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Recombinant Elastin Based Nanoparticles for Targeted Gene Therapy

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Recombinant Elastin Based Nanoparticles for Targeted Gene Therapy

by

Dagmara Anne Monfort

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Engineering Science
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DEDICATION

I would like to dedicate this dissertation to my family including my mother, father, my brothers and my grandmother for their unconditional support and guidance throughout my life. I would also like to thank the Washington State Math Engineering Science Achievements (MESA) staff, University of Washington Post-Baccalaureate Research Education Program (PREP) staff and Mr. Bernard Batson who encouraged me to start and complete my doctoral degree in Engineering Science. I would like to say thank you to my fiancé for his unconditional love and support.

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TABLE OF CONTENTS

LIST OF TABLES	iii
LIST OF FIGURES	iv
ABSTRACT	viii
CHAPTER 1: OVERVIEW OF GENE THERAPY	1
1.1 Gene Delivery Techniques	1
1.1.1 Non-Viral Delivery Methods	1
1.1.2 Viral Delivery Methods	3
1.1.3 Lentiviruses	4
CHAPTER 2: OVERVIEW OF ELASTIN LIKE POLYPEPTIDES	8
2.1 Recombinant ELP Protein Purification	8
CHAPTER 3: DEVELOPMENT OF HETEROGENEOUS ELPS NANOPARTICLES BASED DELIVERY SYSTEM FOR TARGETED GENE THERAPY	12
3.1 Introduction	12
3.2 Materials and Methods	14
3.2.1 Materials	14
3.2.2 Cell Culture	14
3.2.3 Synthesis of LDLR3-ELP and KGF-ELP	15
3.2.4 Purification of LDLR3-ELP	15
3.2.5 LDLR3-ELP Total Protein Assay	16
3.2.6 Characterization of LDLR3-ELP	16
3.2.7 Labeling of the Fusion Proteins	16
3.2.8 Internalization Assay for Fusion Proteins	17
3.2.9 Cytotoxicity Assay	17
3.2.10 Creation of Lentiviral Particles	17
3.2.10.1 Heat Inactive Media	18
3.2.10.2 Viral Plasmid Isolation	18
3.2.10.3 Calcium Phosphate DNA Co-Precipitation	18
3.2.11 Inhibition Assay	19
3.2.12 Transduction Assay	19
3.2.13 Binding Assay	20
3.3 Results	21
3.3.1 Fusion Protein Comprising of the Viral Envelope Binding Domain (VBD) and Elastin Like Peptide (ELP) was Successfully Expressed and Purified Using Inverse Temperature Cycling (ITC)	21
3.3.2 LDLR3-ELP Maintains the Phase Transition Property and Self-Assembles Into Nanoparticles	26

3.3.3	LDLR3-ELP Inhibits Virus Infectivity and Does Not Induce Cell Death	28
3.3.4	Targeted Internalization of LDLR3-ELP Using Growth Factors in High Growth Factor Receptor Expressing Cells	34
3.3.5	Heterogeneous Nanoparticles Comprising of LDLR3-ELP and KGF-ELP Result In Targeted Delivery of the Gene in High Growth Factor Receptor Expressing Cells	36
3.3.6	ELP and KGF-ELP Do Not Bind to the Virus	43
3.3.7	Statistical Analysis	46
3.4	Conclusion	46
CHAPTER 4: DISCUSSION		47
CHAPTER 5: FUTURE DIRECTIONS		50
5.1	Creating a Fusion Protein Consisting of LDLR3 Fused With KGF-ELP	50
5.2	Testing Longevity of Transduction Efficiency	58
5.3	Testing the Internalization Pathway of LDLR3-ELP in the Presence of KGF-ELP	59
5.4	Using Different Growth Factor Receptors	60
5.5	Using the Heterogeneous Nanoparticles for Lung Injuries	71
5.6	Testing for Immunogenicity	73
5.7	Using LDLR3-ELP as an Antiviral Drug for Respiratory Syncytial Virus	74
REFERENCES		76
APPENDIX A: AMINO ACID SEQUENCE FOR KGF, LDLR3, 5-HELIX, AND ELP		85

LIST OF TABLES

Table 1	Advantages and disadvantages of the non-viral vectors	2
Table 2	Advantages and disadvantages of viral vectors	3
Table 3	Lane descriptions for Figure 10	25
Table A	Sequence and molecular weight information of KGF, LDLR3, 5-HELIX and ELP	85

LIST OF FIGURES

Figure 1	Schematic of calcium phosphate transfection	5
Figure 2	GFP expression of transfection efficiency	6
Figure 3	Transition phase of ELPs above and below transition temperatures	9
Figure 4	Schematic of ELP protein purification using Inverse Temperature Cycling (ITC)	10
Figure 5	Schematic of recursive ligation and transformation process of LDLR3-ELP	22
Figure 6	Schematic of LDLR3-ELP successfully inserted into the ELP gene	23
Figure 7	LDLR3 was successfully inserted in the Puc19V40C2 plasmid	23
Figure 8	LDLR3-V40C2 was successfully cut by restriction enzyme PST1	24
Figure 9	LDLR3-V40C2 was successfully inserted in the expression plasmid PET25b+	24
Figure 10	LDLR3-ELP fusion protein was successfully purified using ITC in the 12% SDS-PAGE gel	25
Figure 11	LDLR3-ELP design	26
Figure 12	LDLR3-ELP transitions near 30°C	27
Figure 13	LDLR3-ELP size distribution at the body's physiological temperature 37°C	28
Figure 14	Schematic of LDLR3-ELP binding to the virus in the presence of calcium	29
Figure 15	Green Fluorescent Protein (GFP) expression in A549 cells of LDLR3-ELP inhibiting virus infectivity	30
Figure 16	Quantification of the GFP expression in A549 cells of LDLR3-ELP inhibiting virus infectivity	31
Figure 17	Schematic of the transitioned LDLR-3-ELP along with any bound virus	32

Figure 18	GFP expression in A549 cells of LDLR3-ELP inhibiting virus infectivity at different concentrations	32
Figure 19	Quantification of the GFP expression in A549 cells of LDLR3-ELP inhibiting virus infectivity	33
Figure 20	LDLR3-ELP does not induce cell death	34
Figure 21	KGF-ELP design	35
Figure 22	Schematic of heterogeneous nanoparticle comprising of keratinocyte growth factor-ELP (KGF-ELP) and LDLR3-ELP	35
Figure 23	Targeted internalization of LDLR3-ELP using growth factors in high growth factor receptor expressing cells	36
Figure 24	KGF-ELP enhanced the transduction efficiency blocked by LDLR3-ELP	37
Figure 25	Schematic of heterogeneous NPs containing the virus and KGF-ELP	38
Figure 26	Schematic of heterogeneous nanoparticles with virus that have a higher probability of being internalized by high growth factor receptor expressing cells (A549 cells) than low growth factor receptor expressing cells (H293 cells)	39
Figure 27	GFP expression of heterogeneous nanoparticles that have a higher probability of being internalized by high growth factor receptor expressing cells (A549 cells) than low growth factor receptor expressing cells (H293 cells)	40
Figure 28	Quantification of GFP expression of heterogeneous nanoparticles that have a higher probability of being internalized by high growth factor receptor expressing cells (A549 cells) than low growth factor receptor expressing cells (H293 cells)	41
Figure 29	Heterogeneous nanoparticles resulted in higher geometric mean in high growth factors expressing cells	42
Figure 30	Schematic of ELP not binding to the virus	43
Figure 31	Schematic of KGF-ELP not binding to the virus	43
Figure 32	GFP expression of ELP and KGF-ELP that do not bind to the virus	44
Figure 33	Quantification of GFP expression of ELP and KGF-ELP that do not bind to the virus	45
Figure 34	Schematic of undesired nanoparticles combinations	50
Figure 35	Schematic of desired nanoparticles combinations	51

