DNA stability. To test the efficiency of the lipopolyplexes for intracellular delivery, particle uptake, distribution, and intracellular DNA release in SMC and EC was examined. Additionally, the transfection efficiency of the reporter plasmid EGFP-N1, expressing green fluorescent protein (GFP), was assessed by flow cytometry, enzyme-linked immunosorbent assay (ELISA), and fluorescence microscopy.

Materials and Methods

Materials

Cationic phospholipid, 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), and a fluorescent derivative 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine-B-sulfonyl) (rhodamine-PE) were purchased from Avanti Polar Lipids (Alabaster, AL) and were dissolved in HPLC grade chloroform (Fisher Scientific, Milwaukee, WI). Plasmid DNA expressing GFP (i.e., EGFP-N1, 4.7 Kb) and endothelial
nitric oxide synthase (eNOS pVAX-1, a sample kindly supplied by Dr. Duncan Stewart, Division of Cardiology, University of Toronto, Toronto, Canada) were amplified and purified by Elim Biopharmaceuticals (Hayward, CA)[153]. Phosphate buffered saline (PBS, pH 7.4), Tween® 80, and 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Sigma-Aldrich (St. Louis, MO). CellTiter 96® AQueous one solution cell proliferation assay was obtained from Promega Corporation (Madison, WI). Human aortic EC and SMC were purchased from Cell Applications, Inc. (San Diego, CA) and grown in culture at 37°C and 5% CO₂ cells were grown in endothelial cell and smooth muscle cell growth media. All aqueous solutions and reagents were prepared in deionized distilled water using Barnstead-Thermolyne Nanopure II (Dubuque, IA) system.

**Preparation and Characterization of the Lipopolyplexes**

After evaluating various formulation compositions, the optimized lipopolyplexes were formed by combining PBAE, DNA, and DOTAP in a 1:1:5 weight ratios. For the DNA pre-complexation step, 2.0 μg of PBAE was incubated for 30 minutes with 2.0 μg DNA in 100 μL MES buffer at pH 6.0 as previously described [107, 108]. The buffer was used to keep the pH of the system fixed at 6.0 so the polymer would be positively charged and remain soluble. Cationic liposomes were prepared by evaporating a solution of DOTAP in chloroform at 10 mg/mL using a rotary evaporator (Buchi model number R200) at a pressure of 300 milliTorr, for 30 minutes with a water bath temperature of 50°C. Samples were then placed on a lyophilizer (Labconco Freezone 6 plus) overnight to remove any residual solvent. The lipid film was re-hydrated by adding 1.0 mL of filtered deionized distilled water and vortexed for 30 seconds. After the vortexing step,
hydrated lipid sample was placed in a 50°C water bath for 10 minutes. The lipid was then vortexed for 30 seconds and placed on ice for 10 minutes, this was repeated a total of 3 times. The liposomes were then sonicated on ice with a Sonics Vibracell® (Newtown, CT) probe sonicator for 10 minutes at a power rating of 30% with a 1 second pulse interval. The PBAE-DNA complexes (2 μg of each) were added to 10 μg of DOTAP liposomes and the mixture was allowed to incubate at room temperature for 30 minutes. PBAE-DNA polyplexes and DOTAP-DNA lipoplexes were also made at 1:1 and 5:1 weight ratios, respectively, and were used as controls.

The formed PBAE-DNA polyplexes, DOTAP-DNA lipoplexes, and PBAE-DNA-DOTAP lipopolymplexes were characterized for particle size and surface charge. Hydrodynamic particle diameter was measured by dynamic light scattering at 25°C, at a wavelength of 657.0 nm at an incident angle of 90° using a Brookhaven Instrument’s (Holtsville, NY) ZetaPALS® instrument. Surface charge (zeta potential) was also measured using ZetaPALS instrument at 25°C. The nanovectors were dispersed in deionized water and the zeta potential values were measured at the default parameters of dielectric constant, refractive index and viscosity of water.

Transmission electron microscopy (TEM) images were taken on a JEOL JEM1010 (Peabody, MA). Samples were placed on a grid and negative stained using 1.5% phosphotungstic acid at pH 6.0.

**Stability of DNA in Lipopolymplexes**

Agarose gel electrophoresis was performed to assess the stability of DNA in various formulations after processing and in the presence of DNase-1 (Invitrogen, Carlsbad, CA). Samples were prepared and incubated with DNase-1 according to the
manufacturer’s procedure. After the incubation period, the reaction was stopped by addition of ethylenediaminetetraacetic acid (EDTA). DNA was decomplexed from the lipid / polymer with 5.0 M NaCl and subsequently precipitated in ethanol. One set of samples was treated with DNAse-1 before precipitation and one set was treated after precipitation to show the stability of pre-complexed DNA against DNAse-1 induced degradation.

**Cytotoxicity Evaluations**

To assess the cytotoxicity profile of the lipopolyplexes and the components an MTS assay and an MTT assay were run on ECs and SMCs, respectively since SMCs did not produce any color change with the MTS assay (data not shown). ECs were trypsinized according to the manufacturer’s procedure and placed in 96 well plates at a cell density of 5,000 cells per well in cell growth media as determined by counting viable cells after staining with trypan blue (Biowhittaker Walkersville, MD). After allowing the cells to grow to approximately 70% confluency, the media was removed and the cells were washed once with sterile filtered phosphate buffered saline pH 7.4 (PBS). ECs were treated with DOTAP only liposomes, PBAE, and lipopolyplexes to achieve concentrations similar to the reagent levels in a 1.0 μg dose of lipopolyplexes in 1.0 ml of serum free media. The study was performed in phenol-free Cellgro complete low serum media (Mediatech Herndon, VA). After a 6 hour incubation, the cells were washed thrice with sterile filtered PBS and incubated with CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) From Promega (Madison, WI) for 4 hours. After incubation plate was read for absorbance at 490 nm on a Bio-Tek Synergy HT plate reader (Winooski, VT). Untreated EC’s were used as a control. SMC’s were treated
similarly to EC’s and plated at a cell density of 4,000 cells per well in smooth muscle cell growth media. After 6 hour incubation, the cells were washed thrice with PBS and incubated with MTT formazan (Sigma-Aldrich, St. Louis, MO) for 3 hours. After 3 hours the MTT reagent was removed and the cells were lysed with DMSO (Fischer Scientific Waltham, MA) and the absorbance was measured at 590 nm on a Bio-tek Synergy HT plate reader.

**Cellular Uptake and Distribution Studies**

*Cell Culture Conditions:* Human aortic EC and SMC were purchased from Cell Applications Inc. (San Diego, CA). EC and SMC were grown in specific cell culture media designed for each cell line by Cell Applications at 37°C with 5% CO₂. The cells were only used until passage 6 to minimize the effects of phenotypic alternations with additional passages on the uptake and transfection results.

*Cellular Uptake and Distribution:* To fully understand the uptake of the particles rhodamine-PE incorporated DOTAP liposomes were made by incorporating 0.5% rhodamine-PE into the liposomes during film formation and subsequent hydration. DNA used in this experiment was a plasmid encoding for eNOS since using the EGFP-N1 plasmid used in previous studies could confound the results. Prior to DNA complexation with PBAE the DNA was labeled with PicoGreen® reagent (Invitrogen, Carlsbad, CA). Lipopolyplexes were then made resulting in images showing the tracking of the phospholipid (red) and the DNA (green). In all of the samples, the nuclei were stained blue with Hoechst 33245 dye (Invitrogen, Carlsbad, CA). Images were obtained on an inverted Nikon Eclipse TE 200 microscope fitted with a mercury arc lamp. The excitation (ex) / emission filters (em) were as follows ex: 360/40 em: 460/50 (blue), ex: 470/40, em:
515/30 (green), em: 560/55 ex: 645/75 (red). Cells were plated in a 6-well plate and dosed with 1.0 μg plasmid DNA that was complexed to form lipopolyplexes. The time-points considered for this study were 20 minutes, 40 minutes, 1 hour, 2 hours, 4 hours, and 6 hours. Differential interference contrast (DIC) images were taken for all samples to show morphological changes throughout the experiment. Fluorescence confocal images were acquired using a Nikon TE-2000U scanning fluorescence confocal microscope (Melville, NY) at 2 hours to confirm that the particles are within the cell and not on the cell surface with a slice thickness of 1μm. The Z-axis stack plot was generated using NIH Image-J software (Bethesda, MD). In addition to microscopy, a FACS study with rhodamine-labeled lipopolyplexes was studied over time to get a quantitative measure of the amount of particles taken up over a 6 hour period. Cells were treated with lipopolyplexes and then washed with sterile filtered phosphate-buffered saline (PBS), trypsin-EDTA, and fixed in 4% paraformaldehyde. Using a BD Biosciences FACScaliber (San Jose, CA) equipped with an argon 488 laser. The FL2 channel (585/42 emission) was used to detect cells containing rhodamine-labeled lipopolyplexes. A total of 10,000 events were counted within a gated region. Results obtained were analyzed using CellQuest Pro® software.

**In Vitro Transfection Studies with EGFP-N1 Plasmid**

*Quantitative Transfection Studies:* To assess the ability of the lipopolyplexes to transfect human arterial cells, FACS analysis was carried out. Untreated cells were used as a control to set up the gating and to adjust the forward and side scatter. The cellular debris was gated out of the sample. A total of 10,000 events within the gated region was collected for each sample. Cells were plated in 6-well plates and allowed to grow to 70%
confluency to minimize the effect of cell growth and division on transfection. On day of treatment, the EC and SMC were incubated with each treatment for 6 hours. The cells were then washed with sterile PBS thrice to remove any particles that were not endocytosed and the samples were then placed in an cell culture incubator at 37°C with 5% CO₂. Periodically at 24, 48 and 72 hours post-administration of the control and lipopolyplex samples, the cells were trypsinized and centrifuged. Media and trypsin were removed and the cell pellet was re-suspended in 4% paraformaldehyde/sterile PBS (Fischer Scientific Waltham, MA). The cell samples were placed in sheath fluid and analyzed for transfection on a FACScaliber flow cytometer ((BD Biosciences, San Jose, CA). GFP expression was detected using the FL-1 channel (530/30 emission). Data was analyzed using the Cell Quest Pro® software. The mean fluorescence activity was determined by taking the average fluorescence of the green positive cells. Additionally, an ELISA was used to determine the amount of GFP being produced in the cells. Cells were plated and grown in 6-well plates until approximately 70% confluent. Cells were treated similarly as previously described. Due to the low relative amounts of GFP expected in the Lipofectin® and PBAE samples, one six well plate was used per sample. The samples were lysed with 600 μl of lysis buffer with protease inhibitors on ice, and stored at -80°C until use. Using React-Bind anti-GFP antibody coated plates (Pierce, Rockford, IL) and adding a GFP secondary antibody reactive to alkaline phosphatase, very low levels of GFP can be detected. Briefly, cell supernatants were incubated with the anti-GFP coated plates for 24 hour at 4°C, after washing with PBS - 0.5% Tween®-80, GFP secondary antibody was added to each plate and was allowed to incubate for 2 hours. The plates were washed with PBS-Tween-80® again and alkaline phosphatase was
added and allowed to react for 30 minutes, 0.5 N NaOH was added to stop the reaction after 30 minutes. The plates were read on a Bio-Tek Synergy HT plate reader with KC4 software. The total cellular protein was determined by lysing the cells with Triton®-X100, centrifugation to remove cell membrane, and assaying the cytosolic protein levels with NanoOrange® kit (Invitrogen, Carlsbad, Ca), according to the manufactures instructions.

Qualitative Transfection Studies: To confirm results of the FACS and ELISA fluorescence microscopy was examined in both EC and SMC. The cells were grown in 6 well plates over microscope cover slips. Cells were treated as noted above. After transfection samples were incubated for 48 hours, after 48 hours the cells were washed with sterile PBS. The cover slips were removed and the cells were fixed and mounted onto microscope slides with Fluormount G® (Southern Biotechnology Associates, Inc., Birmingham, AL). Fluorescence and DIC images were taken at 10x magnification for SMC and 20X for EC on an Olympus BX61 (Center Valley, PA). Differential interference contrast (DIC) and fluorescent microscopy images were taken for each time-point with the same exposure time.

Results

Development of Lipopolyplex Formulations

DOTAP liposomes post sonication had a particle size of 93.9 ± 11.0 nm with a zeta potential of 12.9 ± 6.10 mV. PBAE polyplexes had a particle size of 222.7 ± 37.8 nm with a zeta potential value of 17.8 ± 1.3 mV. The DOTAP lipoplexes had a particle size of 157.6 ± 50.0 nm and a zeta potential of 25.4 ± 11.0 mV. The lipopolylex size increases to 673.9 ± 90.8 nm with a zeta potential of 34.1 ± 13.5 mV. Particle size of the
DOTAP liposome, the lipoplex, and the polyplex for size and surface charge are summarized in Table 3. PBAE polyplex TEM image shows a dark core with what is possibly DNA extending from it (Figure 5a). The sizes of these structures are approximately 200 nm. The TEM image in Figure 5b shows a dark core of approximately 250 nm in size, with a lighter corona extending to a total of 500 with a very wide distribution of particle sizes.

**DNA Stability Studies**

To confirm DNA stability of the formulated particles, the complexes were incubated with DNAse-I. The DNAse-I was neutralized with 0.25 mM EDTA and the complexes were destabilized with 5.0 M NaCl, the DNA was precipitated out using ethanol and the pellets were reconstituted in TE buffer. The DNA was analyzed via gel electrophoresis. Figure 6 shows the agarose gel with lanes 3 through 5 depicting DNA that was protected from DNAse. Lanes 7 through 9 showed no bands, indicating that after de-complexation, the DNA is no longer stable in the presence of DNAse.

**Cytotoxicity Studies**

To ensure the formulation is sufficiently biocompatible for cellular transfection studies, the cytotoxicity profile of the formulation and its components were tested in both SMC’s and EC’s (Figure 7). Smooth muscle cell cytotoxicity was measured with an MTT assay. Results from the smooth muscle cells show no cytotoxicity at the doses equivalent to that which would be experienced when a 1.0 μg DNA dose is administered. Endothelial cell cytotoxicity was measured by an MTS assay. Similar to the SMCs no significant toxicity was observed by any of the components in ECs.
Table 3:
Particle size and surface charge of the PBAE-DOTAP-DNA formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle Size (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOTAP Liposomes (post sonication)</td>
<td>93.9 ± 11.0</td>
<td>12.9 ± 6.1</td>
</tr>
<tr>
<td>PBAE : DNA 1:1 (w/w)</td>
<td>222.7 ± 37.8</td>
<td>17.8 ± 1.3</td>
</tr>
<tr>
<td>DOTAP : DNA 5:1 (w/w)</td>
<td>157.6 ± 50.0</td>
<td>25.4 ± 11.0</td>
</tr>
<tr>
<td>PBAE : DOTAP : DNA 1:5:1 (w/w)</td>
<td>673.9 ± 90.8</td>
<td>34.1 ± 13.5</td>
</tr>
</tbody>
</table>

Values are average ± SD (N=3)
Figure 5: TEM images of (A) DNA and PBAE complexed at a 1:1 ratio and, (B) PBAE, DNA and DOTAP complexed at a 1:5:1 ratio.
Figure 6: Gel electrophoresis of Naked DNA, DNA (lane 1), DNAse treated naked DNA (lane 2), DNAse treated then precipitated / extracted PBAE complexed at a 1:1 polymer to DNA ratio (lane 3), DNAse treated then precipitated / extracted DNA and DOTAP complexed at a 5:1 ratio (lane 4), DNAse treated then precipitated / extracted PBAE, DOTAP and DNA complexed at a 1:5:1 ratio (lane 5), precipitated naked DNA (lane 6), Precipitated / extracted then DNAse treated PBAE complexed at a 1:1 polymer to DNA ratio (lane 7), Precipitated / extracted then DNAse treated DNA and DOTAP complexed at a 5:1 ratio (lane 8), Precipitated / extracted then DNAse treated PBAE, DOTAP and DNA complexed at a 1:5:1 ratio (lane 9).
Figure 7: Percent viability of human aortic cell types with assessed by MTT (SMC) and MTS (EC) assays. PBAE was dosed at 1 μg/ml, DOTAP liposomes were dosed at 5 μg/ml and the 1:5:1 PBAE:DOTAP:DNA ratio was dosed at 1 mg PBAE, 5 mg DOTAP liposomes and 1 mg DNA. DNA complexes were formed prior to addition to the cells. NS = no significance by ANOVA, * statistical significance $P = 0.05$ by ANOVA, ** statistical significance $P = 0.01$ by ANOVA. Error bars indicate mean ± standard error ($N = 8$). Bonferroni post hoc test used to assess significance.
Cellular Uptake and Distribution in EC and SMC

To assess the ability of the lipopolyplexes to effectively enter vascular cells, trafficking studies were performed in SMC’s and EC’s (Figure 8). At 20 and 40 minutes post-administration, SMCs did not show very high levels of cell uptake by fluorescent microscopy. As time progressed, the particles accumulated within the SMCs.

