GENE DELIVERY AND TRANSFECTION STUDIES WITH LIPOPOLYPLEXES IN HUMAN ENDOTHELIAL AND SMOOTH MUSCLE CELLS

Luis Brito[1], Saradha Chandrasekhar[1], Steven Little[2], and Mansoor Amiji[1]
[1]Department of Pharmaceutical Sciences, School of Pharmacy, Northeastern University, Boston, MA 02115
[2]Department of Chemical and Petroleum Engineering, University of Pittsburgh, Pittsburgh, PA 15260

ABSTRACT

Non-viral gene delivery and transfection in primary human vascular endothelial cells (EC) and smooth muscle cells (SMC) has tremendous potential for cardiovascular diseases, such as in treatment of coronary restenosis. Using a combination of synthetic cationic poly(beta amino ester) (PBAE) and phospholipids (DOTAP), we have designed a nanovector system that can transfect EC and SMC with very high efficiency. The results of this study show that PBAE-DOTAP-plasmid DNA lipopolyplexes are a promising non-viral vector system for gene delivery and transfection in EC and SMC. Based on these results, there is tremendous potential for use of these lipopolyplexes in cardiovascular diseases, such as coronary restenosis by embedding them onto a stent.

Keywords: Lipopolyplex, restenosis, medical device, gene delivery

1. INTRODUCTION

According to the National Center for Health Statistics, heart disease and related complications remain the number one cause of deaths in the United States (Lethbridge-Çejku *et al* 2006). Over 24 million people have been diagnosed with some type of heart disease in 2004. Atherosclerosis arises from plaque building in the lumen of the coronary artery. As the plaque accumulates the vessel narrows and at some point, the plaque becomes an unstable lesion and can rupture inducing a clotting cascade and blocking the blood vessel. Although this can happen in any blood vessel, it is of particular importance in the coronary artery that supplies blood to the heart. When the plaque is located in the coronary arteries, the obstruction leads to ischemia, myocardial infarction, and can eventually lead to death. Removal of the plaque can be performed by non invasive techniques, such as percutaneous transluminal coronary angioplasty (PTCA), stenting, or atherectomy. These techniques all can lead to a complication called restenosis, where the vessel closes upon itself again, in a different mechanism to atherosclerosis. The placement of stents has become the treatment of choice compared to PTCA due to its lower percentage of restenosis (Kastrati *et al* 2001).

Goldberg *et al* have defined restenosis as a greater than 50% narrowing of the vessel as determined by a follow-up angiogram (Goldberg *et al* 2001). Restenosis is very clinically similar to atherosclerosis, the difference lies in how the vessels are narrowed. Restenosis is typically classified into two distinct steps, smooth muscle cell proliferation, typically called neointimal hyperplasia or intimal hyperplasia, and vessel remodeling (Costa and Simon 2005).

The types of cells involved in the restenotic process where well documented. There are three primary layers in a healthy blood vessel. The tunica intima or intimal layer is the innermost layer and is in contact with the blood flowing through the artery. This layer consists primarily of endothelial cells. Adjacent to the intimal layer is the tunica media or medial layer, consisting of primarily smooth muscle cells (SMC). The outermost layer is the tunica adventitia or adventitial layer, consisting of primarily collagen. Following vascular injury, other cells are recruited including various inflammatory cells such as macrophages, T-cells and a small number of B-cells (Farb *et al* 2002). There is a degree of similarity between restenosis and wound healing. Analysis of the extracellular matrix of a restenotic lesion showed hyaluronan and collagen, which are also both involved in would healing (Farb *et al* 2002; Scott 2006).

Non-viral gene delivery and transfection in primary human vascular endothelial cells (EC) and smooth muscle cells (SMC) has tremendous potential for cardiovascular diseases, such as in treatment of coronary restenosis (Brito and Amiji 2007). Using a combination of synthetic cationic poly(beta amino ester) (PBAE) and phospholipids (DOTAP), we have

designed a nanovector system that can transfect EC and SMC with very high efficiency.

The purpose of this study was to develop and evaluate a gene delivery system for the treatment of restenosis. The desired endpoints for the delivery system were:

1. Ability to transfect EC and SMC effectively with low DNA doses
2. High rate of endocytosis
3. Ability to be coated onto stainless steel
4. Rapid release of system from stent

2. EXPERIMENTAL METHODS

2.1 Lipopolyplex Preparation and Characterization

The lipopolyplexes were made by combining PBAE, DNA, and DOTAP in a 1:1:5 weight ratio as previously described (Brito *et al* 2008). Briefly, DNA was pre-complexed with PBAE in MES buffer at pH 6.0 as previously described (Lynn and Langer 2000; Akinc *et al* 2003). 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). Lipid films were subsequently 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 were added to DOTAP liposomes to achieve a final concentration of 1:1:5 PBAE : DNA : DOTAP. The mixture was allowed to incubate at room temperature for 30 minutes.

