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Improving the efficacy of liposome-mediated vascular gene therapy via lipid surface modifications

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ABSTRACT

Background: We have previously defined mechanisms of intimal hyperplasia that could be targets for molecular therapeutics aimed at vascular pathology. However, biocompatible nanocarriers are needed for effective delivery. Cationic liposomes (CLPs) have been demonstrated as effective nanocarriers *in vitro*. However, *in vivo* success has been hampered by cytotoxicity. Recently, neutral PEGylated liposomes (PLPs) have been modified with cell-penetrating peptides (CPPs) to enhance cellular uptake. We aim to establish CPP-modified neutral liposomes as viable molecular nanocarriers in vascular smooth muscle cells.

Methods: CLPs, PLPs, and CPP-modified PLPs (R8-PLPs) were assembled with short interfering RNA (siRNA) via ethanol injection. Characterization studies determined liposomal morphology, size, and charge. siRNA encapsulation efficiency was measured via RiboGreen assay. Vascular smooth muscle cells were exposed to equal lipid/siRNA across all groups. Rhodamine-labeled liposomes were used to quantify cell association via fluorometry, live/dead dual stain was used to measure cytotoxicity, and gene silencing was measured by quantitative polymerase chain reaction.

Results: R8-PLPs exhibited increased encapsulation efficiency equivalent to CLPs. PLPs and R8-PLP-5 mol% and R8-PLP-10 mol% had no cytotoxic effect. CLPs demonstrated significant cytotoxicity. R8-PLP-5 mol% and R8-PLP-10 mol% exhibited increased cell association *versus* PLPs. R8-PLP-10 mol% resulted in significant gene silencing, in a manner dependent on lipid-to-siRNA load capacity.

Conclusions: The negligible cytotoxicity and enhanced cellular association and gene silencing capacity exhibited by R8-PLPs reveal this class of liposomes as a candidate for future applications. Further modifications for optimizing R8-PLPs are still warranted to improve efficacy, and *in vivo* studies are needed for translational development. However, this could prove to be an optimal nanocarrier for vascular gene therapeutics.

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Introduction

Currently, there are more than 200 million people worldwide with peripheral vascular disease (PVD).[1] As average global life expectancy continues to escalate, the prevalence of PVD will also continue to rise. Endovascular interventions are low risk, minimally invasive procedures that have become increasingly pervasive in the treatment of PVD because of high initial success rates (>90%).[2] Unfortunately, endovascular procedures are linked to higher reintervention rates due to chronic restenosis and injury-induced intimal hyperplasia (IH) development, typically marked by dysfunctional migration and proliferation of vascular smooth muscle cells (VSMCs) in the vessel wall along with delayed vascular endothelial cell recovery.[3] In fact, IH-induced restenosis occurs in greater than 60% of endovascular cases at 12-mo follow-up, often requiring secondary intervention with increased morbidity and mortality rates.[4,5] Establishing an optimal treatment plan to mitigate the development of IH-induced restenosis would improve long-term primary intervention success rates and help relieve the clinical and financial burden of the PVD health epidemic.

RNA interference (RNAi), or the use of short interfering RNA (siRNA) to transiently attenuate cellular protein expression, shows promise as a gene therapy technology to alter vascular pathology.[6,7] Our laboratory has previously defined several molecular mechanisms of IH development that could be potential targets for molecular therapeutics aimed at its attenuation.[8-14] However, the development of molecular nanocarriers is needed to overcome the unfavorable physiochemical properties that limit intracellular siRNA uptake. Various transfection agents have been investigated for their application of gene therapy to vascular cell types, including polymeric nanoparticles, chemical agents, and viral vectors.[15-17] Recently, our group has successfully deployed multiple classes of biodegradable polymers in the delivery of molecular gene therapy in vascular cell types and tissue *in vitro*.[18,19] Although many of these nanocarriers have shown efficacy with *in vitro* transfection, they often fail to translate to an *in vivo* model due to their cytotoxicity, adverse immune response, and inefficient biodistribution.[18,20] Therefore, the development of a biocompatible nanocarrier that can provide targeted vascular gene therapy, without immunological or cytotoxic effects, is needed to provide a drug delivery candidate for future *in vivo* testing aimed at the attenuation of IH development in a postsurgical model.

Liposomes are promising drug delivery vehicles because of their biocompatibility, relatively low cytotoxicity, and flexibility of modification. Liposomal phospholipids are arranged in a bilayer that forms a spherical nanoparticle mimicking the cell membrane. Cationic liposomes (CLPs), made with synthetic lipid components, have been successfully used in the delivery of gene therapeutics *in vitro*, attributed to the high loading capacity of negatively charged phosphate groups present in nucleic acids.[21,22] Also, the positive surface charge of CLPs promotes cellular interaction because of the electrostatic affinity for negatively charged cell membranes. Unfortunately, clinical translatability has been limited, as CLPs are often demonstrated to be cytotoxic and immunogenic when administered *in vivo*, partially attributable to their synthetic constituents.[23]

Neutral liposomes are comprised of naturally occurring phospholipid constituents and have been modified to improve efficacy as targeted nanocarriers while maintaining a lower toxicity profile. Polyethyleneglycol (PEG) can be conjugated to the surface of a liposome to provide "stealth" properties for the nanoparticle. Along with their nanoscale size at <150 nm in diameter, PEGylation allows the nanocarrier to avoid opsonization clearance from the blood stream, increasing the overall half-life of the carrier.[24] The most intriguing aspect of neutral PEGylated liposomes (PLPs) is the flexibility of modification and multifunctional potential provided by the PEG shell on the liposome surface. The PEG molecule can provide a scaffold for conjugation of functional ligands, and various ligand classes can be anchored into the lipid bilayer via stearylated fatty acids. These modifications can provide functional modalities such as enhanced intracellular delivery, cell-type specific delivery, triggered release, imaging capabilities, tissue localization, and so on.[25] Specifically, cell-penetrating peptides (CPPs) are short peptide fragments that can enhance cellular uptake of biomolecular cargo.[26,27] These peptides are typically cationic in nature and can locally and temporarily disrupt the lipid bilayer on the cell surface, leading to direct translocation across the cell membrane. Here, we aim to establish CPP-modified neutral liposomes as efficient molecular nanocarriers in VSMCs, with reduced cytotoxicity and enhanced siRNA delivery compared with standard CLP formulations previously established in the RNAi literature. Improving the efficacy of liposome-mediated molecular therapeutics in vascular cell types, using lipid surface modifications, could lead to a translatable drug delivery platform for future *in vivo* application in vascular injury models of disease aimed at the prevention of IH.

Materials and methods

Neutral and cationic liposome assembly via ethanol injection

All liposome constituent acronyms are defined in Table 1. Lipids and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Octaarginine (R8) peptide, a

Table 1 – Liposome constituent acronyms defined.

Acronym	Lipid derivative
DOPC	1,2-Dioleoyl-*sn*-glycero-3-phosphocholine
DSPE-PEG	1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
DOTAP	1,2-Dioleoyl-3-trimethylammonium-propane
DOPE	1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine
Rho-DOPE	N-(lissamine rhodamine B sulfonyl)-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
Chol	Ovine cholesterol
STR-R8	Stearylated octaarginine