The yellow color observed was due to a co-localization of green (PicoGreen®-complexed DNA) and red (rhodamine-lipids), indicating the co-localization of the lipopolyplexes and the DNA. This can be observed between one and four hours. It can be seen in the images that the particles move towards the nucleus in the cells as time progresses, with the last time-point showing the highest amount of perinuclear red stain. Early time points (1h, 2h and 4h) show some distance between the red / green lipopolyplexes and the blue nucleus. The 6 hour time-point shows a large amount of red surrounding the nucleus. DIC images throughout the experiment show no change in the morphology of the particles, indicating no cellular toxicity, confirming results seen in the MTS and MTT assays. At 6 hours, a high degree of dissociation between the red lipid and the green DNA is observed. There are sections where co-localization of the lipopolyplex and the DNA can be observed, but the majority of the lipopolyplexes were separated since the red lipid and green DNA are not co-localized. Arrows are drawn on the 6 hour EC time-point in Figure 8. The red arrow shows lipid only, the green arrow shows DNA only, and the yellow arrow shows co-localization of the green and red, and the cyan arrow shows co-localization of the DNA and the nucleus.
Figure 8: DIC and fluorescent images of smooth muscle cell and endothelial cell uptake study, time-points taken at 20 minutes, 40 minutes, and 60 minutes 2 hours, 4 hours and 6 hours. Green color is labeled DNA, red color is labeled lipid, yellow color is co-localized DNA and lipid, blue is stained nucleus, and cyan is co-localized DNA and nucleus.
Data from the FACS analysis (Figure 9) confirms the results seen via microscopy. The FACS data for particle uptake shows that there is very little fluorescence at 30 minutes. At longer duration, the amount of fluorescence intensity increased with the greatest amount of fluorescence occurring in the 6 hour time point.

Similar to the SMC’s, the EC’s did not show a very high level of fluorescence at early time points. Up to 1 hour there are low levels of fluorescence observed with high levels of co-localization. Of note is the degree of co-localization for the EC’s is lower than in the SMC’s. Early time points (i.e., 20 minutes, 40 minutes, and 60 minutes) show some green signal, indicating release of the DNA from the lipopolyplex very soon after uptake. To confirm that the particles were indeed taken up and not on the surface a Z-axis stack was performed at 2 hours (Figures 10a and b). From the figure one can see that the particles are indeed within the cell and not on the surface of the cell based on the single bell shaped curve generated. If the particles were not in the center of the cell one would expect a U-shaped curve with a valley between 2 maximum intensities.

At later time-points, there was a higher degree of particles accumulating within the cell. By the 6 hour time point there is still some DNA / lipid co-localization, but for the most part the DNA has separated from the lipopolyplexes. Of note in the 6 hour sample is the presence of cyan in some of the cells, indicating the green co-localized with the blue, indicating that the DNA has successfully penetrated into the nucleus. The FACS data (Figure 9b) for the ECs show that there was very little fluorescence within the cells at early time-points (30 minutes, 1 hour, 2 hours). Later time-points show an increase in fluorescence intensity.
Figure 9: Lipopolyplex uptake in smooth muscle cells (a) and endothelial cells (b) assessed by FACS, the x-axis is FL-2 which is the red channel, the y-axis is the number of cells counted, time-points represented are 30 minutes, 1 hour, 2 hours, 4 hours and 6 hours.
Figure 10: Z-scan intensity profile in smooth muscle cells (a) and endothelial cells (b) showing that most of the fluorescence is coming from center of cell indicating that particles are indeed inside the cell and not on the cell surface; slices 5-13 and 6-21 are the cell interior for SMC and EC respectively. Slice thickness is 1μm.
**In Vitro Transfection Studies with Reporter Plasmid**

*Quantitative Transfection:* Results from these studies show transfection occurring at a variety of doses (0.25 μg to 2 μg DNA), given to the cells (Figure 11). When comparing the mean fluorescence intensity of the cells, smooth muscle cells have the strongest signal intensity at 1.5 μg dose, whereas endothelial cells show a peak at 1 μg dose. Based on these results, a dose of 1.0 μg was chosen for all *in vitro* evaluations of the lipopolyplexes.

SMCs were exposed to lipofectin, 1:1 PBAE polyplexes, 5:1 DOTAP lipoplexes and 1 : 5 : 1 PBAE / DOTAP lipopolyplexes complexed with 1 μg of DNA. After 6 hours of incubation in serum free media, the cells were washed three times with sterile PBS and were incubated for 24, 48 and 72 hours. Figure 12a shows the percent transfected cells and the mean fluorescence intensity of smooth muscle cells over 72 hours, respectively. To show the advantage of the lipopolyplexes, each of the components were also analyzed (PBAE polyplexes and DOTAP lipoplexes). Lipofectin® was used as a comparison.

The graph shows little difference in percent transfection at the first time point. By 72 hours, samples containing Lipofectin® and polyplexes alone have fallen to very low % of cells transfected (~2-3%). Lipopolyplexes and lipoplexes show a very high percentage of transfection at 72 hours ~25% and ~16%, respectively. The mean fluorescence intensity derived from the same samples indicates that cells transfected with lipofectin show very low levels of fluorescent intensity.
Figure 11: Dose range determination for DOTAP-PBAE lipopolyplexes assessed by FACS in endothelial and smooth muscle cells. Data represented as percent transfection and mean fluorescence intensity. 10,000 cells were counted to get the mean values.
Figure 12: Smooth muscle cell transfection assessed by FACS (a). Lipofectin® PBAE polyplexes, DOTAP liposomes and DOTAP PBAE lipopolyplexes % transfection versus time in hours. Bar graph (b) is mean fluorescence intensity for the 4 formulations over 72 hours. 10,000 cells were counted to calculate mean. ELISA data (c) presented in ng of GFP produced per mg of total protein extracted from cells over time (24, 48 and 72 hours) formulations tested were Lipofectin® (white dots, black background), PBAE polyplex (white open), DOTAP liposome (black diagonal lines) and DOTAP PBAE lipopolyplexes (black horizontal lines). * indicates P <0 .001 by ANOVA with Bonferoni post-hoc test. Values are mean ± standard error (N=3)
To assess the quantity of GFP being produced in the SMCs an ELISA was used (Figure 12c). The ELISA data showed that the highest level of GFP per mg of protein was in the early time points with cells treated with DOTAP liposomes and PBAE DOTAP lipopolyplexes. Cells treated with PBAE polyplexes and Lipofectin® produced very little protein.

To confirm that this delivery system will work in ECs as well, the same transfection studies were carried out in SMCs. The DOTAP lipoplexes and the DOTAP-PBAE lipopolyplexes transfected the cells the most effectively with a peak percent transfected cells occurring at 48 hours (Figure 13a). Lipofectin® and PBAE showed very little transfection throughout these experiments. Fluorescence intensity showed results similar to the % transfection, with the DOTAP lipoplexes and the DOTAP PBAE lipopolyplexes exhibiting the highest level of fluorescence and the Lipofectin® and PBAE showing negligible amounts of fluorescence.

To measure the amount of GFP produced per milligram of total protein extracted, an ELISA was performed similarly to the SMCs (Figure 13b and c). The protein expression was very similar as what was observed in microscopy and FACS showing no GFP in Lipofectin® and PBAE transfected cells and very high levels of expression in DOTAP lipoplexes and DOTAP PBAE lipopolyplexes. The expression levels for the DOTAP PBAE lipopolyplexes remained high up to 72 hours but there was a sharp decrease in the DOTAP lipoplex treated sample.

**Qualitative Transfection:** DIC microscopy images of the smooth muscle cells 48 hours after treatment show that morphologically the cells do not seem to be exhibiting
Figure 13: Endothelial cell transfection assessed by FACS (a), Lipofectin® PBAE polyplexes, DOTAP liposomes and DOTAP PBAE lipopolyplexes % transfection versus time in hours. Bar graph (b) is mean fluorescence intensity for the 4 formulations over 72 hours. 10,000 cells were counted to calculate mean. ELISA data (c) presented in ng of GFP produced per mg of total protein extracted from cells over time (24, 48 and 72 hours) formulations tested were Lipofectin® (white dots, black background), PBAE polyplex (white open), DOTAP liposome (black diagonal lines) and DOTAP PBAE lipopolyplexes (black horizontal lines). * indicates P < 0.001 by ANOVA with Bonferroni post-hoc test. Values are mean ± standard error (N=3)
any type of toxicity (Figure 14a). There may be some cell debris in the DOTAP lipoplex sample, but it is not conclusive. The images do show the same trend as the FACS data, the lipofectin and the PBAE polyplex do not have cells with very high intensity. When acquiring these images it was difficult to differentiate between the control auto-fluorescence and the green from the GFP. With the DOTAP lipoplex there is a dim green signal and for the PBAE DOTAP lipopolyplex green cells can be clearly seen. There is a smaller percentage of cells that are fluorescing treated with the DOTAP lipoplexes, compared to the PBAE-DOTAP lipopolyplex.

Images for ECs were taken under a 20x objective 48 hours after treatment to have high numbers of cells in frame compared to 10X for the SMCs (Figure 14b). A higher magnification was used compared to the SMCs since the ECs are smaller in size compared to the SMCs. Morphologically there is no change in the cells after incubation with the formulations. Lipofectin® and PBAE show no fluorescence relative to the control sample. The DOTAP lipoplex and the DOTAP PBAE lipopolyplex each show green cells with more fluorescence in lipopolyplex-treated samples compared to lipoplexes.

Discussion

Pre-complexation with poly(L-lysine) and poly(ethyleneimine) to transfec vascular cells is more effective than non pre-complexed formulations [103-105]. The results from those studies indicated that polymer pre-complexation increases the transfection efficiency within EC and SMC. Using this rationale, we introduced the cationic biodegradable polymer PBAE as a DNA pre-complexing agent. PBAE was chosen because it is biodegradable, biocompatible and also
Figure 14: Smooth muscle cell (a) and endothelial cell (b) microscopy 48 hours after transfection images taken at 10x magnification for SMCs and 20x for ECs, control, Lipofectin® PBAE polyplex, DOTAP liposomes and DOTAP PBAE lipopolyplexes.
has been used in non-viral gene delivery applications due to its unique pH responsive solubility profile [107, 108]. The polymer is insoluble in aqueous medium at pH 6.5 or above. However, once the pH is lowered to below 6.5, such as in the endosomes/lysosomes, the polymer dissolves rapidly to release the encapsulated payload. Based on this property, we hypothesized that the lipopolyplex structure would break apart or loosen the cationic structure within the endosome facilitating endosomal escape and greater fractional accumulation of the plasmid DNA within the nucleus for higher transfection efficiency. The lipid from the lipopolyplex is likely to remain with the negatively charged DNA after release from the endosome, but since the polymer is no longer present, the structure of the lipopolyplex is not as stable and allows for more efficient DNA release from the cationic complex.

To better understand the size of the particles, DLS and TEM were performed. Both methods correlated well with one another. Particle size of the vectors is very important for uptake. It is known that particles under 250 nm in size are endocytosed via clathrin coated pits, whereas particles of 500 nm are endocytosed by calveole [106]. The size of the lipopolyplex is much larger than the lipoplexes or polyplexes alone measured by DLS. The increased particle size is likely due to the destabilization of the lipid bilayer of the liposomes resulting from the negative charge of the DNA. This leads to aggregation of the particles, which contributes to the polydispersity observed in the TEM images and DLS values. The surface charge of the vectors is also an important parameter. The presence of a positive charge allows the particle to enter the cell with high efficiency in vitro. In vivo excess cationic charge has been shown to aggregate when administered systemically [158]. The increased zeta potential was unexpected, but could
be due to the aggregation of charge of the particles. The lipoplexes can have higher
aggregation of charge within the same volume compared to the liposomes. Other groups
have seen similar variability in size and charge and attribute it to time induced
aggregation [105]. DNA stability is a critical parameter, since any degradation in the
DNA could affect the protein product. DNA that has been incubated with the PBAE,
DOTAP and PBAE / DOTAP mixtures are resistant to DNase I mediated degradation.
After de-complexation, the DNA is no longer stable in the presence of DNase. The
double bands observed are likely due to the presence of open circular and supercoiled
DNA after ethanol precipitation.

Since positively charged nanovectors have been associated with toxicity the
toxicity of each of the formulation components and the final formulation were evaluated.
The amount of lipid and polymer to dose 1 μg of DNA does not elicit any toxicity. A
number of groups have demonstrated that DOTAP lipoplexes do not cause inflammation
in a variety of disease states [159, 160]. Our results confirmed these observations. This
is critical in designing a successful gene delivery vector for restenosis since any
inflammatory response would lead to an increased amount of macrophages, which would
exacerbate a restenotic cascade [161].

Since there was no toxicity observed, the trafficking behavior of the cells was
investigated. Based on the microscopy, and the FACS data it is clear that the
lipopoloplexes enter the cell and that the DNA can separate from the lipopoloplex, which
is critical for the success of this formulation. Without separation of the DNA from the
lipopoloplex, the DNA would not be able to enter the nucleus which has been shown to
be critical in transfection [162, 163]. The lipopoloplexes readily enter SMC and EC in a
time dependent manner. Fluorescence intensity was lower in ECs when compared to SMCs. This is believed to be attributed to the size of the ECs compared to the SMCs. The ECs are smaller and are likely to have a smaller dose to cell ratio, resulting in less fluorescent particles taken up by a single cell. Based on these uptake results, it was decided to test the efficiency of transfection in each of these cell types.

Transfection was observed with lipopolyplexes in both SMC and EC with as little as 0.25 μg of DNA(Figure 11). Degree of transfection over time was performed with a 1μg dose of DNA. FACS data showed that the lipopolyplexes are the most efficient at transfecting both EC and SMC. The other vectors do transfect the cells, but it is not nearly as efficient as the lipopolyplexes. ELISA data confirmed this. The FACS data indicates that while lipofectin does transfect cells the levels of protein produced by the cells when transfected with this reagent is low. The same can be said for the polyplexes. The fluorescent intensity in DOTAP lipoplexes increases throughout the experiment. The lipopolyplexes are very high in green intensity likely due to a high level of GFP being produced from the cells. This intensity stays the same throughout the experiment. There does not seem to be a relationship between size of the nano vector and ability to transfect since the DOTAP lipoplexes and the DOTAP PBAE lipopolyplexes each have a very different size 157 nm and 673 nm, respectively. There was a decrease in the amount of GFP as compared to total protein as time progressed. This is attributed not only to a decrease in the amount of GFP in the cells as they double, but also in the amount of total protein extracted. Compared to the 24 hour time-point where an average of 9.6 mg of protein was extracted from the SMCs where the 48 and 72 hour time-points had higher protein extracted 16.1 mg and 13.0 mg, respectively. Since the cells had a longer time to
increase in cell number. At later time points, there was an increase in the amount of total protein extracted from the cells. Endothelial cells were also transfected the most when incubated with lipopolyplexes assessed by FACS and ELISA. One thing to note is the degree of transfection in the dose range transfection studies is higher than from the degree of transfection in the time dependent transfection studies, despite the same procedure. This may be attributed to the use of cells that were on a later passage [164]. When comparing the degree of transfection between the two cell types the SMC’s seem to produce less protein assessed by ELISA despite the similar rates of transfection measured by FACS (Figures 12 and 13). This could be due to a higher percentage of protein per cell in SMCs from actin and myosin, as of this date there are no reports comparing the amounts of protein for HAOSMCs and HAOECs so this cannot be confirmed. Microscopy data confirms the results observed with FACS and ELISA for both SMC and EC. Cells treated with the DOTAP lipoplex show a lower percentage of green cells compared to lipopolyplexes with little to no signal for the Lipofectin® and PBAE polyplex-treated samples.