The PBAE-DNA polyplexes, DOTAP-DNA lipoplexes, and PBAE-DNA-DOTAP lipopolyplexes were characterized for particle size and surface charge. Particle size 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.

2.2 Cellular Uptake and Transfection Studies

Following characterization for size and surface charge, the lipopolyplexes were tested for, cell uptake and trafficking behavior, and transfection efficiency in EC and SMC. EC and SMC were purchased from Cell Applications (San Diego, CA) and were maintained according to the manufacturer's instructions. Trafficking studies were carried out with rhodamine labeled lipids incorporated into the DOTAP liposomes. Additionally pico green reagent (Invitrogen, Carlsbad CA) was added to the DNA prior to complexing, giving the DNA a green fluorescent signal. Cell nuclei were stained with Hoescht dye (Invitrogen, Carlsbad CA). Cells were analyzed by flow cytometry and confocal microscopy at time = 30 minutes, 1 hour, 2 hours, 4 hours and 6 hours.

To assess the ability to transfect, cells were incubated with lipopolyplexes, 1: 1 PBAE : DNA complexes, and 5:1 DOTAP : DNA complexes for 6 hours. After 6 hours the media was removed and the cells were allowed were Quantitative transfection of green fluorescent protein (GFP) was confirmed by flow cytometry analysis and measuring intracellular GFP with enzyme-linked immunosorbent assay (ELISA). Qualitative transfection was determined by fluorescence microscopy.

2.3 Coating and Characterization of Lipopolyplexes on Stainless Steel Mesh

The lipopolyplexes were embedded into a gelatin matrix at 80mg/ml. The lipopolyplexes were made as previously stated, they were then added to dry gelatin at 80mg/ml. Both type A 300 bloom strength and type B 225 bloom strength gelatin was used. The gelatin lipopolyplex solution was placed at 37°C for 15 minutes to allow for the gelatin to dissolve. Once the gelatin was fully dissolved, 50 mm diameter stainless steel mesh (Goodfellow Corp. Oakdale PA) was dip coated in the solution. The procedure was carried out in a sterile environment to minimize the risk of bacterial contamination.

To assess the release of the lipopolyplexes from the gelatin matrix, coated mesh were coated with rhodamine labeled lipopolyplexes and placed in PBS on a rocker at 37°C. Samples were taken (n=3) at 5 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours and 4 hours for both type A and type B gelatin. In

addition to release average coating weight of the mesh was recorded. To assess the coating thickness stainless steel coupons were coated with the gelatin solution. A micrometer screw gauge (Mitutoyo Corp. Tokyo, Japan) was used to assess the thickness of the coating.

2.4 *In Vitro* Evaluation of Uptake and Transfection with Lipopolyplex Coating

To assess the effect on uptake of the nanoparticles embedded within a gelatin matrix Gelatin / rhodamine labeled lipopolyplex coated mesh were incubated with cells for 30 minutes, 1 hour, 2 hours, 4 hours and 6 hours. At each time point, the mesh was removed and fluorescent microscopy and FACS studies were performed.

Transfection experiment was carried out by incubating the mesh with lipopolyplexes containing GFP with cells for 6 hours. After 6 hours the mesh was removed and the samples were washed 3 times with PBS. Samples were analyzed by ELISA and microscopy.

3. RESULTS AND DISCUSSION

Addition of DOTAP to DNA pre-condensed with PBAE resulted in formation of nano-sized lipopolyplexes with a net cationic charge (Table 1). Lipopolyplexes did have a significantly larger size than the PBAE : DNA or DOTAP : DNA complexes. This is believed to be because the DOTAP liposomes are coating the surface of the polyplexes.

Table 1. Particle size and Zeta Potential Values of the Formulations

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

To measure the amount of GFP produced per milligram of total protein extracted an ELISA was performed (Figure 1). Both the EC and SMC showed the highest level of protein with DOTAP lipoplexes and PBAE DOTAP lipopolyplexes. Green fluorescent protein (GFP) expression was very similar as what was observed in microscopy. There was no GFP expressing with Lipofectin® and PBAE transfection in both cell lines. However, we observed high levels of GFP expression with DOTAP lipoplexes and DOTAP-PBAE lipopolyplexes. For ECs, the expression levels for the DOTAP-PBAE lipopolyplexes remained high for up to 72 hours. However, there was a sharp decrease in GFP levels with the DOTAP lipoplex. The lipopolyplexes lead to a significantly ($p<0.001$) higher level of GFP expression as compared to DOTAP lipoplexes.