Figure 36	Schematic of recursive ligation and transformation process of KGF-ELP-LDLR3	52
Figure 37	KGF-ELP-LDLR3 design	53
Figure 38	Schematic of desired nanoparticle, KGF-ELP-LDLR3 at 4°C and 37°C	53
Figure 39	Possible combinations of nanoparticles, KGF-ELP-LDLR3 formation at 37°C in the presence of virus	54
Figure 40	Schematic of heterogeneous nanoparticles of KGF-ELP-LDR3 with virus that have a higher probability of being internalized by high growth factor receptor expressing cells (A549 cells) than low growth factor receptor expressing cells (H293 cells)	55
Figure 41	Schematic of recursive ligation and transformation of KGF-ELP-LDLR3	56
Figure 42	LDLR3-KGF-ELP design	57
Figure 43	Schematic of desired nanoparticle, LDLR3-KGF-ELP, at 4°C and 37°C	57
Figure 44	Possible combinations of nanoparticles, LDLR3-KGF-ELP at 37°C in the presence of virus	58
Figure 45	Comparison of high geometric mean and high expression GFP	59
Figure 46	Schematic of selectivity of nanoparticles to high expression growth factor cells	61
Figure 47	EGF-ELP design	62
Figure 48	NGF-ELP design	63
Figure 49	EGF-ELP-LDLR3 design	64
Figure 50	Schematic of recursive ligation and transformation process of EGF-ELP-LDLR3	65
Figure 51	P-ELP design	66
Figure 52	Schematic of heterogeneous nanoparticle comprising of EGF-ELP-LDLR3 and P-ELP	66
Figure 53	Schematic of recursive ligation and transformation process of P-ELP	67
Figure 54	LDLR3-EGF-ELP-P design	68
Figure 55	EGF-LDLR3-ELP-P design	68

Figure 56	Schematic of recursive ligation and transformation process of LDLR3-EGF-ELP-P	69
Figure 57	Schematic of heterogeneous nanoparticles of EGF-ELP-LDLR3 with virus that have a higher probability of being internalized by high growth factor receptor expressing cells preventing cells from proliferating	70
Figure 58	Schematic of heterogeneous nanoparticles of EGF-ELP-LDLR3-P with virus that have a higher probability of being internalized by high growth factor receptor expressing cells preventing cells from proliferating	71
Figure 59	Schematic of lung injury to elastin degradation	72

ABSTRACT

Gene therapy is a technique used to inactivate, replace or insert a corrective copy of a defective gene in order to help diseased tissues to function properly. Gene therapy is a promising treatment for many diseases cancer, cystic fibrosis, and Parkinson's. There are different methods to introduce a gene to the cell; one of them is the use of viruses. Among viruses, lentiviruses have been popular vectors for gene delivery due to their efficient mode of gene delivery. However, the non-specific delivery of genes associated with viruses may result in undesirable side effects. Here, we propose a heterogeneous nanoparticle delivery system for targeted delivery of lentiviral particles containing a therapeutic gene. The heterogeneous nanoparticles (NPs) consist of the low density lipoprotein receptor 3 (LDLR3) and the keratinocyte growth factor (KGF), each fused to elastin-like-polypeptides (ELPs), LDLR3-ELP and KGF-ELP, respectively. Our results show that while homogeneous nanoparticles comprising of LDLR3-ELP alone blocked viral transduction, heterogeneous nanoparticles comprising of KGF-ELP and LDLR3-ELP enhanced viral transduction in cells expressing high levels of the KGF receptors (KGFR) compared to cells expressing low levels of KGF receptors. Overall, this novel design may help with the targeting of specific cells that overexpressed growth factor such as KGFR.

CHAPTER 1: OVERVIEW OF GENE THERAPY

Gene therapy has been evolving for decades. It is a technique used to deliver DNA into a cell to inactivate mutated gene, replace a mutated gene, or insert a corrective copy of a defective gene in order to help diseased tissues and/or organs to function properly. However, there are many challenges that are encountered in this field. For gene disorders, introducing the missing gene is difficult because many cells have to be able to uptake the gene where the gene has to be activated and gets encoded. When introducing a gene to specific cells, the success of delivering the gene to the targeted cells is crucial. The fact that cells can shut genes down due to exhibiting irregular behaviors causes most disorders to be incurable. Most importantly, creating gene delivery vectors has been challenging because these vectors have to be able to surpass the body's natural immune system to reach the targeted site and also incorporate the gene that can get activated and stay stable.

Gene therapy has been used for diseases such as cancer [1], cystic fibrosis [2], ischemia [3], Alzheimer [4], Parkinson [5] and primary immune-deficiencies [6]. For gene therapy to be successful, the therapeutic gene must reach the targeted cells, then be expressed and sustained in the cells. There are different methods of delivering genes to cells. The most common methods are the use of non-viral vectors and viral vectors.

1.1 Gene Delivery Techniques

1.1.1 Non-Viral Delivery Methods

Non-viral delivery methods deliver the gene of interest into cells by using natural or synthetic compounds or physical forces [7]. This includes lipid mediated gene delivery [8], cationic polymers [9], electroporation [10] and microinjection [11]. Lipid mediated gene delivery is using liposomes DNA complexes to deliver the DNA to the nucleus. Cationic polymers is using positive charged

polymers that bind to the negatively charge DNA and delivers the DNA through endocytosis. Electroporation is using electric pulses to open the pores in the cell membranes to introduce the DNA to the cells. Microinjection is using microneedles to inject a liquid substance containing the DNA to cells. Table 1 below summarizes the advantages and disadvantages of the non-viral vectors.

Table 1 Advantages and disadvantages of the non-viral vectors

Methods	Advantages	Disadvantages
Lipid Mediated gene delivery	Low immunogenicity [12-14] High level of transgene expression after direct administration [15] Low Cost [13] Easy to use in most cell types [12] Simple and fast procedure with high reproducibility [12, 16]	Low efficiency in primary and non-dividing cells [12, 15] Low in vivo efficiency [12]
Cationic Polymers	Poly-ethylenimine (PEI)-high transfection activity in vitro [17, 18] Poly-L-lysine (PLL)-biodegradable [18] Large size of DNA can be transfected [16] Easy to produce and have large scale [15]	PEI-non-biodegradable [17] PLL-low transfection efficiency [18] Cytotoxic [16-18]
Electroporation	High levels of transgene expression in several organs [15]	Can cause local tissue damage and inflammation [15] Parameter optimization for different cell types [15] Depends on high plasmid DNA concentration [15]
Microinjection	Small amount of DNA required of transference [15]	Extremely difficult, low performance [15]

The advantages of non-viral delivery methods compared to viral delivery methods are that the non-viral methods have less immunotoxicity than viral delivery methods and that viral delivery methods have the potential to cause insertional mutagenesis [7, 19]. However, the drawbacks of using non-viral delivery methods are that they have lower transfection efficiency compared to viral delivery methods [19] and that viruses especially lentiviruses can integrate into both dividing and non-dividing cells' genomes.

1.1.2 Viral Delivery Methods

Viral delivery methods are using viral vectors to deliver the DNA into the host cells. Viruses such as herpes simplex viruses [20], adenoviruses [21], adeno-associated viruses [22], and lentiviruses [23] are utilized as vectors for gene delivery. Table 2 below summarizes the advantages and disadvantages of the viral vectors. Due to their efficient mode of gene delivery, viruses such as lentiviruses are popular vectors for gene delivery [24]. Lentiviral vectors are essential because they can infect both dividing and non-dividing cells [25-27], they integrate into the target cell's genome [17, 28, 29], and they have long term stable expression of a transgene [17, 29]. The process of introducing viral particles to cells is called transduction. In clinical trials, the transduction efficiency is critical because higher desired cells that are infected by the virus have a higher chance of expressing the desired gene. However, when the viral particles are not delivered to the desired cells, side effects can occur. In clinical trials, safety is priority. Scientists check for toxicity, immunogenicity, and insertional mutagenesis. Toxicity is the quality of the treatment being toxic to cells that can lead to cell death. Treatments that are targeting cancer cells are beneficial, however if they target healthy cells, that raise concerns. Immunogenicity is an induction of the immune response. When this induction occurs, that can result a loss in efficacy and production of antibodies. Insertional mutagenesis occurs when the insert changes the DNA, hence mutation in the DNA. The mutation in the DNA can cause other diseases.