Conclusions

This study examined the potential of PBAE-DNA-DOTAP lipopolyplexes as gene transfection reagent for human aortic EC and SMC. Our results show that the incorporation of PBAE pre-complexation of DNA followed by DOTAP modification enhances the transfection percentage and more importantly the amount of protein being produced by the EC and SMC as compared to PBAE polyplex or DOTAP lipoplexes alone. The higher charge in the lipopolyplexes compared to the lipoplexes and polyplexes likely enhances the uptake of the nanovector. The difference in size between
the different formulations does vary the route of uptake. Lipoplexes and polyplexes are likely taken up by clathrin coated pits; the lipopolyplexes are likely endocytosed by calveole, this is based on previous studies on the effect of size and particle uptake. To date no group has studied the primary route of uptake in EC and SMC. The delivery system did not show any signs of cytotoxicity at the low levels necessary to induce efficient \textit{in vitro} transfection. These results are very promising and could be used to improve the delivery of a variety of genes previously tested with Lipofectin®, Lipofectamine® 2000, and other non-optimized gene delivery systems for treatment of cardiovascular diseases, including coronary restenosis.
CHAPTER 5: CELLULAR UPTAKE AND TRANSFECTION STUDIES WITH LIPOPOLYPLEXES EMBEDDED IN GELATIN-BASED COATINGS ON STAINLESS STEEL SUBSTRATES

Introduction

We developed a pH-sensitive poly(beta amino ester) (PBAE) to pre-complex with plasmid DNA. After DNA condensation, DOTAP liposomes were added to the DNA/polymer complex to form a lipopolyplex. Due to the high transfection efficiency of these systems in human aortic smooth muscle cells (SMC), we believe that they would be an excellent non-viral vector for gene deliver in coronary artery restenosis lesion. The use of liposomes-coated onto stents has been investigated by a few groups [131-134]. Although these studies employed liposomes as agents to deliver low molecular weight drugs and not genes, the liposomes were directly coated on metal surfaces instead of embedding them in polymer coating. For polymer coating systems, a number of studies have described the use of bovine or porcine collagen [135-142]. For restenosis therapy, viral vectors have been embedded into hydrogel matrices and these constructs showed positive results in preclinical studies [165].

Throughout this study, we have used 50 mm external diameter stainless steel mesh disk as a surrogate for the stainless steel stent. To optimize lipopolyplex uptake and transfection studies upon immobilization in meshes, SMC were used as cells of interest.

The mesh surface was coated with green fluorescent protein (GFP) encoding plasmid DNA (i.e., EGFP-N1) in lipopolyplexes embedded in either type A or B gelatin matrix. As shown in Figure 15, the lipopolyplex-immobilized mesh was then incubated with SMC for evaluation of cellular uptake and GFP transfection efficiency.
Figure 15: Image of a proposed schematic of lipopolyplexes embedded within gelatin matrix. The lipopolyplexes are thought to be well dispersed throughout the entire matrix.
Materials and Methods

Materials

Round stainless steel mesh disks (316L) with a 50 mm external diameter were purchased from Goodfellow Corporation (Oakdale, PA). Plasmid DNA expressing GFP (i.e., EGFP-N1, 4.7 kB) was amplified and purified by Elim Biopharmaceuticals (Hayward, CA). Cationic phospholipid, 1,2-dioleoyl-3-trimethylammonium-propane chloride (DOTAP), and a fluorescent derivative of phosphatidylethanolamine [i.e., 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(Lissamine rhodamine-B-sulfonyl)] (rhodamine-PE) were both purchased from Avanti Polar Lipids (Alabaster, AL). Phosphate buffered saline (PBS, pH 7.4), type A gelatin (275 bloom strength), type B gelatin (225 bloom strength), Tween®80, and 2-(N-morpholino)ethanesulfonic acid (MES), fluorescein isothiocyanate (FITC) were all purchased from Sigma-Aldrich (St. Louis, MO). All other reagents and chemicals were obtained from Fisher Scientific (Milwaukee, WI). Aqueous solutions were prepared using deionized distilled water purified with Nanopure II water purification system (Barnstead/Thermolyne, Dubuque, IA).

Preparation and Characterization of Lipopolyplexes

DOTAP-PbAE-plasmid DNA (encoding for GFP) lipopolyplexes were prepared as previously reported in Chapter 4.

Synthesis of FITC-Labeled Gelatin and Preparation of DNA Entrapped Coatings

Type A or B gelatin was dissolved at 25 mg/mL in 0.1 M borate buffer (pH 8.5) and the amine groups of the protein were conjugated with FITC by reacting 0.75 mg/mL
FITC for 3 hours at room temperature according to a previously published protocol [152]. The resulting FITC-gelatin mixture was extensively dialyzed against deionized distilled water to remove unreacted FITC and freeze-dried. For coating on stainless steel mesh substrates, unlabeled and FITC-labeled gelatins were dissolved in deionized distilled water to a final concentration of 80 mg/mL.

The stainless steel meshes were cleaned thoroughly with deionized distilled water and dip-coated with either unlabeled or FITC-labeled types A and B gelatin. For lipopolyplexes immobilization in the gelatin coating, a known amount of lipopolyplexes was dispersed in the aqueous gelatin solution and the resulting mixture was used for dip-coating on meshes.

**Characterization of DNA-Containing Gelatin Coatings on Meshes**

Following optimization of gelatin coatings to insure uniform coverage, their weights and thickness values were measured both in the wet and dry states. For scanning electron microscopy (SEM) studies, the dried types A and B gelatin-coated meshes were observed with a Hitachi S-4800 (Pleasanton, CA) field emission scanning electron microscope at an accelerating voltage of 3.0 kV and a working distance of 11.3 mm. Epi-fluorescence microscopy images of the FITC-gelatin coated meshes were acquired using an Olympus BX61 (Center Valley, PA) fluorescent microscope. The dissolution of FITC-labeled gelatin coatings from the meshes was evaluated by incubating the meshes in phosphate buffered saline (PBS, pH 7.4) at 37°C and periodically measuring the concentrations of released gelatin by fluorescence spectroscopy using Bio-Tek Synergy® HT (Winooski, VT) microplate reader.
The loading efficiency and stability of plasmid DNA in lipopolyplexes immobilized in the gelatin coatings was a very important concern to insure maximum transfection efficiency in cells. The DNA loading efficiency was evaluated by degrading the gelatin coatings with 0.2 mg/mL protease solution in PBS at 37°C for 30 minutes. The released lipopolyplexes were decomplexed with 5.0 M NaCl solution and the plasmid DNA was precipitated in ethanol. PicoGreen® dsDNA fluorescence assay (Invitrogen, Carlsbad, CA) was used to quantitate the amount of released DNA and reported as both loading capacity (i.e., in μg/mg of gelatin) and efficiency (i.e., percent of added DNA). Loading efficiency was calculated with the following equation.

\[
\frac{\text{(Actual DNA loading Capacity)}}{\text{(Polymer coating weight) × (Theoretical DNA loading)}} = \% \text{ DNA loading efficiency}
\]

**In Vitro Release of Lipopolyplexes from Gelatin Coatings**

The plasmid DNA-complexed lipopolyplexes were prepared, as previously described, except 0.5% (w/w) rhodamine-PE was mixed with DOTAP during the lipid film formation step. PbAE-DNA complexes were then added to this lipid composition and hydrated to form lipopolyplexes. The rhodamine-labeled lipopolyplexes were then immobilized in type A and type B gelatin coatings on stainless steel mesh. Lipopolyplex release from gelatin coatings was evaluated by incubating the meshes in PBS at 37°C. Periodically, samples of the release medium were removed and the fluorescence intensity was measured with the Bio-Tek Synergy® HT microplate reader at the excitation and emission wavelengths of rhodamine.

**Stability of Plasmid DNA in Gelatin Coatings**
EGFP-N1 encoding lipopolyp lexes were embedded in type A and type B gelatin as described above. Meshes were coated with the lipopolyp lex / gelatin solutions and were placed in 10mg/ml protease for 24 hours at 37ºC. DNA decomplexed with NaCl and was precipitated by ethanol as noted previously. Samples were run on a 0.8% agarose gel (Invitrogen, Carlsbad, CA) and imaged on a Kodak imager.

**Particle size and Zeta Potential within the Gelatin Matrix**

Lipopolyp lexes were combined with type A and type B gelatin for a final concentration of DNA at 6.65 μg/mL of solution in 80 mg/ml gelatin. The resulting solutions were then diluted in DI water and measured for hydrodynamic particle diameter by dynamic light scattering at 25ºC, at a wavelength of 657.0 nm at an incident angle of 90º using a Brookhaven Instrument’s (Holtsville, NY) ZetaPALS® instrument. Surface charge (zeta potential) was also measured using ZetaPALS instrument at 25ºC.

**Lipopolyp lexes Uptake and Cellular Internalization in SMC**

*Cell Culture Conditions:* Human aortic SMC were purchased from Cell Applications Inc. (San Diego, CA) and were grown in smooth muscle cell culture media, also obtained from Cell Applications, in 95% O2/5% CO2 environment at 37ºC. For these studies, SMC were only used until passage 6 to minimize the effects of phenotypic alternations with additional passages on the uptake and transfection results.

*Lipopolyp lexes Uptake in SMC:* PbAE-DNA complexes were incorporated into the rhodamine-PE labeled DOTAP liposomes and the formed lipopolyp lexes were immobilized in gelatin coatings on stainless steel meshes. Quantitative cellular internalization of rhodamine-labeled lipopolyp lexes from gelatin coatings was evaluated.
by flow cytometry. SMC were plated in a 6-well microplate to a density of 200,000 cells per well. The lipopolyplexes immobilized meshes were then placed on top of the cells and incubated at 37°C in the cell culture incubator. After 30 minutes, 1 hour, 2 hours, 4 hours, and 6 hours after incubation, the meshes were removed and the cells were washed thrice with sterile PBS to remove any free gelatin or lipopolyplexes. The adherent cells were detached with trypsin-EDTA treatment and fixed with 4% paraformaldehyde. The uptake of rhodamine-labeled lipopolyplexes by SMC was evaluated using BD Biosciences FACScaliber® (San Jose, CA) flow cytometry instrument. A total of 10,000 events were counted for each cell population within the gated region and the results were analyzed using the CellQuest Pro® software.

**In Vitro GFP Transfection Studies in SMC**

*Quantitative Transfection Studies by ELISA:* For quantitative transgene expression in SMC, the cells were plated in a 6-well microplate to a density of 200,000 cells per well. The stainless steel mesh with EGFP-N1 plasmid DNA-containing lipopolyplexes was placed on top of the cells in the well in serum free media for 6 hours to allow for uptake. After 6 hours of incubation, the meshes were removed and regular SMC culture medium with serum was added. After 24 hours, 48 hours, and 72 hours post incubation with lipopolyplexes-immobilized meshes, the cells were detached with trypsin-EDTA treatment and lysed with 600 μL of surfactant based lysis buffer with protease inhibitors. The cytosolic fraction was isolated and stored at -80°C.

GFP specific enzyme-linked immunosorbent assay (ELISA) was used to determine the amount of protein expressed as a function of time. The ELISA plates were prepared with Corning’s Costar® high binding affinity plates (Lowell, MA), which were
incubated with 100μl of 1:24000 anti-eGFP monoclonal antibody (Novus Biologics, Littleton, CO) for 2 hours. After extensive washing and blocking the non-specific binding sites, 100μl of transfected SMC lysates were incubated for 24 hours at 4°C. Wells were washed extensively and an alkaline phosphatase conjugated anti-eGFP secondary antibody (Novus Biologics, Littleton, CO) was added to each plate and was allowed to incubate for 2 hours. Following this treatment, the plates were washed with PBS-Tween-80® and alkaline phosphatase reagent was added and allowed to react for 30 minutes. The reaction was stopped by addition of 0.5 N NaOH and the absorbance of a chromogenic product was quantified with a Bio Tek Synergy® HT microplate reader at a wavelength of 408nm. The total protein in the cell lysates was determined with the BCA (bicinchoninic acid) Protein Assay (Pierce, Rockford, IL) according to the manufactures instructions. GFP expression in SMC as a function of time was reported in ng of GFP per mg of total protein.

**Qualitative Transfection Studies:** To confirm the ELISA results fluorescence microscopy was performed on SMC with mesh coated in type A and type B gelatin containing EGFP-N1 encoding lipopolyplexes. Cells were grown in 6 well plates over microscope cover slips and were treated as noted previously. After transfection, samples were incubated for 48 hours, after 48 hours the cells were washed with sterile PBS. The cover slips were removed and the cells were fixed and mounted onto microscope slides with Fluormount G® (Southern Biotechnology Associates, Inc., Birmingham, AL). DIC and epi-fluorescence images were acquired with an Olympus BX61 microscope (Center Valley, PA).

**Data Analysis**
Error bars represent standard error of the mean. All experiments were repeated 3 times unless otherwise noted. ANOVA or students t-test was employed to assess statistical significance at a p level of 0.05. A Bonferroni post-hoc test was used for all ANOVA calculations. All of the data analysis was performed with Graph Pad Prism® (La Jolla, CA) software.

Results

Gelatin-Coated Mesh as a Model

Gelatin was able to coat the mesh evenly. Type A gelatin coatings formed a film between the mesh wires, type B gelatin films only coated the wires (Figure 16). Both SEM images and fluorescence images confirmed that. SEM images were not taken at high magnification since the beam degraded the coating. Coating weight for type A gelatin (74.01 ± 5.51 mg) was significantly higher than type B gelatin (19.98 ± 6.38 mg). The coating thickness for each of the films was similar, 16.28 ± 1.11 μm for type A and 13.83 ± 3.34 μm for type B.

Lipopolyplexes Immobilization in Gelatin-Coated Mesh

Lipopolyplexes were mixed with the gelatin solution and coated onto mesh. After incubation with protease the total amount of DNA was calculated. Mesh coated with type A gelatin had 1.02 ± 0.06 μg of DNA on the mesh equating to 0.013 μg of DNA per mg of gelatin. Theoretically 6.15μg of DNA could have been loaded onto the mesh, making the total loading efficiency for this system 16.7%. Alternatively, mesh coated with type B gelatin had a DNA loading of 0.99 ± 0.05 μg of DNA. This equates to 0.050 μg of DNA per mg of gelatin. The theoretical DNA loading and loading efficiency for
Figure 16: Fluorescence (top panels) and scanning electron microscopy images (bottom panels) of Type A (left panels) and type B FITC (right panels) gelatin coated onto 316L stainless steel mesh.
Table 4. The properties of gelatin coating on stainless steel meshes and the loading of plasmid DNA

<table>
<thead>
<tr>
<th>Gelatin Coating</th>
<th>Coating Weight (mg)</th>
<th>Coating Thickness (µm)</th>
<th>Theoretical DNA Loading Capacity (µg)</th>
<th>Actual DNA Loading Capacity (µg)</th>
<th>Percent DNA Loading Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type A Gelatin</td>
<td>74.01 ± 5.51*</td>
<td>16.28 ± 1.11</td>
<td>6.15</td>
<td>1.02 ± 0.06</td>
<td>16.70%</td>
</tr>
<tr>
<td>Type B Gelatin</td>
<td>19.98 ± 6.33</td>
<td>13.83 ± 3.34</td>
<td>1.67</td>
<td>0.99 ± 0.05</td>
<td>59.50%</td>
</tr>
</tbody>
</table>

*Mean ± S.D. (n = 3)
Figure 17: In-vitro release of FITC gelatin (A) and rhodamine lipopolyplexes (B) in 37°C PBS over 2 hours. Filled black squares represent release from type A gelatin, open white squares represent release from type B gelatin. Error bars indicate standard error of the mean (N = 3). * indicates (P<0.05) by T-test.
this system was 1.66 μg DNA and 59.5%, respectively. Table 4 summarizes the physical characterization and DNA efficiency.