Lipopolyplexes were coated onto type A and type B gelatin. Lipopolyplex release from the mesh occurred within the first 5 minutes. The gelatin hydrogel layer was not crosslinked facilitating the DNA release. The coating thicknesses were 2.8 μm and 2.9 μm for type A gelatin and type B gelatin, respectively. Average coating weight was 57.4 mg for type A gelatin and 14.5 mg for type B. The high variability was probably due to the uneven coating of the mesh. The difference in weight for type A and type B gelatin was likely due to the differences in bloom strength. Type A gelatin used was 300 bloom strength, whereas type B gelatin had a bloom strength of 225. The difference in bloom strength effects how the gelatin coats in between the wires on the mesh. Type B gelatin closely wraps around each mesh wire whereas the type A gelatin bridges between them leading to a higher coat weight.

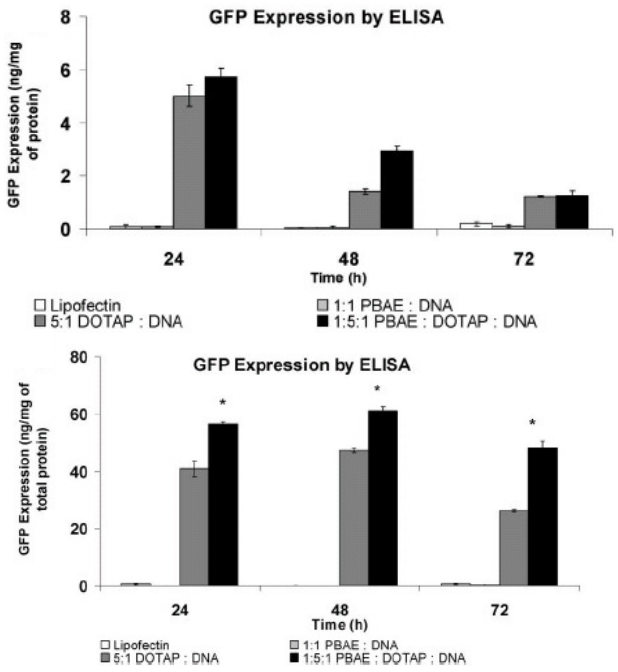

Figure 1. GFP ELISA data after 6 hours of incubation with various transfection reagents. Endothelial and smooth muscle cells were lysed at 24 hours, 48 hours, and 72 hours and assayed for amount of GFP. GFP amount is expressed in terms of ng GFP / mg total protein. (* indicates significance of $p < 0.001$)

To assess whether the lipopolyplexes were changed after coating onto a mesh microscopy and FACS were used to assess particle uptake. FACS data shows that lipopolyplexes embedded within both type A and type B gelatin were internalized in SMC by 4 hours (Figure 2). There does appear to be a difference between the samples at 2 hours. Particles are taken up faster when embedded within type B gelatin. This was also confirmed by fluorescence microscopy (data not shown). Transfection was evaluated by ELISA and microscopy (data not shown). The ELISA data showed that after 24 hours lipopolyplexes embedded within both type A and type B gelatin lead to transfection levels comparable as previously observed with non-embeded lipopolyplexes. By 48 hours, the level of expression in type A gelatin-coated mesh fell sharply.

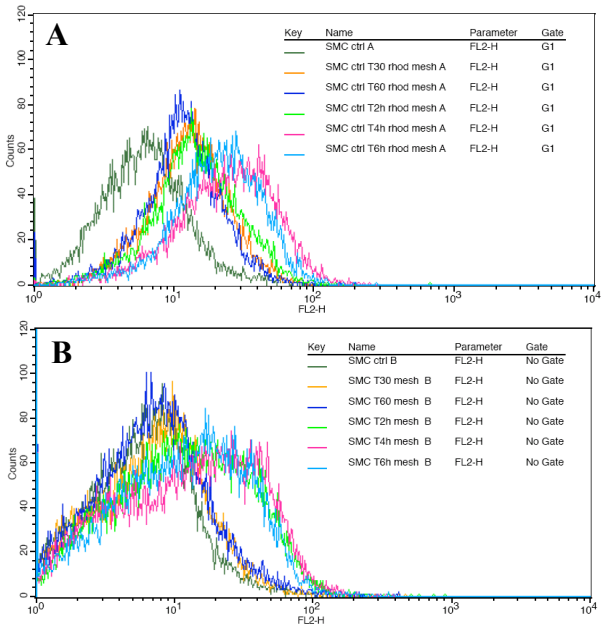

Figure 2. FACS data for rhodamine-labeled lipopolyplex uptake from type A coated mesh (A) and type B coated mesh (B). Samples analyzed at time = 0 (dark green), 30 minutes (orange), 60 minutes (blue), 2 hours (lime green), 4 hours (pink) and 6 hours (cyan).

4. CONCLUSIONS

The results of this study show that PBAE-DOTAP-plasmid DNA lipopolyplexes coated onto a stainless steel mesh are a promising non-viral vector system for gene delivery and transfection in EC and SMC. Based on these results, there is tremendous potential for use of these lipopolyplexes in cardiovascular diseases, such as coronary restenosis. Studies are underway to assess the *in vivo* potential of this system using stainless steel stents.

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