Table 2 Advantages and disadvantages of viral vectors

Methods	Advantages	Disadvantages
Adenovirus	Can transduce in both dividing and non-dividing cells [15, 17, 29] Well suited as oncolytic vector [17, 21] Able to deliver large DNA particles [17, 30]	Does not integrate into cell's genome [29] Immunogenic [6, 15, 29, 30] High levels of pre-existing immunity [29]

Table 2 (Continued)

Adeno Associated Virus	Integrate into specific site on chromosome 19 [6, 17]	Limited transgene capacity [6, 15, 17, 29] Need co-infection by helper virus [29]
Herpes Simplex Virus	Able to deliver large DNA particles [17, 29, 30] Well suited as neurotropic vector [17]	Difficult to keep virus action under control [30] High levels of pre-existing immunity [29]
Lentivirus	Can integrate into host cell genome [17, 29] Wide cellular tropism [29] Ability to infect both dividing and non-dividing cells [17, 25-27, 30] Long term stable expression of a transgene [17]	High risk of insertional mutagenesis [29] Concerns of biosafety [30]

1.1.3 Lentiviruses

Lentiviruses are diploid viruses (two strands of RNA) from the retroviridae family [31]. Lentiviral plasmids are modified to prevent the formation of replication competent lentiviruses. The three plasmids that are used to create lentiviruses are an envelope plasmid, a packaging plasmid, and a transfer plasmid. The envelope is a vesicular stomatitis virus glycoprotein envelope plasmid which is used to target and infect cells structural proteins. The packaging plasmids include a GAG, POL, and REV. The GAG, a polyprotein that codes the core structural proteins; the POL, a reverse transcriptase that generates the cDNA from RNA; and the REV is regulator of expression of virion proteins needed to produce viral like particles. Finally, the transfer plasmid which is a plasmid that transfer the desired gene. Calcium phosphate transfection protocol is one of the methods that is used to create lentiviral particles (Figure 1) [32].

Figures are not drawn to scale

Figure 1: Schematic of calcium phosphate transfection.

For transfection, we used human embryonic kidney 293 (H293) cells because they are popular for producing recombinant proteins [33]. The transfection efficiency is about 70% (Figure 2).

Figure 2: GFP expression of transfection efficiency.

Lentiviruses are used because their abilities to integrate in to the cells' genome of both dividing and non-dividing cells. The problem with lentiviruses is their lack of specificity, which can result in indiscriminate transduction [34]. The non-specific delivery of therapeutic genes may lead to undesirable side effects such as toxicity [35, 36], insertional mutagenesis [35, 36], and immunogenicity [35, 36]. The lentiviral vectors have been used successfully to engineer CART19 cells for patients with advanced leukemia [37]. However, this study is ongoing because the first part of it has shown a serious toxic effect in which patients develop cytokine-release syndrome [37]. Targeted gene therapy is imperative because it may lower the probability of these undesirable side effects and increase the probability of delivering the gene of interest to the

desired site. Developing an efficient and safe gene delivery vector has continually been a major obstacle to gene delivery [38].

CHAPTER 2: OVERVIEW OF ELASTIN LIKE POLYPEPTIDES

Elastin like polypeptides (ELPs) which are temperature protein-based polymers have been used as novel drug carriers [38-40]. ELPs hold beneficial characteristics which are: biocompatible, biodegradable, non-immunogenic, and thermo-responsive. They are composed of repeating sequences of pentapeptides, $(\text{Val-Pro-Gly-X-Gly})_n$ where X can be any amino acids except for Proline [41] because it destroys the inverse phase transition [42]. This sequence of pentapeptides are derived from the hydrophobic chain of tropoelastin, a soluble form of elastin [43]. Also, ELPs are genetically encodable biopolymers that can be fused with any peptides or proteins. ELPs have an interesting physical property where they undergo an entropically driven phase transition where they are soluble below transition temperature and insoluble above transition temperature [43]. This property enables ELPs expressed in bacteria host (such as *Escherichia coli*) to be purified rapidly using inverse temperature cycling (ITC) [44]. ELPs chimeric fusion sequence self-assembled above transition temperature into nanostructures such as nanoparticles [43, 45] or into cross-linked materials [46]. These physical properties of ELPs have been fully elucidated by several researchers [47-51]. The benefits of the characteristics of ELPs have led their use for medical applications [52] such as scaffolds for tissue engineering [53] and drug delivery [54].

2.1 Recombinant ELP Protein Purification

Inverse temperature cycling (ITC) is used to purify recombinant proteins by a series of cold and hot temperatures where the ELPs are soluble below transition temperature (usually in the cold spin) and insoluble above transition temperature (usually in the hot spin) (Figure 3). Before starting ITC, proteins were constructed. Then for protein production, the gene encoding the protein was expressed in the BLRD competent cells which were grown overnight in an agar plate.

Elastin like polypeptide (ELP)

Figure 3: Transition phase of ELPs above and below transition temperatures. Below transition temperature ($T < T_t$), ELPs are soluble and above transition temperature ($T > T_t$), ELPs are insoluble forming aggregates.

A single colony was selected from the streaked agar plate containing carbenicillin (75uL of 1 mg/mL stock) for starting a culture of 75 mL of terrific broth media (1 L solution: deionized (DI) water, 12 g of tryptone, 24 g of yeast extract, 2.31 g of potassium phosphate monobasic, 12.54 g of potassium phosphate dibasic, and 4 mL of 100% Glycerol). This culture was inoculated overnight and transferred to a one liter culture the next day (1mL of 1 mg/mL stock of carbenicillin was added). This one liter culture was grown overnight and the pelleted bacteria was collected by centrifuging the liter culture at 4°C at 3,000 G for 20 minutes. Once the pelleted bacteria is collected, bacteria was re-suspended in 160 mL 4°C phosphate buffered saline (PBS) and were lysed using a sonicator. The settings were for a total of 12 minutes three cycles with 59 seconds pulse on and 59 seconds pulse off, and a 50% amplitude. After each sonicating cycle the bacteria

solution was put on ice in a 4°C fridge for 30 minutes. Once the sonication process was completed, cellular debris were removed by a centrifugal process of 20,000 G at 4°C for 20 minutes. Since ELPs are soluble at 4°C, the supernatant was collected and the pellet was discarded. ITC process (Figure 4) was proceeded once the supernatant containing the ELPs was collected.

Figure 4: Schematic of ELP protein purification using Inverse Temperature Cycling (ITC).

Since salt is a supplement that enhanced the aggregation of ELPs, 2 M of sodium chloride (NaCl) was added to the supernatant. The supernatant salt solution was vortexed and incubated in a water bath at 42°C for 45 minutes. Above transition temperature ELPs are insoluble. Therefore, in the water bath, the solution becomes cloudy which means that the ELPs are insoluble. After the incubation time, the ELPs pellet was collected by centrifuging the hot solution at 20,000 G at 40°C for 15 minutes. The supernatant from this hot spin was discarded. The pellet was re-suspended in 100 mL 4°C PBS. If the ELPs contain cysteines, to prevent disulfide bonds, dithiothreitol (DTT) was added in the re-suspended pellet solution. This solution was cooled for one hour to allow ELPs to dissolve because they are soluble at 4°C. Then a cold spin was performed by centrifugation at 20,000 G at 4°C for 20 minutes. The supernatant was collected

and the ITC cycle of hot and cold spins was repeated at least two more times. After the last hot spin, the pellet containing the ELP was re-suspended in 50 mL 4°C DI water. This solution was then dialyzed against DI water for a total 48 hours (after 24 hours the old water was discarded and fresh water was added) to remove any remaining DTT or salts present. After dialysis, the ELPs were lyophilized for 72 hours.