**In Vitro FITC-Gelatin and Lipopolyplex Release Studies**

Both type A and type B gelatin displayed a burst release (Figure 17a). Type A gelatin took approximately 10 minutes to fully dissolve whereas type B gelatin dissolved within 5 minutes. Rhodamine-labeled lipopolyplexes displayed a more gradual release than gelatin alone (Figure 17b). Lipopolyplexes embedded within type B gelatin were released at a faster rate compared to lipopolyplexes embedded in type A gelatin.

**Stability of Immobilized DNA**

Lipopolyplexes maintained DNA integrity after embedding and being released into PBS. Figure 18 is a gel electrophoresis of DNA extracted from lipopolyplexes. Lane 1 is the ladder, lane 2 is eGFP-N1 plasmid, lane 3 is DNA from lipopolyplexes released from type A gelatin into PBS, lane 4 is DNA from lipopolyplexes released from type B gelatin into PBS. No degradation of DNA is observed after release of the lipopolyplexes from the gelatin matrix. The bands released from the gelatin travel down the gel at a slower rate than non-embedded plasmids.

**Particle Size and Zeta Potential of Lipopolyplexes in Gelatin Matrix**

The particle size of the lipopolyplexes was similar to what was previously observed, 511.6 ± 50.3 nm. When embedded in type A and type B gelatin the particle size changed to 711.5 ± 108.9 nm and 236.2 ± 73.5 nm, respectively. Zeta potential for the lipopolyplexes was 24.1 ± 3.0 mV. When embedded in type A and type B gelatin the
Figure 18: Gel electrophoresis (0.8% agarose) of DNA after release from gelatin hydrogel. Lane 1 is a supercoiled DNA ladder, lane 2 is eGFP-N1 plasmid, lane 3 is eGFP-N1 lipopolyplexes embedded in type A gelatin with no protease digestion, lane 4 is eGFP-N1 lipopolyplexes embedded in type B gelatin with no protease, lane 5 is eGFP-N1 lipopolyplexes embedded in type A gelatin 24 hours of protease digestion at 37°C, lane 6 is eGFP-N1 lipopolyplexes embedded in type A gelatin 24 hours of protease digestion at 37°C.
Table 5. Particle size of lipopolypelexes in gelatin solutions

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFP LPP</td>
<td>$511.6 \pm 50.3$</td>
<td>$24.1 \pm 3.0$</td>
</tr>
<tr>
<td>eGFP LPP / type A gelatin</td>
<td>$711.5 \pm 108.9$</td>
<td>$10.2 \pm 0.7$</td>
</tr>
<tr>
<td>eGFP LPP / type B gelatin</td>
<td>$236.2 \pm 73.5$</td>
<td>$7.9 \pm 3.9$</td>
</tr>
</tbody>
</table>

*Mean ± S.D. (n = 3)
zeta potential was 10.2 ± 0.7, mV and 7.9 ± 3.9 mV, respectively. Table 5 summarizes the size and charge data.

**Cellular Uptake and Distribution Studies**

Fluorescence microscopy was carried out to assess the ability of the lipopolyplexes to enter SMC after being embedded (Figure 19a). Particles embedded within either type A and type B gelatin did not show any appreciable uptake at 30 minutes. After 60 minutes particles began to enter the cell regardless of the type of gelatin they were embedded in. There was a much higher degree of uptake when embedded within type B gelatin during the early time points (60 minutes, 2 hours). By 4 hours both formulations showed uptake in all cells, samples incubated with type B coated mesh appeared to have a higher intensity than cells incubated with type A coated mesh. By 6 hours both samples were similar in intensity.

Flow cytometry was used to confirm the results seen in microscopy (Figures 19b and 19c). Cells incubated with type A mesh exhibited a gradual increase in the fluorescence intensity as time progressed. Cells incubated with type B gelatin coated mesh showed limited uptake initially, but by 2 hours there was a dramatic shift in fluorescence intensity. As time progressed there was an additional increase in intensity. As with the microscopy study samples there was a difference in the rate at which the lipopolyplexes were taken up. By 2 hours (lime green line) cells incubated with type A mesh show a much lower fluorescence intensity than cells incubated with type B mesh.

**Quantitative and Qualitative Transfection Studies**

Gene expression assessed by ELISA 24 hours after the start of transfection showed no difference in EGFP-N1 levels between lipopolyplexes embedded in either...
Figure 19: Particle uptake assessed by FACS (A) and microscopy (B). FACS data is color coded $T = 0$ - dark green, $T = 30$ minutes – orange, $T = 1$ hour – blue, $T = 2$ hours – lime green, $T = 4$ hours – pink, $T = 6$ hours – cyan. Lipopolyplexes embedded within type A gelatin is on the left, lipopolyplexes embedded within type B gelatin is on the right. Representative microscopy images from rhodamine lipopolyplexes embedded within type A mesh and type B mesh taken at 30 minutes, 1 hour, 2 hours, 4 hours and 6 hours. All images taken with the same exposure time.
Figure 20: Transfection of eGFP with lipopolyplexes embedded within type A and type B mesh 24, 48 and 72 hours after the start of transfection assessed by ELISA (A). Samples with no treatment (small black squares), type A gelatin embedded lipopolyplexes (large black squares), and type B gelatin embedded lipopolyplexes (horizontal lines). Error bars indicate standard error (n=3), statistics calculated by ANOVA with a Bonferroni Post hoc test. Representative microscopy images (B) taken 48 hours after the start of transfection. DIC images (left panels) and fluorescent images (right panels) of a no treatment group (top row), a group treated with lipopolyplexes embedded within type A gelatin (middle row), and a group treated with type B gelatin (bottom row). All images taken with the same exposure time.
type A or type B gelatin (Figure 20a). By 48 hours, there was a significant drop (P = 0.02) in the expression levels when embedded in type A gelatin. There was no change in expression levels with type B gelatin embedded lipopolyplexes. By 72 hours, GFP expression was reduced in both systems, but was higher than the background levels.

Microscopy taken at 48 hours showed cells with a higher intensity of GFP in cells incubated with the type B gelatin coated mesh (Figure 20b). These results corroborated with those seen with the ELISA.

Discussion

There have been a variety of different approaches to deliver the therapeutic genes for the treatment of restenosis, including nanoparticle based administration with a porous catheter, immobilization of naked DNA plasmid onto a stent, and immobilization of viral vectors onto a stent [83, 86, 95, 117, 119, 129]. It has been shown that the placement of a stent after removal of the atherosclerotic plaque reduces vessel recoil and keeps the vessel patent during early vessel remodeling highlighting the need for the placement of a stent to prevent restenosis ultimately changing the physiological response from the collapsing of the blood vessel with angioplasty to the growth of neo-intima with stents [166]. Approaches using naked plasmid were able to show transfection with very high levels of DNA (500 μg to 1 mg per stent), but these only considered reporter plasmid [118]. The use of viral vectors on a stent shows significant promise although the ability to develop a commercial product like this is limited because of the known immunogenic problems associated with viral vectors [95, 167].

Coatings applied to stainless steel for drug release have been extensively studied for cardiovascular applications. We have used stainless steel mesh as a platform to
develop a novel hydrogel-coated DNA delivery platform. In these studies, we investigated the effect of different types of gelatin and how they affect the potency of the lipopolyplexes. Gelatin was chosen because of its ability to promote endothelialization [168]. Previous to these studies there has been a lot of work being done with viral vectors or naked plasmid DNA embedded into fibrinogen, collagen, and gelatin [118, 119, 165]. Liposomes have been used to deliver drugs coated on medical devices such as catheters and stents [132-134]. Gene delivery using collagen matrices with lipoplexes and polyplexes has been used for tissue engineering [135-142].

The SEM and fluorescence microscopy images showed that both types of gelatin coated the mesh evenly. Type A gelatin was shown to bridge between the mesh wires where as type B gelatin only coated the individual wires. Coating thickness was similar between each of the gelatin types. The significant difference in coating weights is due to this extra gelatin that was sitting between the mesh wires effectively increasing the total surface area of the coating. Interestingly, the amount of lipopolyplexes loaded onto each of the mesh systems was similar. This is attributed to a charge repulsion between the cationic gelatin and the cationic delivery system [169].

The gelatin coating dissolved very rapidly from the surface of the mesh for both types of gelatin. In the scope of this study this is an advantage, as it is well understood that excess polymer at the site of a restenotic lesion has been attributed to an increase in inflammation [170]. There are between 1 and 2 RGD sequences within gelatin strands depending on where the parent collagen molecule is hydrolyzed. This presence of RGD allows gelatin strands to bind to α5β3 integrin receptors which are present on activated endothelial cells and have been shown to interact with SMC [171]. Any unbound gelatin
will be washed away in solution reducing the chance of an acute inflammatory response. The gelatin remaining at the surface of the cells promote a seeding layer for endothelial cells [168].

The lipopolyplexes followed a similar trend to the gelatin coating. Lipopolyplexes did release slower than gelatin alone. Possible explanations for this could be ionic interactions between the gelatin and lipopolyplexes or settling of the lipopolyplexes during the release [135]. In vitro release of this type of system may not accurately show what would happen in the presence of negatively charged cells or in-vivo.

To assess how the gelatin coatings affected endocytosis of lipopolyplexes into SMC, rhodamine-labeled lipids were used. Despite the little differences in the release of the two coating systems, there was a significant increase in the rate and extent of particle uptake. Particles embedded in the cationic gelatin exhibited a slower particle uptake. One possible rationale for this is that excess positive charge from the type A gelatin could be interfering with the ability of the cells to interact with the positively charged lipopolyplexes. The interaction between the negatively charged gelatin and the lipopolyplexes did not affect particle uptake. The rate uptake can also be explained by the particle size data. The vectors return to a non-agglomerated size of approximately 250 nm when embedded within the anionic gelatin. This smaller size likely contributes to the increase in the rate endocytosis. Lipopolyplexes embedded in cationic gelatin are larger than non embedded lipopolyplexes. Zeta potential values for both of the formulations were lower than non-embedded lipopolyplexes. This is believed to be due to the
viscosity of the gelatin slowing the movement of the particles. By 6 hours, the amount of particles endocytosed from both formulations was similar.

Initial rates of transfection were not affected by the difference in particle uptake since the samples were not washed until 6 hours after the start of the experiment. Six hours was chosen to be able to compare to previously published results. Twenty four hours after the start of transfection, protein levels were comparable to results obtained to lipopolyplexes not embedded within mesh [169]. Protein expression levels fell significantly in mesh coated with type A verses type B gelatin (p = 0.05). Cells incubated with type B gelatin coated mesh did not show a significant drop in protein expression levels. The microscopy data shows cells that fluorescence with a higher intensity when incubated with type B mesh. The total number of green positive cells is similar between the two formulation types. The additional cationic charge from the type A gelatin may be interacting with the lipopolyplex preventing full plasmid DNA decomplexation. The lack of decomplexation reduces the total amount of DNA available in the nucleus to be translated into protein. As the cells propagate the plasmid DNA in the nucleus is halved, reducing the amount of transfection. By 72 hours both of the expressed protein concentrations are similar to what was previously reported.

Conclusions

We have reported a novel way to deliver genes from stainless steel matrices. This approach is directly applicable to gene mediated restenosis treatment. A much larger amount of type A gelatin was able to be coated on the surface since the material bridged between the mesh wires. Despite the higher amount of gelatin deposited on the type A mesh, similar amounts of DNA were found on the mesh surface. The particles were still
able to be endocytosed though there was a difference in the rate of endocytosis between the two types of coatings. Coatings composed of type B gelatin allowed for a faster uptake of the particles. In a system where it will be exposed to a high shear environment, there is a clear advantage to a faster rate of endocytosis to reduce the amount of material washed away. Transfection results also showed an advantage to lipopolyplexes that were embedded in type B gelatin.

These results are the first report of a non-viral vector physically embedded within a polymer coated onto stainless steel. This has implications in developing a gene eluting stent without the need for a viral vector or excessive amounts of DNA.
CHAPTER 6: OPTIMIZATION OF STENT COATING AND PRELIMINARY IN VIVO EVALUATIONS OF GENE DELIVERY AND TRANSFECTION

1. Introduction

Based on the success of the in vitro coating work performed with type B gelatin, a similar coating system was developed for in vivo evaluation of gene delivery and transfection in rabbit iliac artery restenosis model. There are a number of changes that must be made to the type B gelatin coating for it to be successful as a stent coating. First, the coating must be stable for at least 24 hours prior to implantation since it would be too difficult logistically to implant the stents the same day that they are coated. Second, even though lipopolyplexes are highly potent transfection reagent, we will still require a higher dose than the 1 μg DNA dose that was used for in vitro studies to achieve sustained in vivo transfection and therapeutic efficacy. Based on previous studies, we anticipated that at least 10 μg of DNA dose would be required within the stent coating, as many groups used a much higher DNA dose [61, 83, 172]. The small surface area of the stent makes it difficult to embed a high DNA dose with lipopolyplexes in the liquid state within the coating system. Lastly, the coating must be stable during in vivo insertion and stent deployment to allow for sustained DNA release at the arterial lumen site over a prolonged period.

To overcome these issues, we lyophilized the lipopolyplexes in the presence of mannitol, a cryo-protective agent, in order to concentrate the DNA and allow for loading at higher concentrations. Additionally, we have investigated the role of poly(D,L-lactide-co-glycolide) (PLGA) coating on top of lipopolyplex-embedded type B gelatin layer to minimize loss during implantation in the iliac artery and allow for slow release over time.
2. Materials and Methods

2.1 Materials

Cationic phospholipid, 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), and a fluorescent derivative 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine-B-sulfonyl) (rhodamine-PE) were purchased from Avanti Polar Lipids (Alabaster, AL) and were dissolved in HPLC grade chloroform (Fisher Scientific, Milwaukee, WI). Plasmid DNA expressing GFP (i.e., EGFP-N1, 4.7 Kb) and endothelial nitric oxide synthase (eNOSpVAX-1, 9.5 Kb), (original sample kindly supplied by Dr. Duncan Stewart, Division of Cardiology, University of Toronto) were amplified and purified by Elim Biopharmaceuticals (Hayward, CA) [153]. Phosphate buffered saline (PBS, pH 7.4), Tween® 80, and 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Sigma-Aldrich (St. Louis, MO). CellTiter 96® AQueous one solution cell proliferation assay was obtained from Promega Corporation (Madison, WI). Human aortic EC and SMC were purchased from Cell Applications, Inc. (San Diego, CA) and grown in culture at 37°C and 5% CO₂ cells were grown in endothelial cell and smooth muscle cell growth media. All aqueous solutions and reagents were prepared in deionized distilled water using Barnstead/Thermolyne Nanopure II (Dubuque, IA) system.

2.2 Preparation and Characterization of the Lipopolyplexes

Lipopolyplexes were made as previously described in Chapter 4. After formation of the lipopolyplexes, the lipopolyplex solution was combined with different concentrations of mannitol, flash frozen and lyophilized for 48 hours.
2.3 *In Vitro* Transfection Studies with EGFP-N1 Plasmid

Lipopolyplex stability and potency after lyophilization was evaluated by ELISA as previously outlined. Briefly, SMCs were plated and grown in 6-well plates until approximately 70% confluent and were treated with 2 μg DNA. The samples were lysed with 600 μl of lysis buffer with protease inhibitors on ice, and stored at -80°C until use. Costar high binding affinity plates (Corning Incorporated Life Sciences, Lowell, MA) were incubated for 2 hours with anti-eGFP monoclonal antibody (Novus Biologics, Littleton, CO), after extensive washing and blocking, cell lysates were incubated for 24 hours at 4°C. Wells were washed extensively and an anti-eGFP secondary antibody (Novus Biologics, Littleton, CO) was added to each plate and was allowed to incubate for 2 hours. Plates were washed with PBS-Tween-80® again and alkaline phosphatase was added and allowed to react for 30 minutes, 0.5 N NaOH was added to stop the reaction after 30 minutes. Plates were read on a BioTek Synergy® HT (Winooski, VT) plate reader with KC4 software. Total protein was determined with the Pierce’s (Rockford, IL) bicinchoninic acid protein assay, according to the manufactures directions.

2.4 Stent Coating and Characterization

Balloon-mounted Legend-Mini® stainless steel coronary stents (10 cm x 2.5 cm) were purchased from Relysis Medical Devices Limited (Hyderabad, India). On the day prior to implantation, the stents were removed from the sterile packaging and coated in a sterile tissue culture hood. Stents were coated with reconstituted lipopolyplex encoding eGFP-N1 plasmid DNA with type B gelatin. Using a 200 μl pipet, 40 μl of the formulation to be coated was directly deposited on the surface of the balloon mounted stent. Stents were allowed to dry and placed in sterile bags and stored overnight at 4°C.
To assess coating integrity a rhodamine-labeled lipopolyplex solution with gelatin was coated on to a balloon mounted stent. The stent was mounted on a microscope slide and evaluated on an Olympus BX61 (Center Valley, PA) epi-fluorescent microscope. Fluorescent images were taken with a rhodamine filter set. White light images were taken with an external light source illuminating the stent from above. Images were taken at 2X magnification.

2.5 Modification of In Vitro Release Profiles

To study the effect of a PLGA coating on the stability of gelatin and lipopolyplexes, stainless steel coupons (small 2 cm square sections of stainless steel) were used to assess the release kinetics. Coupons were cleaned with acetone and warm water prior to coating. Using a micropipette, 40 μl of solution (either 80 mg/ml FITC labeled gelatin with 80 mg/ml mannitol or rhodamine-labeled lipopolyplexes (638 μg/ml DNA in 80 mg/ml gelatin solution) was placed on the surface of the coupon and allowed to dry. PLGA was dissolved in acetone (100 mg/ml and 200 mg/ml) and coated onto the dried coatings. A variety of different coating amounts were applied to assess the effect on release. After the second coating was allowed to dry, coupons were placed in 6-well plates and were incubated with 4 ml of 37°C PBS on a rocker for 6 hours to assess release of FITC gelatin and lipopolyplexes. Stents were coated similarly with 40 μl of rhodamine-labeled lipopolyplex in gelatin solution and allowed to dry at room temperature. Once the gelatin coating was completely dry, a PLGA layer was applied to the stent surface and allowed to dry as well. The release of lipopolyplexes from the gelatin-PLGA double coated stents was assessed as previous using stainless steel coupons.
2.6 DNA Loading and Stability on Stent Surfaces

Stents were coated with 40 μl of 638 μg/ml eNOS encoding plasmid DNA (eNOSpVAX1) in lipopolyplexes mixed with 80 mg/ml mannitol and 80 mg/ml type B gelatin as described above. The coated stents were placed in deionized distilled water with 2 mg/ml protease and left at 37ºC overnight. DNA precipitation was performed as previously described. Briefly, 5 M NaCl was added to the lipopolyplex solution resulting in a 0.5 M NaCl solution followed by 2 parts of ethanol and placed at -20ºC overnight. The precipitated DNA suspension was centrifuged for 15 minutes and the supernatant was removed. The pellet was reconstituted in TE buffer and analyzed using the PicoGreen® DNA assay according to the manufacturer’s directions. Fluorescence emission values were read on a Biotek Synergy® HT plate reader.

Stents for the DNA stability study were coated as described above. To assess the effect of PLGA coating on DNA release and stability, three different formulations were tested: (1) no PLGA, (2) 12 mg PLGA and (3) 24 mg PLGA per stent. The DNA for the stability study was extracted in a similar manner as the loading study, except that stents were briefly dipped in acetone to remove residual PLGA coatings. Extracted DNA was run on a 0.8% agarose gel and the bands were imaged with a Kodak imager.

2.7 Stent Implantation and Tissue Harvesting

Animal studies were performed according to an experimental protocol approved by the Institutional Animal Care and Use Committee at Northeastern University (Boston, MA, USA). Male New Zealand White rabbits (2.5 - 3.5kg body weight) were purchased from Millbrook Farms (Amherst, MA). The animals were housed singly and had access
to food and water *ad libitum* prior to surgery. Drinking water was supplemented with 0.07 mg/ml of aspirin (Sigma Chemical Co., St. Louis, MO) for all animals to reduce sub-acute thrombosis. Animals were allowed to acclimate for at least 48 hours prior to surgical procedure.

The rabbits were randomly divided into 2 groups for implantation of bare metal stents (*n* = 4) and eGFP-N1 lipopolyplex-coated stent (*n* = 6). Denudation of the iliac arterial endothelial layer and stent implantation was performed according to the published procedure of Welt *et al.*, [9]. Briefly, the animals were anesthetized with a combination of xylazine (5 mg/kg) and ketamine (35 mg/kg) administered by intramuscular injection into the anterior thigh. If necessary, additional xylazine and ketamine was administered at 1/3rd of original dose to ensure the animal remained sedated throughout the procedure. The animal was shaved and the surgical area was prepped with Betadine (7.5% Povidone®-iodine) and isopropyl alcohol. At the time of stent implantation, the animal was given an intravenous bolus of heparin (100U/kg) to prevent acute thrombosis. The femoral artery was exposed and a 1 cm section was isolated and ligated. An arteriotomy was performed in the femoral artery. Using a vein lifter (Becton-Dickinson, Franklin Lakes, NJ), the vessel was propped open and a three French Fogarty balloon catheter (Edwards Life Sciences, Irvine, CA) was inserted and fed up to the abdominal aorta, passing the iliac artery. The balloon was filled with 0.6 ml of air and passed through the iliac artery 3 times to ensure full endothelial denudation. The balloon-mounted stent catheter was fed in through the femoral artery and placed in the iliac artery. The stent was expanded with 8 atmospheres of pressure and left in place for 30 seconds; the balloon was deflated and subsequently removed.
2.8 Quantitative and Qualitative Evaluations of GFP Transfection

On day of sacrifice, the animals were first sedated with ketamine/xylazine and sacrificed with high dose pentobarbital (50 mg/kg). Immediately following sacrifice, the abdominal aorta was ligated and phosphate buffered saline was flushed through the bottom half of the animal for at least 10 minutes. Stent implanted iliac arteries were harvested from the animals for quantitative and qualitative evaluation of GFP transfection. Transfection efficiency at the mRNA level was determined by rtPCR. Briefly, RNA was extracted from the vessels with TRI reagent (Sigma-Aldrich) according to the manufacturer’s instructions. The primers used to amplify the cDNA were AGCTGGACGGCGACGTAAAC and TCAGCTCGATGCGGTTCACC. cDNA synthesis was performed with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). The PCR cycle was as follows: 95°C for 1 minute, 59°C for 1 minute and 72°C for 1 minute. This was repeated 50 times and the resulting DNA segment was run on a 1.2% agarose gel and imaged. The expected fragment size was 400bp.

Qualitative evaluations of GFP expression was examined by using the whole vessel and tissue cryosections. The vessel was imaged using an Olympus BX61 (Center Valley, PA) epi-fluorescent microscope. GFP expression was evaluated with a FITC filter set. All images were taken with the same exposure time. After images were taken, the stent was removed and the vessel was halved, one section was flash frozen and used for rt-PCR, the other section was fixed and used for histology. Additionally, formalin-fixed vessels were embedded, sectioned and placed on glass microscope slides. Sections were imaged on an Olympus BX61 (Center Valley, PA) epi-fluorescent microscope. To minimize auto-fluorescence, the images were taken at the same exposure time.
3. Results

3.1 Stability of Lyophilized Lipopolyplexes

The lipopolyplexes embedded within type B gelatin were able to transfect when stored at 4°C and -20°C for 24 hours (Figure 21). At room temperature there was complete loss of ability to transfect when assessed by ELISA. Samples stored at 4°C did not lose any potency when compared to samples that were analyzed immediately after coating. Samples stored at -20°C showed a significant decrease in potency as compared to samples stored at 4°C.

To assessed the in vitro plasmid DNA stability and transfection potential in lipopolyplexes after lyophilization a series of 3 experiments were conducted. For all of these experiments, the SMC received a 1 μg dose of DNA. The first experiment looked to assess the effect of mannitol, used as a cryoprotective agent with lipopolyplexes during lyophilization, on transfection. A total of 5 formulations were tested and the DNA:polymer:lipid weight ratio remained consistent throughout this procedure at 1:1:5 as examined previously. The formulations contained 53.2 μg/DNA with no mannitol, 26.6 μg/ml DNA with 160 mg/ml mannitol, 26.6 μg/ml DNA with 80mg/ml mannitol, 26.6 μg/ml DNA with 40 mg/ml mannitol, 53.2 μg/ml DNA with 80 mg/ml mannitol, and 79.8 μg/ml DNA with 160 mg/ml mannitol.

The results in Figure 22a show that formulations containing higher amounts of mannitol were able to transfec cells most efficiently. The second experiment was to
Figure 21: Transfection of eGFP with lipopolyplexes embedded within type B gelatin coated mesh 24 hours after the start of transfection assessed by ELISA (A). Samples with stored at 20°C (small black squares), 4°C (large black squares), and -20°C (horizontal lines). Error bars indicate standard error (n=3), statistics calculated by ANOVA. * = (P = 0.05) by Bonferini post-hoc test.
assess how high of a DNA concentration can be used and still achieve transfection. All formulations for this study contained 160 mg/ml mannitol. The DNA concentrations were 79.8 μg/ml, 156.9 μg/ml, 319.2 μg/ml and 638.4 μg/ml. The data from this study showed no difference between the four formulations tested (Figure 22b), indicating that DNA could be concentrated up to 638.4 μg/ml without any loss of activity. The final experiment in this study was to evaluate the role of mannitol in lyophilized formulations with high DNA concentrations. Since the 79.8 μg/ml DNA with 160 mg/ml mannitol formulation was run for the previous experiments, it was again used as an internal standard for this experiment (Figure 22c). Results from this study showed that as mannitol concentration increased, the ability to transfect cells also increased. The transfection levels for this study were markedly lower than for the previous study. It should be noted that the cells used for this study were in their 6th passage. The results in Figure 22d show the relationship between theoretical stent loading and DNA concentrations.

3.2 Stent Coating Evaluations

When observed using a bright-field and fluorescence microscope (Figure 23), the stent coated with lipopolyplexes and gelatin showed a very nice even coating. Light microscopy indicates that the gelatin-embedded lipopolyplex coating covers the stent struts and the balloon. The fluorescence microscopy shows that the rhodamine-labeled lipopolyplexes are evenly distributed throughout the stent coating.
Figure 22: Effect of lyophilization on transfection of lipopolyplexes. ELISA performed for 14 formulations in 3 experiments. Experiment 1 (A) formulations, no mannitol 53.2 μg/ml DNA (small checkers), 160 mg/ml mannitol 26.6 μg/ml DNA (large checkers), 80 mg/ml mannitol 26.6 μg/ml DNA (horizontal lines), 40 mg/ml mannitol 26.6 μg/ml DNA (vertical lines), 80 mg/ml mannitol 53.2 μg/ml DNA (diagonal upward lines), 160 mg/ml mannitol 79.8 μg/ml DNA (diagonal downward lines). Experiment 2 formulations, 160 mg/ml mannitol 79.8 μg/ml DNA (large checkers), 160 mg/ml mannitol 159.8 μg/ml DNA (horizontal lines), 160 mg/ml mannitol 318.2 μg/ml DNA (vertical lines), 160 mg/ml mannitol 638.4 μg/ml DNA (diagonal upward lines). Experiment 3 (C) formulations, 160 mg/ml mannitol 79.8 μg/ml DNA (small checkers), 40 mg/ml mannitol 638.4 μg/ml DNA (large checkers), 80 mg/ml mannitol 638.4 μg/ml DNA (horizontal lines), 160 mg/ml mannitol 638.4 μg/ml DNA (vertical lines). Graph D is the theoretical load of DNA on a stent at different lipopolyplex concentrations.

Error bars indicate standard error (n=3).
Figure 23: Brightfield (left panels) and fluorescent (right panels) microscopy images of a bare metal stent (top panels) and a stent coated with type B gelatin, mannitol and rhodamine labeled lipopolyplexes (bottom panels).
3.3 Modification of Gelatin and Lipopolyplex Release by PLGA Coating

PLGA was able to modify FITC gelatin release from stainless steel coupons as shown in Figure 24. Dip coating of the coupon in a 200 mg/ml PLGA acetone solution yielded a marginal delay in release of FITC from the stainless steel. Direct deposition of 20 µl PLGA at either 100 mg/ml or 200 mg/ml yielded similar results to the dip coating. When 30 µl and 40 µl of PLGA solutions in acetone were deposited on the gelatin film, there were variable results. The additional PLGA retarded the release of the gelatin, but not in a reproducible manner. The 40 µl of 100 mg/ml solution of PLGA was found to delay the release of the gelatin more than the other formulations tested. Increasing the volume to 60 µl for both PLGA solutions a shifted release for the 200 mg/ml solution with very little change to the release with the 100 mg/ml solution.

The release of lipopolyplexes from a stainless steel coupon was performed with a 200mg/ml PLGA solution only (Figure 25). These results were similar to what was seen with the FITC gelatin except that the release was spread over a longer period of time. The stainless steel coupons released lipopolyplexes at the fastest rate when these were dip-coated. As the PLGA amount was increased from 4 mg to 8 mg, and then to 12 mg, the release of the lipopolyplexes was delayed in an amount dependent manner.

The results of lipopolyplex release from a stent differed from the results obtained with the stainless steel coupons (Figure 26). Although the release was delayed directly in relation to the amount of PLGA deposited, the overall delay was only a few minutes as compared to 2 hours with the stainless steel.
**Figure 24:** In-vitro release of FITC gelatin coated stainless steel coupons dip coated in 200 mg/ml PLGA/acetone (black diamonds), and after deposition of 3 mg of PLGA deposited at 100 mg/ml PLGA/acetone (gray squares), 4 mg of PLGA deposited at 100 mg/ml PLGA/acetone (dark gray triangle), 6 mg of PLGA deposited at 100 mg/ml PLGA/acetone (black X), 4 mg of PLGA deposited at 200 mg/ml PLGA/acetone (gray star), 6 mg of PLGA deposited at 200 mg/ml PLGA/acetone (light gray circle), 8 mg of PLGA deposited at 200 mg/ml PLGA/acetone (gray +), 12 mg of PLGA deposited at 200 mg/ml PLGA/acetone (light gray line). All samples run in triplicate, error bars indicate standard error of the mean.
Figure 25: In-vivo release of rhodamine labeled lipopolyplexes from type B gelatin coated onto a stainless steel coupon. Samples received no additional PLGA coating (gray diamonds), 4 mg of PLGA (light gray squares), 8 mg of PLGA (gray triangle), 12 mg of PLGA (dark gray X), and a dip coating in 200 mg/ml PLGA/acetone (gray star). All samples run in triplicate, error bars indicate standard error of the mean.
Figure 26: In-vitro release of rhodamine labeled lipopolyplexes from type B gelatin coated onto a stainless steel stent. Samples received no additional PLGA coating (diamonds), 24 mg of PLGA (triangles), 8 mg of PLGA (squares). All samples run in triplicate, error bars indicate standard error of the mean.
3.4 DNA Loading and Stability on Stents

The amount of DNA found on the stent was 15.85 ± 0.15 μg DNA / stent. This equates to a loading efficiency of 62.11% ± 0.60%. The extracted DNA does appear to be stable after coating onto the stent with or without PLGA (Figure 27). The stability results showed that the DNA bands were running slower than the control eNOSpVAX1 plasmid band. This may be due to the complexation of plasmid with excess gelatin.