CHAPTER 3: DEVELOPMENT OF HETEROGENEOUS ELPS NANOPARTICLES BASED DELIVERY SYSTEM FOR TARGETED GENE THERAPY

3.1 Introduction

Genetic diseases caused by a defective gene, single modification in a gene or mutations affect millions of people worldwide. People with genetic diseases such as cystic fibrosis [2], cancer [37], and Parkinson's [5] are getting treatments to improve their quality of life but there are no cures for these diseases. Gene therapy that aims to deliver a gene of interest to replace, inactivate or correct the defective gene to help the diseased tissue function properly is the most promising approach for curing these debilitating genetic diseases. Scientists have used multiple gene delivery techniques such as lipid mediated gene delivery [8], cationic polymers [9], electroporation [10], microinjection [11], and viruses [55]. Due to their efficient mode of gene delivery, viruses such as lentiviruses are popular vectors for gene delivery [24]. Lentiviral vectors are attractive because they can infect both dividing and non-dividing cells [25-27], and they integrate into the cells' genome [28]. The problem with viruses is their lack of specificity, which can result in indiscriminate transduction [34]. The non-specific delivery of therapeutic genes could lead to undesirable side effects such as toxicity [35, 36], insertional mutagenesis [35, 36], and immunogenicity [35, 36]. The need for targeted therapy is imperative because it could lower the probability of these undesirable side effects and increase the probability of delivering the gene of interest to the desired site.

Most recombinant lentiviral vectors in use for gene therapy are pseudotyped by the Vesicular stomatitis virus (VSV) glycoprotein G VSV-G [56]. This is primarily because VSV exhibits a very robust and pantropic infectivity and also it has been extensively studied and characterized [57]. VSV-G pseudotyped lentiviruses exhibit remarkable stability, high transduction efficiency and the

same broad tropism as VSV. Thus, currently they are the gold standard for several gene therapy procedures [58]. Recent studies have indicated that VSV-G interacts with the low density lipoprotein receptor (LDLR) thereby enabling the entry of the virus into the cells [59]. The widespread expression of LDLR accounts for the broad applicability of VSV-G pseudotyped viral vectors for gene therapy.

Elastin like polypeptides (ELPs) are gaining popularity as drug delivery vectors [60, 61] due to their ability to be genetically encodable and to undergo phase transition [62]. ELPs are protein-based polymers that have been used as novel drug carriers [38-40]. They are composed of repeating sequence of pentapeptides, $(\text{Val-Pro-Gly-X-Gly})_n$ where X can be any amino acids except for Proline [41] because it destroys the inverse phase transition property of ELPs [42]. This sequence of pentapeptides is derived from the hydrophobic chain of tropoelastin, a soluble form of elastin [43]. ELPs have an interesting physical property where they undergo an entropically driven phase transition rendering them insoluble above the transition temperature [43]. This property enables ELPs to be expressed in a bacterial host (such as *Escherichia coli*) and to be purified rapidly using inverse temperature cycling (ITC) [44]. Moreover, since ELPs are genetically encodable, chimeric fusion proteins comprising of biologically active motifs and ELPs can be synthesized easily [63, 64]. These fusion proteins retain the biological activity of the fused motif as well as the phase transitioning property of ELPs, thereby enabling the self-assembly of nanostructures such as nanoparticles above the transition temperature [64]. These characteristics of ELPs make them attractive targeted delivery vehicles for viral gene delivery.

Our lab has previously described a heterogeneous nanoparticle delivery system based on ELPs that focuses on selective delivery of peptides through selective enhancement of macropinocytosis via growth factors [65]. Here we describe the application of the same delivery system for targeted delivery of lentiviral particles to high growth factor receptor expressing cells. Specifically, we report the construction of heterogeneous nanoparticles comprising of two chimeric ELP fusion proteins, namely the low density lipoprotein receptor repeat 3 (LDLR3)-ELP

and keratinocyte growth factor (KGF)-ELP. We report that VSV-G pseudotyped lentiviral particles bind to the heterogeneous nanoparticles via LDLR3-ELP thereby preventing viral entry in cells. We further show that the heterogeneous nanoparticles containing the bound virus are internalized in cells overexpressing the keratinocyte growth factor receptor (KGFR) via KGF-ELP, thereby resulting in successful targeted transduction. We believe that this approach of selective transduction will lead to successful gene therapy treatment with minimum side effects. Moreover, the modular nature of the chimeric ELP fusion protein system ensures that this approach can easily be used to target any growth factor receptor thereby broadening the applicability of this strategy to multiple cell types.

3.2 Materials and Methods

3.2.1 Materials

Dulbecco modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies. A549, H1650, H23, and H292 cells were kindly donated by Dr. Hara from Moffit Cancer Center. Peptide genes and growth factors were purchased from GenScript (Piscataway, NJ). H293 cells were purchased from ATCC (Manassas, VA).

The gel extraction, miniprep, and midiprep kits were purchased from QIAGEN (Valencia, CA). RT-PCR reagents were purchased from Bio-Rad (Hercules, CA). The restriction enzymes and other enzymes used for cloning were purchases from New England Biolabs (Ipswich, MA). The lentiviral plasmids psPAX2 and pMD2.G were a gift from Didier Trono (Addgene plasmid #12259 and #12260, respectively). The lentiviral gene plasmid, pLVTHM-syndecan-1 shRNA GFP, was kindly donated by Dr. Ralph Sanderson from the University of Alabama.

3.2.2 Cell Culture

Human lung carcinoma cells, A549 and H292, and human lung adenocarcinoma cells, H23 and H1650, and human embryonic kidney cells, H293, were cultured in DMEM supplemented with 10% FBS and 1% antibiotic antimycotic (AA) in a humidified incubator at 37°C and 5% CO₂.

3.2.3 Synthesis of LDLR3-ELP and KGF-ELP

The PUC57 plasmids containing the genes (VPGVG)₂VPGCCG(VPGVG)₂, LDLR3, and KGF were purchased from GenScript (Piscataway, NJ). Recursive direction ligation method was used to create V40C2 encoding gene as previously described [66]. PflMI and BglI enzymes were used to cut the LDLR3 and KGF genes. A 1.0% agarose gel was used to run these genes. The genes were then extracted using QIAquick gel extraction kit. The PflMI was used to linearize the pUC19 vector containing the ELP sequence and the removed LDLR3 or KGF genes were cloned in with the ELP gene.

The pUC19 vector containing LDLR3-ELP or the KGF-ELP fusion protein gene situated by PflMI and BglI sites. PflMI and BglI enzymes were used to remove the sequence encoding fusion proteins from the pUC19 vector. As mentioned above, a gel extraction was performed to recover the sequence encoding fusion proteins. The pET25b+ expression vector was modified to incorporate a SfiI cloning site for the LDLR3-ELP or the KGF-ELP fusion protein gene to be cloned. Through heat shock at 42°C, the pET25b+ expression vector containing the LDLR3-ELP or the KGF-ELP fusion protein gene was transformed into BLRD competent cells.

3.2.4 Purification of LDLR3-ELP

For protein production, the BLRD competent cells were grown overnight in an agar plate. A single colony was selected from the streaked agar plate containing carbenicillin for starting a culture of 75 mL. This culture was inoculated overnight and transferred to a one liter culture the next day. This one liter culture was grown overnight and inverse temperature cycling was used to purify LDLR3-ELP or the KGF-ELP fusion as previously described [41]. Since LDLR3-ELP have six cysteine residues, double amount of the reducing agent dithiothreitol (DTT) was added to the cold centrifugation to reduce the formation of disulfide bonds. After the ITC process, the ELP fusion proteins solution were dialyzed for 48 hours. They were then lyophilized for 72 hours to be stored at room temperature.

3.2.5 LDLR3-ELP Total Protein Assay

The fusion protein LDLR3-ELP purity was conducted using total protein stain assay with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The lyophilized LDLR3-ELP was dissolved in 4°C PBS to a final concentration of 2 μ M and 10 μ M. Both the bacterial lysates and the dissolved lyophilized LDLR3-ELP were analyzed by SDS-PAGE (12% acrylamide gels).

3.2.6 Characterization of LDLR3-ELP

The Dynamic light scattering (DLS) instrument (Zetasizer Nano S, Malver, UK) was used to analyze the transition temperature from 4°C to 40°C at a step of 2°C and size of the LDLR3-ELP at the body's physiological temperature, 37°C. Three readings were performed in each temperature point with an equilibrium time of 10 minutes. For both transition temperature and size, 2 μ M of LDLR3-ELP was prepared in 1X phosphate buffered saline (PBS) and 1 mL of the solution was inserted into a cuvette for readings. For the transition temperature, readings were based on scattered light intensity versus temperature. For the size, readings were based on mean intensity versus diameter size at 37°C.