3.5 In Vivo GFP Expression

Reverse transcriptase PCR confirmed the results observed with microscopy (Figure 28). It should be noted that there were problems with repeatability within this study. During stent insertion a significant amount of the coating was washed away with by arterial blood. Gross examination of the vessels under fluorescent microscopy showed varying amounts of transfection within the vessel. Some vessels displayed a high level of fluorescence while others showed little to none. All images were taken at the same time of exposure to reduce the possibility of auto-fluorescence. Figure 29 shows the differences in vessel transfected with eGFP-N1 expressing lipopolyplexes and a bare metal stent control vessel. Additionally, 10 μm cryosections of the vessels in Figure 30 show a very high intensity of GFP directly below the stent strut. There was also significant fluorescence signal in the area adjacent to the stent strut. These sections show that there was a high concentration of fluorescence in the medial layer within the vessel. No fluorescence signal was observed in the adventitia.
Figure 27: Gel electrophoresis (0.8% agarose) of pVAX-1 eNOS plasmid DNA after release from gelatin matrix coated on a stent. Lane 1 is a supercoiled DNA ladder, lane 2 is pVAX-1 eNOS plasmid, lane 3 is pVAX-1 eNOS lipopolyplexes embedded in type b gelatin with no PLGA coating, lane 4 is pVAX-1 eNOS lipopolyplexes embedded in type b gelatin with a 12mg PLGA coating, lane 5 is pVAX-1 eNOS lipopolyplexes embedded in type b gelatin with a 24mg PLGA coating. Samples were exposed to 24 hours of protease digestion at 37°C.
Figure 28: eGFP rtPCR gel electrophoresis after 30 cycles. Lane A – DNA ladder, Lane B – GFP transfected vessel, Lane C – control.
Figure 29: Fluorescent microscopy of stented vessels after bare metal stent implantation and gelatin lipopolyplex-coated stent implantation.
Figure 30: Representative bright field and fluorescence microscopic images (4x and 10x) of 10 μm cryo-sections from stented rabbit iliac vessels.
4. Discussion

To successfully develop a gene eluting stent, there were a number of criteria that had to be met. Coating stability was one of key endpoints to developing a successful coating since the stents had to be coated well in advance of implantation. Coatings stored at 4°C showed no changes in transfection efficiency when compared to results from previous chapters. The freezing of the coating likely produced ice crystals that broke apart the lipoplex structure, flash freezing may have avoided this but would have been difficult to do aseptically [173].

The second key endpoint for developing a successful stent coating was to concentrate the DNA to coat sufficient amounts on the stent. The stainless steel mesh had a total of surface area of 2,500 mm². This compares with the surface area of a balloon mounted stent of only 68 mm². Lipopolyplexes were lyophilized with mannitol since it is a well known cryoprotectant. Results from the first experiment showed that increased amounts of mannitol preserved transfection efficiency at all DNA concentrations (Figure 22). The second experiment from this study indicated that the lipopolyplexes are able to be concentrated up to 638 μg/ml of DNA without any loss in transfection efficiency. The third experiment studied the effect of mannitol concentration with 638 μg/ml of DNA. The transfection efficiency for this experiment was much lower than for the previous experiments, despite having 2 formulations that were repeated. The results indicated that there was a small change in transfection efficiency when the mannitol concentration was reduced from 160 mg/ml to 80 mg/ml to 40 mg/ml. Despite the higher transfection efficiencies with 160 mg/ml mannitol, the high concentration of sugar would yield a coating that was very flaky and dissolved very rapidly. The most
concentrated DNA formulation was chosen for the coating since the theoretical amount of DNA that could be loaded onto the stent was at least 2 fold higher than any other formulations. The target amount of DNA loaded onto the stent was between 10 and 20 μg. This is between 50 to 100 fold lower than previous studies that utilized plasmid DNA on a stent [118, 119]. Assuming a loading efficiency of 60% based on the mesh studies the only concentration tested that would provide that amount of DNA on the stent was the 638 μg/ml DNA formulation.

Stents coated with 40 mg/ml mannitol, 40 mg/ml type B gelatin and 638 μg/ml DNA lipopolyplexes displayed a very even coating with very homogeneous distribution of lipopolyplexes with no visible crystals of mannitol within the stent coating as observed by fluorescence microscopy (Figure 23). During femoral insertion of the coated stents a significant amount of the coating was washed away by arterial blood. Problems with reproducibility have been reported by other groups who have used lipoplexes and polyplexes embedded within collagen sponges for sustained release [135]. GFP was observed microscopically in transfected vessels. Vessel sections showed that the media was the area of highest transfection, with the highest level of transfection near the stent strut. These results are comparable to previous studies that used plasmid DNA at 50 to 100 fold higher concentrations and viral based systems [95, 114, 117-119, 124, 125, 136]. rtPCR confirmed the results from the microscopy studies indicating that there was eGFP-N1 mRNA being produced by the transfected vessels. An ELISA was performed on the vessels and a statistically significant amount of eGFP-N1 was found, but it was well below the limit of detection of the assay, hence the data was not presented.
Despite these promising results, there was still a lot of variability within the data. To minimize a source of variability a secondary coating of 50:50 PLGA was used to evaluate the effect on burst release. Other groups have used this approach with stents to reduce burst release [118, 119]. As expected an additional layer of PLGA prevented the release of the gelatin and lipopolyplexes in a concentration dependent manner. A coating applied by dip coating did not change the release profile of the gelatin or lipopolyplexes. The most reproducible gelatin release results were from the series containing 200 mg/ml PLGA in acetone. This series was then used to evaluate the effect of lipopolyplex release from the matrix. There was a very gradual decrease in the rate of release as the amount of PLGA on the coupon was increased. Based on these studies a PLGA concentration of 200mg/ml was chosen and coated onto a stent. The change in release from the stent is much less than on the stainless steel coupon. This can be attributed to the geometry of the stent. The cylindrical shape of the stent and the smaller surface area to coat the gelatin onto changes the efficiency of the PLGA. Any areas that degraded faster allowed for a greater percentage of released lipopolyplexes.

The addition of PLGA to the stent coating did not affect the amount of DNA coated on the stent. The loading efficiency of the stent is similar to what was observed previously on the mesh and well within the target goal of 10-20 μg of DNA per stent. DNA released from the stents after PLGA coating showed no plasmid degradation. The bands migrated slower than expected, but this was most likely due to gelatin’s viscosity impeding the migration of the band on the gel.
5. Conclusions

The transition from an *in-vitro* model for the treatment of restenosis to an *in vivo* model required three key characteristics.

1. The coating must be stable for at least 24 hours prior to implantation.
2. The stents would need to have at least 10 μg of DNA within the coating.
3. The stent coating must be stable until the stent is deployed within the animal

We have shown that stability of the lipopolyplexes at 4°C is appropriate for a stent coating. Using lyophilization and mannitol concentrated lipopolyplexes can be made at 638 μg DNA / ml of solution. When combined with gelatin and coated onto a stent there is sufficient amount of DNA / vector on the stent to assure transfection with no anticipation of cytotoxicity either locally or systemically. Despite promising results coating stability was an issue, by adding PLGA we were able to modify the release of the lipopolyplexes ensuring that they are able to be deployed at the target site. Based on these criteria the optimized coating of 638 μg DNA/ml, with 80 mg/ml mannitol, and 80 mg/ml gelatin with 12 mg of PLGA should be ideal for use as a therapeutic in restenotic vessels in rabbits.
CHAPTER 7: IN VIVO ENDOTHELIAL NITRIC OXIDE SYNTHASE ENCODING GENE DELIVERY AND TRANSFECTION EFFICIENCY USING LIPOPOLYPLEX-IMMOBILIZED STENTS

Introduction

Previous results showed that 5:1:1 DOTAP : PBAE : DNA lipopolyplexes embedded within a type B gelatin matrix coated onto a stent had limited success. The addition of a PLGA exterior showed excellent lipopolyplex release profiles. This coating allows for more of the lipopolyplex dose to reach the site of stent expansion without being washed away. The gene encoding for eNOS has been shown to inhibit restenosis in a number of studies [94, 99-102, 174, 175]. This chapter focuses on the in-vivo evaluation of transfection with eNOS lipopolyplexes in the New Zealand rabbit model of restenosis.

Materials and Methods

Materials

Cationic phospholipid, 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), and a fluorescent derivative 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine-B-sulfonyl) (rhodamine-PE) were purchased from Avanti Polar Lipids (Alabaster, AL) and were dissolved in HP LC grade chloroform (Fisher Scientific, Milwaukee, WI). Plasmid DNA expressing endothelial nitric oxide synthase (eNOS pVAX-1, kindly supplied by Dr. Duncan Stewart, Division of Cardiology, University of Toronto, Toronto, Canada) was amplified and purified by Elim Biopharmaceuticals (Hayward, CA) [153]. Phosphate buffered saline (PBS, pH 7.4), Tween® 80, acetone and 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Sigma-Aldrich (St.
Mannitol was purchased from Acros Organics (Morris Plains, NJ). Lactel 50 : 50 PLGA (0.17 dL / g viscosity) was purchased from Durect (Pelham, AL). All aqueous solutions and reagents were prepared in deionized distilled water using Barnstead/Thermolyne Nanopure II (Dubuque, IA) system.

**Preparation of Lipopolyplexes**

Lipopolyplexes were made as outlined previously [169]. Briefly, 2.0 μg DNA was pre-complexed with 2.0 μg of PBAE was incubated for 30 minutes with DNA in 100 μL MES buffer at pH 6.0 as previously described [107, 108]. Cationic liposomes were prepared by evaporating a solution of DOTAP in chloroform at 10 mg/mL using a rotary evaporator (Buchi model number R200) at a pressure of 300 milliTorr, for 30 minutes with a water bath temperature of 50°C. Samples were then placed on a lyophilizer (Labconco Freezone 6 plus) overnight to remove any residual solvent. The lipid film was re-hydrated and then sonicated on ice with a Sonics Vibracell® (Newtown, CT) probe sonicator for 10 minutes at a power rating of 30% with a 1 second pulse interval. The PBAE-DNA complexes (2 μg of each) were added to 10 μg of DOTAP liposomes and the mixture was allowed to incubate at room temperature for 30 minutes. Twenty-four ml of the lipopolyplex solution was added to 250 μl of a 160 mg/ml mannitol solution. The solution was flash frozen in liquid nitrogen and lyophilized for 48 hours. Post lyophilization the resulting powder was reconstituted with 250 μl DI water and 250 μl of 160 mg/ml type B gelatin solution resulting in a 80mg/ml gelatin solution containing 638 μl of DNA / ml with 80 mg/ml mannitol.
Stent Coating

Balloon mounted Legend Mini stents (10cm x 2.5cm) were purchased from Relysis Medical Devices Limited (Hyderabad, India). On the day prior to implantation the stents were removed from the sterile packaging and coated in a sterile tissue culture hood. Stents were coated with one of 3 coatings, 80 mg/ml gelatin with 80 mg/ml mannitol (gelatin only), 80 mg/ml gelatin with 80 mg/ml mannitol and 638 μg/ml of eNOS plasmid DNA (gelatin plasmid), and reconstituted lipopolyplex solution encoding eNOS (LPP gelatin). Using a 200 μl pipet, 40 μl of the formulation to be coated was directly deposited on the surface of the balloon mounted stent. Stents were allowed to dry and then were coated with 12 mg of PLGA dissolved in acetone at 200 mg/ml concentrations. The second coatings were allowed to air dry under sterile conditions and the coated stents were placed in sterile bags and stored overnight at 4°C.

Stent Expansion and Coating Integrity

To assess coating integrity a rhodamine lipopolyplex solution was coated on to a balloon mounted stent. The stent was expanded while mounted on a microscope slide on an Olympus BX61 (Center Valley, PA) epi-fluorescent microscope. Fluorescent images were taken with a rhodamine filter set. White light images were taken with an external light source illuminating the stent from above. Images were taken at 2X and 4X magnifications expanded at 0 atm, 2 atm, 4 atm, 8 atm after 5 seconds and 8 atm after 30 seconds. The stent was expanded with Basix IN3125 inflation device filled with DI water (Merit Medical, South Jordan, UT). Scanning electron microscopy images of the expanded stent were also taken to confirm what was observed with the epi-fluorescent images. The sample was sputter coated with approximately 10 nm platinum / palladium.
(80:20) using a Denton DV502 Vacuum Evaporator. Images of the stent were taken with a Hitachi S-4800 (Pleasanton, CA) field emission SEM at 3.0 kV with a working distance of 10mm.

**Stent Implantation and Tissue Harvesting**

Animal studies were performed according to the procedure in Chapter 6 with the following changes.

Animals received stents bilaterally in the iliac artery. This study contained 4 groups; a bare metal stent group (n = 10 vessels), a gelatin only coated (gelatin only) stent group (n = 10 vessels), a naked eNOS plasmid embedded in gelatin (plasmid gelatin) coated stent group (n = 10 vessels), and an eNOS lipopolyplexes embedded in gelatin (LPP gelatin) coated stent group (n = 10 vessels).

On day of sacrifice animals were sedated with ketamine / xylazine. Animals were sacrificed with 0.5 ml/kg of pentobarbital (Nembutal®) given intravenously. Immediately following sacrifice, the abdominal aorta was ligated and phosphate buffered saline was flushed through the bottom half of the animal for at least 10 minutes. Vessels were harvested, the stents were removed, the vessels were sectioned in half and immediately flash frozen in liquid nitrogen and stored at -80°C for transfection evaluation.

**Measurement of eNOS mRNA by Quantitative PCR**

Quantitative RT-PCR was performed on the other half of the iliac artery tissue to assess the levels of mRNA transcript after transfection. The sequences of the primers used are shown in Table 6. Primers for the rabbit housekeeping gene HPRT were designed as previously reported [176]. RNA was extracted by a single step extraction
Table 6: Primers for Quantitative PCR

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>Human eNOS 1</td>
<td>5'-CCCACCAGCGCCAGAACACA-3'</td>
</tr>
<tr>
<td>Human eNOS 2</td>
<td>5'-CCCCTCTGGGGGCTGGGTAA-3'</td>
</tr>
<tr>
<td>Rabbit HPRT 1</td>
<td>5'-CTCAACCTTAACCTGGAACAATGTC-3'</td>
</tr>
<tr>
<td>Rabbit HPRT 2</td>
<td>5'-CCTTTTCACCAGCAGGCT-3'</td>
</tr>
</tbody>
</table>
method [177]. cDNA was then made with the First Strand III synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Real time PCR (ABI 7900HT; Applied Biosystems, Foster City, CA) was performed on the cDNA using SYBR Green RT-PCR kit (Applied Biosystems). The results obtained from the RT-PCR reaction were then analyzed by comparative $C_t$ analysis for determination of relative human eNOS in the samples. PCR products were visualized on agarose gels.

**Measurement of Transfected eNOS by ELISA**

Vessels were harvested from the animals at 1 day ($N = 20$ vessels) or 5 days ($N = 20$ vessels). Half of the vessel was homogenized in lysis buffer (T-Per, Promega) and centrifuged to remove non-soluble cellular components. A Quantikine sandwich ELISA (R&D Systems, Minneapolis, MN, USA) for human eNOS was used to determine eNOS protein levels according to the manufacturer’s directions in triplicate for each sample. Human eNOS levels were normalized to total protein levels assessed with a BCA (bicinchoninic acid) Protein Assay (Pierce Rockford, IL).

**Measurement of Nitric Oxide by Griess Reaction**

Extracts generated for the ELISA were also used to determine nitric oxide levels indirectly. Nitric oxide levels were measured using a commercial Griess reaction kit (Cayman Chemicals, Ann Arbor, Michigan). The assay was preformed according to the manufacturer’s directions in triplicate for each sample. Nitric oxide concentrations were normalized to total protein levels in the extracts.
Data Analysis

Error bars represent standard error of the mean. All experiments were repeated 3 times unless otherwise noted. ANOVA was employed to assess statistical significance with a Bonferroni post-hoc test. All analysis was performed in Graph Pad Prism® (La Jolla, CA).

Results

Stent Expansion and Coating Integrity

Microscopic evaluation of the stent under bright field and fluorescence microscopy showed an even coating prior to stent expansion. As the balloon pressure increased no changes to the coating under bright field or fluorescence was observed up to 4 atmospheres (Figure 31a to f). After 5 seconds at 8 atmospheres, the standard deployment pressure, the ends of the stents began to expand (Figure 31g and h). After 30 seconds at 8 atmospheres the stent was fully expanded. Bright-field microscopy showed that the coating remained primarily on the stent struts after expansion. Some bridging of the coating was observed between the stent struts. Fluorescence microscopy showed an even distribution of lipopolypexles on the stent struts, though some lipopolypexles were found to be between the struts.

The even distribution of the coating on the balloon mounted expanded stent was confirmed by scanning electron microscopy (Figure 32). Most of the coating covered the stent struts, but there are areas where the coating spanned the entire distance between the struts. High magnification images showed areas of smooth coating with small crystals protruding from the coating surface.
Figure 31: pVAX-1 eNOS rhodamine lipopolyplexes embedded in type b gelatin with 12mg PLGA coated on a balloon mounted stent. Images (2x) were taken pre-expansion (A, B), at 2 ATM (C, D), 4 ATM (E, F), 8 ATM (after 5 seconds) (G, H) and 8 ATM (after 30 seconds) (I, J). 4x magnification images were taken after expansion (K, L, M, N).
Figure 32: pVAX-1 eNOS rhodamine lipopolyplexes embedded in type b gelatin with 12mg PLGA coated on a balloon mounted stent. SEM images were taken post expansion, at 30x (A), 400x (B), 900x (C), and 3000x (D).
Measurement of eNOS mRNA by Quantitative PCR

Quantitative PCR results are summarized in Figure 33a. At 1 day there is a 100-fold increase in eNOS mRNA levels in gelatin LPP coated stents compared to the eNOS mRNA in the bare metal stent group. All other groups show no increase in mRNA levels. This was also confirmed by the gel electrophoresis of the PCR products (Figure 33d). At 5 days the mRNA levels fall for gelatin LPP formulations. Plasmid gelatin formulations show an increase in mRNA transcripts. This is similar to what is seen in the gel electrophoresis (Figure 33e). Individual qPCR results for the 1 day (Figure 33b) and the 5 day (Figure 33c) were found to have a wide distribution of sample values for the 1 day gelatin LPP and the 5 day plasmid gelatin and gelatin LPP.

Measurement of eNOS by ELISA

An ELISA was used to measure eNOS protein levels after 1 and 5 days of transfection. Figure 34 summarizes eNOS protein expression levels. Negligible amounts of eNOS protein were found in the 1 day bare metal and gelatin only groups and the 5 day bare metal, gelatin only and gelatin plasmid groups. These amounts were below the limit of detection of this assay. At 1 day 50 pg eNOS / mg total protein was found in the gelatin plasmid group. At both 1 day and 5 days after transfection LPP gelatin stents were found to have approximately 125 pg eNOS / mg total protein. One day after transfection there was a significant amount of eNOS in gelatin lipopolyplex embedded stents (p = 0.05) when compared to stents coated with gelatin only. After 5 days there was a significant amount of eNOS present after lipopolyplex mediated transfection when compared to bare metal (p = 0.05), gelatin only (p = 0.01), and naked
Figure 33: Fold increase of eNOS mRNA levels in iliac vessel extracts assessed by qPCR (A). Samples ($N = 5$ per group) were analyzed 1 day and 5 days after implantation of bare metal stents (black bar), gelatin coated stent (gray bar), eNOS plasmid embedded in gelatin coated stent (dark gray bar), eNOS lipopolyplexes embedded in gelatin coated stent (light gray bar). Individual sample variability at 1 day (B) and at 5 days (C). Gel electrophoresis of the PCR products for 1 day (D) and 5 days (E). * indicates $P = 0.05$ assessed by ANOVA.
Figure 34: eNOS protein levels in iliac vessel homogenates normalized to total mg protein assessed by ELISA (A). Samples (N = 5 per group) were analyzed 1 day and 5 days after implantation of bare metal stents (black bar), gelatin coated stent (gray bar), eNOS plasmid embedded in gelatin coated stent (dark gray bar), eNOS lipopolyplexes embedded in gelatin coated stent (light gray bar). Individual sample variability at 1 day (B) and at 5 days (C). * indicates P = 0.05, ** indicates P = 0.01, *** indicates P = 0.001 assessed by ANOVA.
eNOS plasmid in gelatin ($p = 0.001$). Figures 34b and 34c show the variability of the ELISA with respect to each sample. The graphs show a high variability at 1 day for all formulations. By 5 days the variability falls for all of the formulations, particularly the lipopolyplex embedded in gelatin sample, which clearly shows high levels of transfection compared to the other groups.

**Measurement of Nitric Oxide Production**

To assess if the transfected nitric oxide synthase was active and producing nitric oxide a Griess reaction was used. Since NO has a short half life, the Griess reaction measures NO metabolites, nitrate and nitrite. **Figure 35** summarizes the Griess data. One day after transfection there was approximately 200 $\mu$M nitrite / mg protein for gelatin LPP stents compared to 110, 112 and 100 $\mu$M nitrite / mg protein for the bare metal, gelatin only and naked eNOS plasmid groups respectively. **Figure 35b** shows the sample variability between each of the groups. All groups displayed high sample variability. At 5 days the amount of NO in the bare metal, gelatin only, gelatin plasmid and gelatin LPP was approximately 90, 210, 100 and 150 $\mu$M nitrite / mg protein respectively. **Figure 35c** shows the variability for the Griess reaction at 5 days. The variability for the bare metal, gelatin plasmid and gelatin LPP groups was similar to the 1 day time point. The gelatin only group shows a very high variability with samples values ranging between 25 and 345 $\mu$M nitrite / mg protein.

**Discussion**

In-vivo stent based non-viral vector transfection has not been reported in the literature. A number of groups have used viral vectors and naked plasmid to transfected
Figure 35: Nitrite levels in iliac vessel homogenates normalized to total mg protein assessed by the Griess reaction (A). Samples (N = 5 per group) were analyzed 1 day and 5 days after implantation of bare metal stents (black bar), gelatin coated stent (gray bar), eNOS plasmid embedded in gelatin coated stent (dark gray bar), eNOS lipopolyplexes embedded in gelatin coated stent (light gray bar). Individual sample variability at 1 day (B) and at 5 days (C).
vessels from a stent [94, 95, 117-119, 123-127, 130, 178, 179]. Groups have looked at
the incorporation of liposomes on the surface of a stent for drug delivery [131, 133, 134].
Other groups have looked at embedding lipoplexes and polyplexes into collagen matrices
for gene therapy for tissue engineering applications [135-142]. We previously showed
that the addition of a PLGA layer on the stent surface allowed for a change in release
characteristics of the coating. Microscopy images showed an even distribution of the
lipopolyplexes within the stent that remained on the stent struts after normal expansion.
Scanning electron microscopy images showed a smooth coating that contained small
crystals. The crystals are likely due to mannitol that has crystallized after coating.
When compared to commercial coatings the coating we developed is not as smooth, is
thicker, and bridges between the stent struts [180]. The bridging of the coating can be
remedied by using a different coating method on a non-balloon mounted stent. Other
defects in the coating are similar to defects found in commercial coatings.

During insertion we previously saw coating loss without PLGA. The addition of
PLGA allowed for the insertion of a stent without loss of the gelatin coating. One day
and five days after transfection eNOS protein levels were found to remain constant for
LPP gelatin containing stents. Since eNOS must undergo a number of post translational
modifications a Griess reaction was run to ensure the protein was being encoded and
modified properly [93]. The Griess data for the LPP gelatin stents showed similar results
indicating an increased ability to produce NO. The eNOS mRNA levels for the gelatin
LPP were variable; the values were on average higher than the other groups. There are
reports of mRNA transcript variability after transfection with viral vectors encoding for
eNOS and iNOS [181]. Despite the variability the researchers still saw a significant
decrease in the neointima/media ratio. The results from these three assays indicate that eNOS plasmid, when coated onto stents bound to lipopolyplexes, is efficiently entering and transfecting the cells, producing sufficient amounts of protein that is able to increase nitric oxide levels.

The other experimental groups did not show any significant increases in protein, mRNA or NO levels. Naked plasmid embedded within gelatin did show a slight increase in protein and mRNA levels compared to the bare metal stent. Other groups did show that naked plasmids are able to transfect cells when embedded within gelatin albeit at much higher concentrations [118, 119, 136]. The stent coated in gelatin only showed an increase in NO levels at 5 days. When closely examined there was very high variability with that time point. Since gelatin is well known to attract endothelial progenitor cells (EPCs) the increased NO levels could be due an increase in EPCs [168]. The ELISA kit that was used for protein quantification would not have picked up rabbit derived eNOS. Another possible reason for increased NO levels in the presence of macrophages producing NO from iNOS though this was not confirmed in these studies.

There was no toxicity detected locally. At 5 days there was a decrease in the LDH levels for eNOS LPP coated stents, this may be attributed to an increased apoptosis of vascular cells after eNOS transfection observed by another group though the authors of that study acknowledge that these results are not consistent with the proposed mechanism of eNOS [182].

Conclusions

We have successfully developed a non-viral vector that can be coated onto a stent and delivered locally with no loss of transfection. The overall coating was able to remain
intact during implantation. The lipopolyplexes were well distributed over the stent coating. The transfection data indicate that human eNOS mRNA is being detected in the rabbit vessel at 1 and 5 days when transfected with eNOS lipopolyplexes embedded in gelatin. The ELISA results show human eNOS protein after transfection with the eNOS lipopolyplex coated stent. The presence of NO in the transfected vessels was confirmed by the Griess reaction. These results warrant evaluation to evaluate in-vivo efficacy of an eNOS lipopolyplex coated stent.
CHAPTER 8: IN VIVO THERAPEUTIC EFFICACY OF TRANSFECTED ENDOTHELIAL NITRIC OXIDE SYNTHASE IN RABBIT ILIAC ARTERY RESTENOSIS MODEL

Introduction

In vivo transfection results with eNOS lipopolyplexes embedded within a type B gelatin matrix coated on a stent with an additional PLGA layer showed very good results as shown in Chapter 7. The primary endpoint for this work is to evaluate a stent for efficacy in a relevant pre-clinical model. This chapter focuses on the in vivo evaluation of therapeutic efficacy of transfected eNOS with lipopolyplexes for the inhibition of restenosis in the New Zealand rabbit model of restenosis established in the iliac artery.

Therapeutic efficacy of transfected eNOS was measured by evaluating by suppression of smooth muscle cell (SMC) proliferation and re-endothelialization of the arterial lumen.

Materials and Methods

Materials

Cationic phospholipid, 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), and a fluorescent derivative 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine-B-sulfonyl) (rhodamine-PE) were purchased from Avanti Polar Lipids (Alabaster, AL) and were dissolved in HPLC grade chloroform (Fisher Scientific, Milwaukee, WI). Plasmid DNA expressing endothelial nitric oxide synthase (eNOS pVAX-1, kindly supplied by Dr. Duncan Stewart, Division of Cardiology, University of Toronto, Toronto, Canada) and pVAX-1 vector (Invitrogen, Carlsbad, CA) was amplified and purified by Elim Biopharmaceuticals (Hayward, CA)[153]. Phosphate buffered saline
PBS, pH 7.4), bromo-deoxyuridase (BrdU), acetone and 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Sigma-Aldrich (St. Louis, MO). Mannitol was purchased from Acros Organics (Morris Plains, NJ). Lactel 50 : 50 PLGA (0.17 dL / g viscosity) was purchased from Durect (Pelham, AL). All aqueous solutions and reagents were prepared in deionized distilled water using Barnstead-Thermolyne Nanopure II (Dubuque, IA) system.

**Preparation of the Lipopolyplexes**

Lipopolyplexes were made as outlined in previous chapters with the following changes. Post lyophilization the resulting powder was reconstituted with 250 μl deionized distilled water and 250μl of 160 mg/ml type B gelatin solution resulting in a 80 mg/ml gelatin solution containing 638 μl of eNOS DNA/ml with 80 mg/ml mannitol. Lipopolyplexes encoding pVAX-1 plasmid were prepared in the same manner as the eNOS plasmid lipopolyplexes.

**Stent Coating**

Stents were coated as outlined in chapter 7 with one of 3 coatings, 80 mg/ml gelatin with 80 mg/ml mannitol, reconstituted lipopolyplex encoding empty p-VAX1 vector, and reconstituted lipopolyplex with plasmid DNA encoding eNOS (pVAX1 eNOS). Once coated with gelatin the stents were allowed to dry and then were coated with 12 mg of PLGA dissolved in acetone (200 mg/ml). After the second coating was allowed to air-dry, the stents were placed in sterile bags and stored overnight at 4°C.
In Vivo Stent Implantation and Tissue Harvesting

Animal studies were performed according to previous chapters. Animals received stents bilaterally. This study contained 4 groups; a bare metal stent group (n = 5 vessels), a gelatin only coated stent (gelatin only) group (n = 5 vessels), a pVAX-1 empty vector lipopolyplex embedded in gelatin coated stent (empty vector LPP) group (n = 5 vessels), and an eNOS lipopolyplex embedded in a gelatin coated stent (eNOS LPP) group (n = 5 vessels).

Stent implantation and iliac denudation was performed as outlined in chapter 6. On day of sacrifice animals were sedated with ketamine / xylazine. Animals were injected with bromodeoxyuridine (BrdU, 50 mg/kg intravenous, Sigma Chemical Co) 30 minutes prior to euthanizing. A subset of the animals (n = 1 / group) were injected with Evans blue dye (Sigma Chemical Co) 30 minutes prior to sacrifice. Animals were sacrificed with 0.5 ml/kg of pentobarbital (Nembutal) intravenously. Immediately following sacrifice, the abdominal aorta was ligated and phosphate buffered saline was flushed through the bottom half of the animal for at least 10 minutes. Vessels were harvested and stored overnight at 4°C in formalin for efficacy evaluation by histology.

Measurement of Local Toxicity by Lactate Dehydrogenase (LDH) Assay

Local arterial toxicity was assessed by measuring lactate dehydrogenase levels at the tissue level. Supernatant from the homogenized transfection vessels were assayed using a QuantiChrom™ lactate dehydrogenase kit (Bioassay Systems, Hayward, CA) according to the manufacturer’s instructions. LDH levels were normalized to total protein in the supernatant.
Assessment of Re-endothelialization

After removal of Evans blue dyed vessels images were taken with a Powershot SD850 IS digital camera (Canon, Lake Success, NY) in macro mode. Images were taken of a fully denuded vessel, a vessel with an intact endothelium, a vessel with a bare metal stent, a vessel with a gelatin coating, a vessel with a pVAX-1 empty vector lipopolyplex gelatin coating and a vessel with an eNOS lipopolyplex gelatin coating (n = 1 for all groups) two weeks after balloon injury. Image analysis was performed by selecting pixels of similar color using the “magic wand” tool in Adobe Photoshop CS2 (San Jose, CA) at a threshold of 20. Images were then imported into Image J and converted to binary images. Area was measured using the measure particle command within Image J.

Vessel Sectioning and Histology

Vessels were removed 14 days after implantation. Formalin fixed stented vessels were transferred to 70% acetone for 24 hrs at 4°C. Next, vessels were then dehydrated in graded acetones (90% - 1X, 100% - 2X, for 1 hr each). All steps were carried out at 4°C as described by Kacena, et al [183]. Following dehydration vessels were placed in infiltration medium containing 85% destabilized MMA (Sigma, St. Louis, MO), 15% dibutyl phthalate (Sigma), and 0.15% benzoyl peroxide (Polysciences, Inc., Warrington, PA). This mixture was stirred for at least 2 hrs and filtered through CaCl2 prior to use. Vessels in infiltrate mixture were placed in a vacuum dessicator at 4°C for two to three days to facilitate infiltration.