3.2.7 Labeling of the Fusion Proteins

For the particle internalization experiments, maleimide chemistry was used to label the cysteines that are present in the fusion proteins. Fluorescein-5-maleimide (AnaSpec Inc cat # 81405) was added to the fusion proteins. The mixture was incubated at room temperature for one hour on an orbital shaker. Then it was incubated at 4°C overnight. A series of hot and cold cycles were then performed to remove unconjugated fluorescein on a centrifuge at 20,000 G for 10 minutes each time. In the hot spin, the pellet is collected because above transition temperature the protein aggregates. In the cold spin, the supernatant is collected because below transition temperature the protein is soluble. Overall, after the hot spin, the supernatant was discarded and equal amount of volume of cold sterile PBS was added. Once the pellet is dissolved, the

supernatant was collected at 4°C. The hot and cold cycles were repeated at least two times to remove all of the unconjugated fluorescein.

3.2.8 Internalization Assay for Fusion Proteins

Cells (20,000/well) were seeded in a 48-wells plate and cultured in DMEM supplemented with 10% FBS and 1% AA. They were grown until 30% confluency was reached and were starved for 24 hours then were treated in serum free media with the indicated treatments (labeled 2 μ M LDLR3-ELP and combination of labeled 2 μ M LDLR3-ELP and ELP and KGF-ELP, and LDLR3-ELP, respectively) for 48 hours. Flow cytometry was used to quantify the data. Before analysis, trypan blue was used in each sample suspension to capture only the internalized labeled fusion proteins and not the ones that are bound to the periphery of the cells. Flow cytometry was used to quantify the data and the normalized fold increase of cell uptake was reported.

3.2.9 Cytotoxicity Assay

Cells (20,000/well) were seeded in a 48-wells plate and cultured in DMEM supplemented with 10% FBS and 1% AA. They were grown until 30% confluency was reached and were starved for 24 hours then were treated in serum free media with the indicated treatments for 48 hours. After their respective treatments, the plate was put on ice for five minutes to dissolve fusion proteins, then cells were washed three times with ice-cold sterile PBS. Thiazolyl blue tetrazolium bromide (MTT, Acros Organics cat # 298-93-1) was added to each well to a final concentration of 1.2 mM. Cells were then incubated for three hours at 37°C. After the incubation time, cells were washed once with room temperature sterile PBS to remove the excess MTT medium. Dimethyl sulfoxide (DMSO, Fisher Bioreagents cat # BP231-1) was added to solubilize the formazan crystals. For data analysis, the EON microplate spectrophotometer (BioTek, Winooski, VT) was used to read the absorbance at 570 nm.

3.2.10 Creation of Lentiviral Particles

Lentiviral particles were created using calcium phosphate transfection protocol. Transfection is the process of creating the lentiviral particles.

3.2.10.1 Heat Inactive Media

FBS was thawed at 37°C. Once thawed, it was incubated in a water bath at 55°C for 30 minutes. Then filtered with 0.22 µm. Aliquots of 10 mL were made and stored at -20°C.

3.2.10.2 Viral Plasmid Isolation

The psPAX2, pMD2.G (Vesicular Stomatitis Virus Glycoprotein-G (VSVG)) and pLVTHM-syndecan-1 shRNA GFP, were inoculated and grown overnight. The plasmid DNAs were isolated as described by QIAGEN midiprep kit.

3.2.10.3 Calcium Phosphate DNA Co-Precipitation

The generation of virus from a transient transfection using calcium phosphate precipitation protocol as previously described [67] was optimized to create lentivirus particles pseudo typed with the VSV-G envelope. Briefly, the day prior transfection, early passage of H293 cells (1 x 10⁶/well) were seeded in a 6-well plate and cultured in DMEM supplemented with 10% FBS and 1% AA. They were grown until 70% to 80% confluency is reached.

Calcium phosphate precipitates were prepared in a 15mL conical tube in master mixes sufficient for transfect a 10 mm cell culture dish. Each dish received a solution containing 6 ug of DNA (2.90 ug of psPAX2; 0.10 ug of pMD2.G; and 3.0 ug of pLVTHM-syndecan-1 shRNA GFP), 124 µL of 2 M calcium chloride, 1 mL of 2X HEPES-buffered saline solution (2X HBS), and 858.9 uL of nuclease free water in a total volume of 2000 µL. DNA was added to nuclease free water in the order of psPAX2, pMD2.G, and pLVTHM-syndecan-1 shRNA GFP, then calcium chloride was added dropwise to that solution and was left to react for 5 minutes at room temperature. 2X HBS was then added at the bottom of the test tube then a 1mL pipette was used to blow bubbles for 15 seconds. This overall solution was left in room temperature for 30 minutes for precipitates to form.

Prior to adding the calcium phosphate precipitates to the wells, to enhance transfection, 5 µL of 50 mM of chloroquine was added to the dish and incubated at 37°C for five minutes. The calcium phosphate precipitates (2 mL/dish) were then added to the dish dropwise. After 8 hours,

the media was extracted and 10 mL of fresh 37°C DMEM supplemented with 10% FBS and 1% AA was added to each well.

Once 100% confluency is reached between 24 and 48 hours, 10 mL of heat inactive DMEM media was added to the dish. The supernatant containing the lentiviral particles was collected after 24 hours and filtered with a sterile 0.45 µm filter. Lentiviral particles can be immediately used for various assays or stored at -80°C. Transfection efficiency was about 70%.

3.2.11 Inhibition Assay

Cells (10,000/well) were seeded in a 24-wells plate and cultured in DMEM supplemented with 10% FBS and 1% AA. They were grown until 30% confluency was reached. Prior to treatment, 8 µg of 10 mg/mL of polybrene (Santa Cruz Biotechnology cat# sc-134220) was added to the supernatant containing the viral particles to neutralize the virus to allow the cells to uptake them. The viral particles were incubated with the NPs prior to cell treatment. Then cells were incubated for 24 hours with the virus containing NPs. After 24 hours, the treatment was removed and fresh media, DMEM supplemented with 10% FBS and 1% A, was added to each well. Then, 72 hours later, the transduction efficiency was quantified using flow cytometry and the normalized fold increase GFP-expressing cells was reported.

3.2.12 Transduction Assay

Cells (20,000/well) were seeded in a 48-wells plate and cultured in DMEM supplemented with 10% FBS and 1% AA. They were grown until 30% confluency was reached and were starved for 24 hours. Prior to treatment, 8 µg of 10 mg/mL of polybrene (Santa Cruz Biotechnology cat# sc-134220) was added to the supernatant containing the viral particles to neutralize the virus to allow the cells to uptake them. The viral particles were incubated with the NPs prior to cell treatment. Then the cells were incubated for 24 hours with the virus containing NPs. After 24 hours, the old media was discarded and fresh 37°C media containing DMEM supplemented with 10% FBS and 1% AA was added to each well. Then, 72 hours later after their respective treatments, each plate was put on ice for five minutes to dissolve fusion proteins, then cells were washed three times

with ice-cold sterile PBS and were trypsinized using 0.25% trypsin (200 μ L). The trypsin reaction was neutralized by an addition of 200 μ L of DMEM supplemented with 10% FBS and 1% AA. Solution was then transferred into micro-test tubes and cells were pelleted and re-suspended in 250 μ L of PBS. Then cells were analyzed using flow cytometry and the normalized fold increase GFP-expressing cells was reported.

3.2.13 Binding Assay

A549 cells (20,000/well) and H293 cells (30,000/well) were seeded in a 48-wells plate and cultured in in DMEM supplemented with 10% FBS and 1% AA, respectively. A549 cells were grown until 30% confluency was reached and were starved for 24 hours, but not the H293 cells. H293 cells were cultured with 10% FBS throughout the experiment since they are sensitive in serum free media. Prior to treatment, 8 μ g of 10 mg/mL of polybrene was added to virus. The virus was incubated at 4°C for 10 minutes. Only LDLR3-ELP was added to the virus and the micro-test tubes were incubated at 4°C for 15 minutes. Afterwards, all vials were incubated at 37°C for 30 minutes. Virus with the treatments were centrifuged at 10,000 G at 37°C for 1 minute. A negative control containing the virus was not centrifuged to determine if the centrifuge affected the virus throughout the protocol. Supernatant was collected and the pellet was re-suspended in an equal amount of 4°C heat inactive media. Both supernatant and re-suspended pellet were incubated at 4°C for 15 minutes. Then, ELP, KGF-ELP, and LDLR3-ELP were added respectively.

All samples, supernatant and re-suspended pellet were then incubated for 30 minutes at 37°C. Treatments were added to cells. Then the cells were incubated for 24 hours with the virus containing NPs. After 24 hours, the old media was discarded; for the A549 cells fresh 37°C serum free media containing DMEM supplemented and 1% AA was added to each well; for the H293 cells fresh 37°C serum media containing DMEM supplemented with 10% FBS, and 1% AA was added to each well. Then, 72 hours later after their respective treatments, the plates were put on ice for five minutes to dissolve fusion proteins, cells were then washed with ice-cold sterile PBS.

For visualization purposes fluorescent pictures were taken before analysis of flow cytometry. Nuclei were counterstained with NucBlue (Life Technologies R37605) and fluorescent pictures were taken (20X magnification; bar 200 μ m) using EVOS fluorescence microscope (Life technologies). Cells were trypsinized using 0.25% trypsin (200 μ L). The trypsin reaction was neutralized by an addition of 200uL of DMEM supplemented with 10% FBS and 1% AA. Solutions were transferred into micro-test tubes and cells were pelleted and re-suspended in 250 μ L of PBS. Then cells were analyzed using flow cytometry and the normalized fold increase GFP-expressing cells and geometric mean of cells were reported.

3.3 Results

3.3.1 Fusion Protein Comprising of the Viral Envelope Binding Domain (VBD) and Elastin Like Peptide (ELP) was Successfully Expressed and Purified Using Inverse Temperature Cycling (ITC)

Previous studies have shown that the virus pseudotyped with the vesicular stomatitis virus glycoprotein-G (VSV-G) envelope enters the cell by binding with the low density lipoprotein (LDL) receptor [59]. This binding prevented the virus from infecting the cells. In order to create a fusion protein that binds to the virus to prevent the non-selective infection of untargeted cell types, a fragment of the LDL receptor gene, LDL receptor repeat 3 (LDLR3), was cloned upstream of the elastin like peptide gene (Figure 5).

Throughout the recursive ligation process, we checked if the LDLR3 gene was successfully inserted in the PuC19V40C2 gene after performing a gel electrophoresis; this was done after performing the colony polymerase chain (PCR) (Figures 6 and 7). This showed a band at 1000 base pairs (bp). The self-ligation is below the 1000 bp mark. To make sure that LDLR3-ELP was expressed, we cut it with the restriction enzyme PST1 and performed a gel electrophoresis.

*Gel extraction was performed to extract peptide

Figures are not drawn to scale

Figure 5: Schematic of recursive ligation and transformation process of LDLR3-ELP.

Figure 6: Schematic of LDLR3-ELP successfully inserted into the ELP gene.

Figure 7: LDLR3 was successfully inserted in the Puc19V40C2 plasmid. A gel electrophoresis was ran after performing PCR in the cloning competent cells. ELP=V40C2. The self-ligation has a band below 1000 bp while LDLR3-V40C2 has a band approximately 1000pb.

Once the LDLR3-ELP plasmid is cut, a single band at about 4 kb is shown (Figure 8). Finally, we showed that LDLR3-ELP was successfully inserted in the expression plasmid PET25b+ after performing the colony PCR in the expression competent cells BLRD and running a gel electrophoresis (Figure 9).

Figure 8: LDLR3-V40C2 was successfully cut by restriction enzyme PST1. An agarose gel electrophoresis was ran and a band is shown about 4 kb.

Figure 9: LDLR3-V40C2 was successfully inserted in the expression plasmid PET25b+. A colony PCR was performed and a gel electrophoresis was ran. The red rectangle shows the colony that was pick to express in the expression competent cells BLRD.

We then performed a transformation for the plasmid to be expressed in the expression competent cells BLRD. BLRD is the bacterial host (*E. Coli*) that carries T7 RNA polymerase as a

λ lysogen [68]. T7 RNA polymerase allows DNA to be transcribed and is highly specific for T7 phage promoters. The gene encoding the fusion protein LDLR3-ELP (where ELP is V40C2) was then expressed in a bacterial host (*E. coli*) and purified by inverse temperature cycling (ITC).

After three ITC cycles, isolated protein was stained with simply safe blue and analyzed for purity by SDS-PAGE gel electrophoresis. The single black band with a molecular weight of 50 kDa shown in figure 10 (with description of each lane in table 3) confirms purity of the LDLR3-ELP fusion protein. Different concentrations were used to clearly show the black band, a darker band at 10 μ M and a faded band at 2 μ M.

Figure 10: LDLR3-ELP fusion protein was successfully purified using ITC in the 12% SDS-PAGE gel.

Table 3 Lane descriptions for Figure 10

Lane	Description
1	Ladder
2	10uM LDLR3-ELP
3	2uM LDLR3-ELP
4	Bacterial Lysate
5	Bacterial Lysate

3.3.2 LDLR3-ELP Maintains the Phase Transition Property and Self-Assembles Into Nanoparticles

Since we were successfully able to purify LDL3-ELP using ITC, it suggests that LDLR3-ELP fusion protein retained the physical phase transition property characteristic of ELPs (Figure 11).

Figure 11: LDLR3-ELP design. The top figure represents the sequence of LDLR3-ELP gene inserted into the expression vector. The bottom figure represents the LDLR3-ELP fusion protein holds the characteristics of ELPs where below transition temperature it is soluble and above transition temperature it is insoluble.

To further characterize the phase transitioning property of LDLR3-ELP, dynamic light scattering (DLS) was used to analyze the transition temperature of LDLR3-ELP at a concentration of 2 μM . The results show that LDLR3-ELP transitions near 30°C (Figure 12). Previous studies described for other ELP based fusion proteins [41] state that at the transition temperature the fusion protein form fairly monodispersed aggregates with radii in the nanometer range. To ascertain whether this is the case with LDLR3-ELP, we measured the particle size and dispersity with DLS at a concentration of 2 μM , at homeostatic physiological temperature 37°C. The

dispersity of proteins was tightly distributed around a mean size of 150 nm with respective minimum and maximum sizes of 12 nm and 615 nm with 96.4% of peak 1 area intensity is 204 nm and 3.6% of peak 2 area intensity is 3.6 nm (Figure 13). A concentration of 2 μ M of LDLR3-ELP was characterized because that is the total concentration used for the experiments mentioned in this paper.

Figure 12: LDLR3-ELP transitions near 30°C. Dynamic light scattering (DLS) was used to analyze the transition temperature of LDLR3-ELP at a concentration of 2 μ M (in PBS) from 5°C to 40°C.

Figure 13: LDLR3-ELP size distribution at the body's physiological temperature 37°C. DLS was used to analyze the size distribution of LDLR3-ELP at a concentration of 2 μ M (in PBS) at 37°C. The size distribution is presented as mean intensity (%).

3.3.3 LDLR3-ELP Inhibits Virus Infectivity and Does Not Induce Cell Death

Previous studies have shown that recombinant soluble LDLR (sLDLR) inhibited transduction by a lentivirus pseudotyped with the VSV-G envelope [59]. To test if LDLR3-ELP fusion protein maintained the activity of the fused LDLR-3 domain (Figure 14), a transduction assay using lentiviral particles pseudotyped with the VSV-G envelope and containing the green fluorescent protein (GFP) gene was performed. The virus and LDLR3-ELP fusion protein were incubated together and then introduced to cells. Indeed, 20 μ M of LDLR3-ELP inhibited the viral transduction efficiency of more than 99% (Figures 15 and 16).

Figures are not drawn to scale

Figure 14: Schematic of LDLR3-ELP binding to the virus in the presence of calcium.