After 2 to 3 days under vacuum, vessels were removed from infiltration MMA and transferred into 20 ml scintillation vials with pre-polymerized bases containing 3 ml of
catalyzed MMA (cMMA). cMMA was prepared in a manner identical to MMA infiltrate, the only modifications were increasing the concentration of benzoyl peroxide catalyst to 5% and stirring for 3 hrs prior to filtration. Vessels on the pre-polymerized bases were covered with fresh cMMA, the vials were capped tightly and placed in a container with dH2O covering 1/3rd of the scintillation vials (dissipates heat created by polymerization), and incubated for 2 to 3 days at 37°C in a radiant heat oven. Glass vials were removed from oven, incubated at -20°C for 1 hour and glass removed by breaking.

Specimen blocks were then trimmed and sanded on a metallurgical sander (Metaserve, Buehler, Germany), and 6 mm sections were obtained on an automated microtome (Leica 2165 Microtome, Leica, Heidelberg, Germany) using a D-profile tungsten-carbide knife (Dorn and Hart Microedge, Villa Park, IL). Sections were then placed on charged slides, covered with individual plastic sheets, clamped tightly and incubated at 60°C for 48 hours to properly adhere sections to slides. Vessel sections were stained with Verhoffs elastin stain and counter stained with Van Giesons stain and were examined with Olympus BX61 (Center Valley, PA). Bright-field images were taken at 4X and 10X. Morphometric analysis of the vessels was performed at 4x using Image J software to measure the intima and media.

**Vessel Injury Score**

Vessel injury scores were calculated according to a previous report by Schwartz et al., [184]. Vessel sections were examined at each stent strut and were given a numerical score (0-3) depending on the severity of the injury. **Table 7** summarizes the scoring system.
<table>
<thead>
<tr>
<th>Vessel score</th>
<th>Description</th>
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<tbody>
<tr>
<td>0</td>
<td>Intact internal elastic lamina (IEL), denuded endothelium. Media may be compressed with no lacerations.</td>
</tr>
<tr>
<td>1</td>
<td>IEL is lacerated with compressed media with no lacerations.</td>
</tr>
<tr>
<td>2</td>
<td>IEL and media lacerated, external elastic lamina may be compressed but not lacerated.</td>
</tr>
<tr>
<td>3</td>
<td>EEL lacerated, some stent struts may be residing in adventitia.</td>
</tr>
</tbody>
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**Tissue Immunohistochemistry**

Arterial tissue immunohistochemistry was performed on 5 μm methacrylate embedded sections (n = 3 per group) according to previously published reports [9, 165]. Briefly, antibodies for Ram-11, BRD-U, CD-31 and alpha-actin (Dako, Carpinteria, CA) were incubated with the sections after heat induced antigen retrieval in citrate buffer after protein blocking. A secondary anti-mouse HRP-linked antibody was incubated with the samples after removal of endogenous peroxide activity. Slides were developed with a Metal Enhanced DAB Substrate Kit for visualization (Pierce Rockford, IL). Specimens were counterstained with H&E and bright-field images were taken with an Olympus BX61 (Center Valley, PA) at 20x and 40x. Area with positive staining was counted and the values were tabulated at 40X magnification. Total cell counts were calculated for each region. All values are reported as positive stained cells divided by total number of cells.

**Data Analysis:**

Error bars represent standard error of the mean. All experiments were repeated 3 times unless otherwise noted. ANOVA was employed to assess statistical significance with a Bonferroni post-hoc test. All analysis was performed in Graph Pad Prism® (La Jolla, CA).

**Results**

**Measurement of Local Toxicity**

To assess local toxicity of all of the formulations LDH was measured from the homogenized vessel supernatant. For both 1 day and 5 days there was no significant change in LDH between any of the groups. **Figure 36** summarizes the LDH data.
Local LDH activity

Figure 36: LDH levels in iliac vessel homogenates normalized to total mg protein assessed by the Griess reaction (A). Samples (N = 5 per group) were analyzed 1 day and 5 days after implantation of bare metal stents (black bar), gelatin coated stent (gray bar), eNOS plasmid embedded in gelatin coated stent (dark gray bar), eNOS lipopolymers embedded in gelatin coated stent (light gray bar). NS: P ≥ 0.05 analyzed by ANOVA.
Vessel Sectioning and Histology

To assess therapeutic efficacy of eNOS lipopolyplex mediated transfection neointimal / media ratio was calculated for each of the treatment groups (n = 5 / group). Vessels were stained with Verhoffs elastin stain and counter stained with Van Giesons stain. Figure 37 shows representative images from each of the treatment groups at 4X and 10X magnification. Two weeks after injury the neointimal / media ratio for each of the groups was 1.6 ± 0.55, 2.4 ± 0.16, 1.2 ± 0.42, and 1.8 ± 0.16 for the bare metal, gelatin only, empty vector LPP, and eNOS LPP groups respectively (Figure 38). Vessel injury scores for the bare metal, gelatin only, empty vector LPP, and eNOS LPP groups were 0.61 ± 0.17, 0.87 ± 0.09, 0.83 ± 0.14, 0.97 ± 0.04 (± SEM) respectively (p = 0.26 assessed by ANOVA).

Assessment of Re-endothelialization

Two weeks after injury re-endothelialization was assessed with Evans blue dye. Areas within the vessel exhibiting blue color indicate a lack of endothelium. Figure 39 shows an intact (A) and denuded (B) blood vessel after Evans blue staining. The denuded vessel is a bright blue with small areas of white tissue. The non-denuded vessel is white. Figure 40 shows images of the four experimental groups after Evans blue staining. The bare metal stent has small areas of white tissue, when analyzed the white area represents 8% of the total area (Figure 40a). Gelatin only coated stent has a much larger amount of white tissue, accounting for 40% of the total area (Figure 40b). Upon examination of the empty vector LPP a moderate amount of white tissue was present,
Figure 37: 10μm polymethylacrylate embedded iliac vessel sections stained with Verhoffs elaisin stain and counter stained with Van Gieson's stain. Images were taken at 4x and 10x magnification.
Figure 38: Neointima / media ratio of methacrylate embedded iliac vessels (N = 5 per group) 14 days after implantation of bare metal stents (small checkers), pVAX-1 empty vector lipopolyplexes embedded in gelatin coated stent (large checkers), gelatin coated stent (horizontal lines), eNOS lipopolyplexes embedded in gelatin coated stent (vertical lines).
Figure 39: Rabbit iliac vessel stained with Evans blue dye 14 days after implantation before (A) and after (B) balloon denudation.
Figure 40: Rabbit iliac vessel stained with Evans blue dye 14 days after implantation of a bare metal stent (A), gelatin coated stent (B), p1AX-1 empty vector lipopolyplexes embedded in gelatin coated stent (C), eNOS lipopolyplexes embedded in gelatin coated stent (D) 14 days after implantation. Graph E summarizes the % re-endothelialization (N=1) of bare metal stents (small checkers), p1AX-1 empty vector lipopolyplexes embedded in gelatin coated stent (large checkers), gelatin coated stent (horizontal lines), eNOS lipopolyplexes embedded in gelatin coated stent (vertical lines).
when analyzed the vessel was determined to be 28% re-endothelialized (Figure 40c). The eNOS LPP vessel shows a large portion of white tissue; when analyzed 45% of the vessel was determined to be re-endothelialized (Figure 40d).

**Tissue Immunohistochemistry**

Tissue immunohistochemistry for cell proliferation, smooth muscle cell growth, macrophages, and the presence of endothelial cells was performed on 5 μm sections. Analysis was performed on vessels with two week old injuries. Cell counts were performed to quantify cellular changes and were normalized to total cell counts. **Figures 41a** through **d** are representative images at 20X magnification of cells positively stained for BrdU-U. The bare metal stent, gelatin only stent, and empty vector stent each showed high levels of diaminobenzidine staining within the intima indicating continued cell growth at 14 days for these groups. The eNOS lipopolyplex group had much less percentage of stained cells. This observation is reflected in **Figure 41d**. The % positive stained cells for each group (n = 3) are summarized in **Figure 41e**.

CD-31 was used to stain endothelial cells. **Figure 42a** through **d** are images taken at 20X showing endothelial cells. **Figures 42a** (bare metal) and **42c** (empty vector LPP) show a small percent of brown stained cells whereas **Figures 42b** (gelatin only) and **42d** (eNOS LPP) display a higher percentage of stained cells. Cell counts for these samples (n = 3) show the gelatin only and eNOS LPP coated stents have a higher percentage of CD-31 positive cells compared to the bare metal and empty vector stents (**Figure 42e**).

The presence of macrophages within the intima was evaluated with RAM-11 antibody. **Figures 43a** through **d** are images stained for the presence of macrophages. All of the groups exhibited similar percentage of macrophages, the bare metal and gelatin
Figure 41: Representative immunohistochemistry images (20x) to assess the effect of a bare metal stent (A), gelatin coated stent (B), p14AX1 empty vector lipopolyplexes embedded in gelatin coated stent (C), eNOS lipopolyplexes embedded in gelatin coated stent (D) on cellular proliferation 14 days after implantation. Graph E summarizes the positively stained cells (N=9) of bare metal stents (smell checkers), p14AX1 empty vector lipopolyplexes embedded in gelatin coated stent (large checkers), gelatin coated stent (horizontal lines), eNOS lipopolyplexes embedded in gelatin coated stent (vertical lines).
Figure 42: Representative immunohistochemistry images (20x) to assess the effect of a bare metal stent (A), gelatin coated stent (B), p1AX-1 empty vector lipopolyplexes embedded in gelatin coated stent (C), eNOS lipopolyplexes embedded in gelatin coated stent (D) on CD-31 positive cells 14 days after implantation. Graph E summarizes the positively stained cells (N=5) of bare metal stents (small checkers), p1AX-1 empty vector lipopolyplexes embedded in gelatin coated stent (large checkers), gelatin coated stent (horizontal lines), eNOS lipopolyplexes embedded in gelatin coated stent (vertical lines).
Figure 43: Representative immunohistochemistry images (20x) to assess the effect of a bare metal stent (A), gelatin coated stent (B), p14AX-1 empty vector lipopolypexes embedded in gelatin coated stent (C), eNOS lipopolypexes embedded in gelatin coated stent (D) on RAM-11 positive cells 14 days after implantation. Graph E summarizes the positively stained cells (N=5) of bare metal stents (small checkers), p14AX-1 empty vector lipopolypexes embedded in gelatin coated stent (large checkers), gelatin coated stent (horizontal lines), eNOS lipopolypexes embedded in gelatin coated stent (vertical lines).
only groups have a slightly higher degree of staining compared to the other groups. Cell counts for macrophage staining are summarized in Figure 41e.

To assess smooth muscle cell proliferation an antibody for alpha-actin was used. Figures 44a through d show representative images of SMC staining for each of the experimental groups tested. SMC was only counted within the intima. The empty vector and eNOS LPP groups showed a lower percentage of positive cells compared to the bare metal or gelatin only groups (Figure 44e).

Discussion

Current clinically approved therapies for the inhibition of restenosis all use cytostatic or cytotoxic drugs that inhibit smooth muscle cell growth in the lumen [20, 23, 24, 116, 185-187]. These stents all do an excellent job in stopping cellular growth, but do not allow for the re-endothelialization of the vessel, a key step in long term treatment of this disease [188, 189]. Nitric oxide is known to play a role in re-endothelialization and inhibition of smooth muscle cell growth. Studies have been conducted that look at the effect of nitric oxide release from a polymer [190-192]. Other studies have focused on either viral or non-viral routes of transfection of eNOS and iNOS [85, 94, 95, 98-100, 102, 174, 176, 181, 182, 193-196]. By developing a non-viral delivery system in-vitro we were able to select a potent combination of pH responsive polymer and lipid. We have shown the combination of 5 : 1 : 1 DOTAP : PBAE : DNA transfects vascular cells efficiently in-vitro. By embedding the lipopolyplexes into a gelatin matrix we were able to coat the lipopolyplexes onto stainless steel with no loss of activity. The addition of a thin PLGA coating over the gelatin provided excellent transfection results with eNOS.
Figure 44: Representative immunohistochemistry images (20x) to assess the effect of a bare metal stent (A), gelatin coated stent (B), p147AX-1 empty vector lipopolyplexes embedded in gelatin coated stent (C), eNOS lipopolyplexes embedded in gelatin coated stent (D) on R440-11 positive cells 14 days after implantation. Graph E summarizes the positively stained cells (N=5) of bare metal stents (small checkers), p147AX-1 empty vector lipopolyplexes embedded in gelatin coated stent (large checkers), gelatin coated stent (horizontal lines), eNOS lipopolyplexes embedded in gelatin coated stent (vertical lines).
LDH results show there is no apparent toxicity from this system, which is normally a concern for non-viral cationic delivery systems.

The efficacy of this system was measured by calculating the neointima / media ratio. The gelatin only and eNOS LPP groups showed a decrease in the ratio, indicating a lower amount of intimal growth compared to the bare metal stent and empty vector LPP. A number of studies have had limited controls to reach their conclusions, Fishbein et al., looked at the effect of a viral vector coated on a stent and saw a significant reduction in neointima-to-media ratio of the experimental stent compared to a bare metal stent only [130]. That study did not look at the effect of the polymer coating, which contained PEI, or the effect of an empty viral vector. Other groups have taken similar approaches, only looking at either a bare metal stent, the polymer coating or an empty vector [94, 127, 178]. Sharif et al., coated a viral vector onto a phosphorylcholine coated stent [94]. Their results showed a non significant decrease in % stenosis when a reporter vector or coating was compared to eNOS encoding viral vector in a normocholesterolemic rabbit model. When a hypercholesterolemic rabbit model was used, they did see a significant increase between their experimental and control groups. The small differences in efficacy between groups seen in this study are common for this animal model since the vessels do not restenosis as much as other models.

The efficacy data is further supported with the Evans blue data. The stents coated with gelatin only or eNOS LPP show a higher % of re-endothelialization compared to the bare metal or empty vector groups. A study employing viral vectors encoding for eNOS showed 80% re-endothelialization after 14 days [94]. Cooney et al., compared local delivery of a viral vector encoding for eNOS and iNOS and saw 90% and 75% re-
endothelialization respectively, 2 weeks after injury [181]. The IHC for CD-31 also confirms this indicating that eNOS transfection is causing re-endothelialization. The CD-31 staining protocol was based on previous reports [165]. The staining pattern observed for all of the IHC is consistent with balloon injured rabbits [197]. The gelatin only group also showed re-endothelialization by Evans blue and IHC. This could be explained by an increased percentage of EPCs seeding on the gelatin. Gelatin or collagen are common substrates for seeding different cell types [136, 141, 155]. The Griess data from the previous chapter showed an increase in NO production for the gelatin only group indicating either re-endothelialization or macrophage recruitment. IHC results do not show an increase in macrophages that can be attributed to an increased NO production.

Cellular proliferation assessed by BrdU immunostaining decreased in proliferating cells two weeks after injury in vessels receiving eNOS LPP stents. The other groups showed similar amounts of cellular proliferation, indicating continuing cell proliferation in all but the eNOS LPP stents. RAM-11 positive cells were slightly higher in the bare metal and gelatin only groups though not significant. This could indicate a possible inflammatory response in the empty vector group compared to the other groups. Interestingly there were fewer cells stained positive for alpha-actin in the empty vector LPP and eNOS LPP groups. The IHC data indicates that the eNOS LPP group does inhibit SMC growth, while promoting EC growth, without an inflammatory response.

Conclusions

The data presented in this chapter shows in-vivo efficacy of a lipopolyplex coated stent that encodes for endothelial nitric oxide synthase. These results confirm what has
been observed with catheter delivered viral and non-viral vectors, in addition to stent based therapies [94, 98, 102, 174, 181, 182, 193, 194, 198]. Additional pre-clinical transfection and efficacy studies in a hypercholesterolemic rabbit or porcine model may allow for a further differentiation between the gelatin and eNOS LPP groups. Further investigation as to the re-endothelialization properties of gelatin are also warranted based on this work. This work detailed the first time a non-viral vector has been coated onto a stent and successfully transfected cells with a gene product to inhibit restenosis.
REFERENCES


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