Figure 15: Green Fluorescent Protein (GFP) expression in A549 cells of LDLR3-ELP inhibiting virus infectivity. A549 cells (20,000/well) were seeded in a 48-wells plate and cultured in DMEM supplemented with 10% FBS and 1% AA. They were grown until 30% confluency was reached and were starved for 24 hours. Prior to treatment, 8µg of 10mg/mL of polybrene was added to the supernatant containing the viral particles to neutralize the virus to allow the cells to uptake them. The viral particles were incubated with the NPs prior to cell treatment. Then the cells were incubated for 24 hours with the virus containing NPs. After 24 hours, the old media was discarded and fresh serum free 37°C media containing DMEM supplemented and 1% AA was added to each well. 72 hours later, Nuclei were counterstained with NucBlue and fluorescent pictures were taken (20X magnification; white bar is 200 µm) using EVOS fluorescence microscope.

Previous studies have reported that sLDLR inhibits virus infectivity by binding to the VSV-G envelope of the lentivirus. sLDLR binds to the virus in the presence of calcium. Though our body contains calcium, the binding affinity of calcium and LDLR3-ELP was not addressed, we showed that the LDLR3-ELP binds with the lentivirus pseudotyped with VSV-G envelope. Then, we incubated the virus with different concentrations of LDLR3-ELP fusion protein. The transitioned LDLR-3-ELP along with any bound virus was then pelletized by centrifugation and the supernatant was collected (Figure 17). The collected supernatant was then put atop of the cells and the remaining virus in the supernatant was quantified by measuring the transduction efficiency. Indeed, we observed a decrease in transduction efficiency of more than 90% suggesting that the most of the virus was bound to the LDLR-3-ELP (Figures 18 and 19). We further performed an MTT assay to make sure that LDLR3-ELP was not toxic to cells. Our results indicate that LDLR3-ELP with a concentration as high as 20 µM is not toxic to cells (Figure 20).

Figure 16: Quantification of the GFP expression in A549 cells of LDLR3-ELP inhibiting virus infectivity. Average $\pm$ SD of the normalized GFP Expression. A549 cells (20,000/well) were seeded in a 48-wells plate and cultured in DMEM supplemented with 10% FBS and 1% AA. They were grown until 30% confluency was reached and were starved for 24 hours. Prior to treatment, 8 μ g of 10mg/mL of polybrene was added to the supernatant containing the viral particles to neutralize the virus to allow the cells to uptake them. The viral particles were incubated with the NPs prior to cell treatment. Then the cells were incubated for 24 hours with the virus containing NPs. After 24 hours, the old media was discarded and fresh serum free 37°C media containing DMEM supplemented and 1% AA was added to each well. 72 hours later, the transduction efficiency was quantified using flow cytometry. Treatments were normalized to control (virus). These experiments were repeated two more times with triplicates. ***indicates $P < 0.001$ when compared to control (virus). Error bars represent $\pm$ SD.

Figure 17: Schematic of the transitioned LDLR-3-ELP along with any bound virus.

Figure 18: GFP expression in A549 cells of LDLR3-ELP inhibiting virus infectivity at different concentrations. A549 cells were starved for 24 hours and a binding assay was performed. Cells were treated for 24 hours with the virus containing NPs. After 24 hours, the old media was discarded, and fresh 37°C serum free media was added to each well. Then, 72 hours later Nuclei were counterstained with NucBlue and fluorescent pictures were taken (20X magnification; white bar is 200μm) using EVOS fluorescence microscope.

Figure 19: Quantification of the GFP expression in A549 cells of LDLR3-ELP inhibiting virus infectivity. Average $\pm$ SD of the normalized GFP Expression. A549 cells (20,000/well) were seeded in a 48-wells plate and cultured in in DMEM supplemented with 10% FBS and 1% AA. They were grown until 30% confluency was reached and were starved for 24 hours. Prior to treatment, 8µg of 10mg/mL of polybrene was added to virus. The virus was incubated at 4°C for 10 minutes. Only LDLR3-ELP was added to the virus and the micro-test tubes were incubated at 4°C for 15 minutes. Afterwards, all vials were incubated at 37°C for 30 minutes. Virus with the treatments were centrifuged at 10,000g at 37°C for 1 minute. A negative control containing the virus was not centrifuged to determine if the centrifuge affects the virus throughout the protocol. Supernatant was collected and cells were treated with the supernatant. Then the cells were incubated for 24 hours with treatment in the supernatant. After 24 hours, the old media was discarded, and fresh 37°C serum free media containing DMEM supplemented and 1% AA was added to each well. 72 hours later, the transduction efficiency was quantified using flow cytometry. Treatments were normalized to control (virus). These experiments were repeated two more times with triplicates. ***indicates $P < 0.001$ when compared to control (virus). Error bars represent $\pm$ SD.

Figure 20: LDLR3-ELP does not induce cell death. A549, H1650, H292, and H23 cells (20,000/well) were seeded in a 48-wells plate and cultured in DMEM supplemented with 10% FBS and 1% AA. They were grown until 30% confluency is reached and were starved for 24 hours then were treated in serum free media with the indicated treatments (0.2 μ M, 2 μ M, 10 μ M and 20 μ M LDLR3-ELP) for 48 hours. After 48 hours, a MTT assay was performed and a spectrophotometer was used to read the absorbance at 570nm. Treatments were normalized to control (no treatment). These experiments were repeated two more times with triplicates. ***indicates $P < 0.001$, **indicates $P < 0.05$, and *indicates $P < 0.1$ when compared to control (no treatment). Error bars represent $\pm$ SD.

3.3.4 Targeted Internalization of LDLR3-ELP Using Growth Factors in High Growth Factor Receptor Expressing Cells

Our results clearly indicate that LDLR3-ELP binds to the virus and thus prevents the virus from entering and infecting the cells. We reasoned that the virus can selectively be delivered to the cells by targeted delivery of LDLR3-ELP to the cells. We have previously described a

heterogeneous ELP based nanoparticle delivery platform containing growth factors that selectively delivers payload in high growth factor receptor expressing cells. We reasoned that targeted gene therapy can be achieved using this platform. To test this hypothesis we created heterogeneous nanoparticles (NPs) comprising of keratinocyte growth factor-ELP (KGF-ELP which has been previously created in our lab Figure 21) and LDLR3-ELP (Figure 22). Only 2 μ M of LDLR3-ELP were labeled with maleimide chemistry. Indeed, these heterogeneous NPs were selectively able to deliver LDLR-3-ELP in the high KGFR expressing A549 cells (Figure 23).

Figure 21: KGF-ELP design. KGF-ELP fusion protein holds the characteristics of ELPs where below transition temperature it is soluble and above transition temperature it is insoluble.

Figure 22: Schematic of heterogeneous nanoparticle comprising of keratinocyte growth factor-ELP (KGF-ELP) and LDLR3-ELP.

Figure 23: Targeted internalization of LDLR3-ELP using growth factors in high growth factor receptor expressing cells. Average $\pm$ SD of the normalized fold increase of cell uptake. A549 cells were starved for 24 hours then were treated in serum free media with the indicated treatments labeled 2 μ M LDLR3-ELP and a combination of labeled 2 μ M LDLR3-ELP and ELP and KGF-ELP, and LDLR3-ELP respectively, for 48 hours. After 48 hours, flow cytometry was used to quantify the data. Before analysis of using the flow cytometry, trypan blue was used in each sample suspension to capture only the internalized labeled fusion proteins and not the ones that are bound to the periphery of the cells. These experiments were repeated two more times with triplicates. ***indicates $P < 0.001$ when compared to control (2 μ M LDLR3-ELP). Error bars represent $\pm$ SD.

3.3.5 Heterogeneous Nanoparticles Comprising of LDLR3-ELP and KGF-ELP Result in Targeted Delivery of the Gene in High Growth Factor Receptor Expressing Cells

Next, we tested whether the heterogeneous NPs comprised of LDLR-3 ELP and KGF-ELP can selectively deliver the VSV-G pseudotyped virus to high growth factor receptor expressing

A549 cells thereby resulting in selective transduction. We first performed a transduction assay, where we added the virus, LDLR3-ELP, and KGF-ELP together. Figure 24 shows that LDLR3-ELP blocks the virus infectivity, however, in the presence of KGF-ELP, the transduction efficiency increased. Since we did not want free floating virus, we performed a binding assay to test if the LDLR3-ELP binds to the virus. Also, we performed a transduction efficiency assay to test different concentrations of LDLR3-ELP in the presence of the virus.

Figure 24: KGF-ELP enhanced the transduction efficiency blocked by LDLR3-ELP. A549 Cells (10,000/well) were seeded in a 24-wells plate and cultured in DMEM supplemented with 10% FBS and 1% AA. They were grown until 30% confluency was reached. Prior to treatment, 8μg of 10mg/mL of polybrene (Santa Cruz Biotechnology cat# sc-134220) was added to the supernatant containing the viral particles to neutralize the virus to allow the cells to uptake them. The viral particles were incubated with the NPs prior to cell treatment. Then cells were incubated for 24 hours with the virus containing NPs. After 24 hours, the treatment was removed and fresh media, DMEM supplemented with 10% FBS and 1% A, was added to each well. 72 hours later, the transduction efficiency was quantified using flow cytometry and the normalized fold increase GFP-expressing cells were reported. These experiments were repeated one more time with duplicates. **indicates $P < 0.05$ when compared to control (virus). Error bars represent $\pm$ SD.

To this end, the virus was incubated with 1 μM LDLR3-ELP and the LDLR3-ELP bound with the virus was collected by centrifugation. The pellet containing the LDLR3-ELP bound with the virus was re-suspended in cold media and was then mixed with 1 μM KGF-ELP, resulting in the formation of heterogeneous NPs containing the virus and KGF-ELP (Figure 25).

Figures are not drawn to scale

Figure 25: Schematic of heterogeneous NPs containing the virus and KGF-ELP.

These heterogeneous nanoparticles have a higher probability of being internalized by high growth factor receptor expressing cells (A549 cells) than low growth factor receptor expressing cells (H293 cells) (Figures 26 and 27).

Figure 26: Schematic of heterogeneous nanoparticles with virus that have a higher probability of being internalized by high growth factor receptor expressing cells (A549 cells) than low growth factor receptor expressing cells (H293 cells).

Figure 27: GFP expression of heterogeneous nanoparticles that have a higher probability of being internalized by high growth factor receptor expressing cells (A549 cells) than low growth factor receptor expressing cells (H293 cells). A549 cells (20,000/well) and H293 cells (30,000/well) were seeded in a 48-wells plate and cultured in in DMEM supplemented with 10% FBS and 1% AA, respectively. A549 were grown until 30% confluency was reached and were starved for 24 hours, but not the H293 cells. H293 cells were cultured with 10% FBS throughout the experiment since they are sensitive in serum free media. Prior to treatment, 8μg of 10mg/mL of polybrene was added to virus. The virus was incubated at 4°C for 10 minutes. Only LDLR3-ELP was added to the virus and the micro-test tubes were incubated at 4°C for 15 minutes. Afterwards, all vials were incubated at 37°C for 30 minutes. Virus with the treatments were centrifuged at 10,000g at 37°C for 1 minute. The pellet was re-suspended in an equal amount of 4°C heat inactive media. The re-suspended pellet was incubated at 4°C for 15 minutes. Then, ELP, KGF-ELP, and LDLR3-ELP were added respectively. Then, all samples of the re-suspended pellet were then incubated for 20 minutes at 37°C. Pelleted treatments were added to cells. Then the cells were incubated for 24 hours with the virus containing NPs. After 24 hours, the old media was discarded, and for A549 cells fresh 37°C serum free media containing DMEM supplemented and 1% AA was added to each well, but for the H293 cells fresh 37°C serum media containing DMEM supplemented with 10%FBS, and 1%AA was added to each well. 72 hours later, Nuclei were counterstained with NucBlue and fluorescent pictures were taken (20X magnification; white bar is 200 μm) using EVOS fluorescence microscope.

These nanoparticles resulted in a 5.5 fold increase of transduction efficiency in high KGFR expressing A549 cells. (Figure 28). On the other hand there was a modest increase of about 1.5

fold in H293 cells which have low levels of the KGFR (Figure 28). To determine the number of gene copies introduced per cell we quantified the geometric mean of the fluorescence intensity and found that there was a 30 % increase in the intensity in A549 cells compared to H293 cells (Figure 29), suggesting that multiple viral particles were introduced in the high KGFR expressing A549 cells line.

Figure 28: Quantification of GFP expression of heterogeneous nanoparticles that have a higher probability of being internalized by high growth factor receptor expressing cells (A549 cells) than low growth factor receptor expressing cells (H293 cells). Average $\pm$ SD of the normalized GFP Expression in A549 cells and H293 cells. A549 cells were starved for 24 hours and H293 cells treatment started when their confluency reached 30%. A binding assay was performed. The pellet was re-suspended in an equal amount of 4°C heat inactive media. Then, 1 μ M of ELP, KGF-ELP, and LDLR3-ELP were added respectively. Re-suspended pelleted treatments were added to cells. Then the cells were incubated for 24 hours with the virus containing NPs. After 24 hours, the old media was discarded, and for A549 cells fresh 37°C serum free media was added to each well, but for the H293 cells fresh 37°C serum media was added to each well. 72 hours later, the transduction efficiency was quantified using flow cytometry. Treatments were normalized to control (1 μ M LDLR3-ELP + 1 μ M LDLR3-ELP + Virus). These experiments were repeated two more times with triplicates. ***indicates $P < 0.001$ and **indicates $P < 0.05$ when compared to control (1 μ M LDLR3-ELP + 1 μ M LDLR3-ELP + Virus). Error bars represent $\pm$ SD.

Figure 29: Heterogeneous nanoparticles resulted in higher geometric mean in high growth factors expressing cells. Average normalized geometric mean of A549 and H293 cells. A549 cells (20,000/well) and H293 cells (30,000/well) were seeded in a 48-wells plate and cultured in in DMEM supplemented with 10% FBS and 1% AA, respectively. A549 were grown until 30% confluency was reached and were starved for 24 hours, but not the H293 cells. H293 cells were cultured with 10% FBS throughout the experiment since they are sensitive in serum free media. Prior to treatment, 8µg of 10mg/mL of polybrene was added to virus. The virus was incubated at 4°C for 10 minutes. Only LDLR3-ELP was added to the virus and the micro-test tubes were incubated at 4°C for 15 minutes. Afterwards, all vials were incubated at 37°C for 30 minutes. Virus with the treatments were centrifuged at 10,000g at 37°C for 1 minute. The pellet was re-suspended in an equal amount of 4°C heat inactive media. The re-suspended pellet was incubated at 4°C for 15 minutes. Then, ELP, KGF-ELP, and LDLR3-ELP were added respectively. Then all samples of the re-suspended pellet were incubated for 20 minutes at 37°C. Pelleted treatments were added to cells. Then the cells were incubated for 24 hours with the virus containing NPs. After 24 hours, the old media was discarded, and for A549 cells fresh 37°C serum free media containing DMEM supplemented and 1% AA was added to each well, but for the H293 cells fresh 37°C serum media containing DMEM supplemented with 10%FBS, and 1%AA was added to each well. 72 hours later, the transduction efficiency was quantified using flow cytometry. Treatments were normalized to control (1µM LDLR3-ELP + 1µM LDLR3-ELP + Virus). These experiments were repeated two more times with triplicates. ***indicates P<0.001 and **indicates P<0.05 when compared to control (1µM LDLR3-ELP + 1µM LDLR3-ELP + Virus). Error bars represent ± SD.

3.3.6 ELP and KGF-ELP Do Not Bind to the virus

To confirm that the increase in transduction efficiency demonstrated by the heterogeneous NPs is not because of KGF binding with the viral particles, we tested whether KGF-ELP or ELP binds to the virus (Figures 30 and 31). To this end, the virus and either 12 μ M ELP or KGF-ELP were incubated together and centrifuged. Cells were then treated with the supernatant and the transduction efficiency was assessed. Indeed, neither the ELP nor the KGF-ELP treatment of the virus resulted in a decrease in transduction efficiency of the viral supernatant (Figures 32 and 33) thus suggesting that neither of them bound to the virus.

Figure 30: Schematic of ELP not binding to the virus.

Figure 31: Schematic of KGF-ELP not binding to the virus.

Figure 32: GFP expression of ELP and KGF-ELP that do not bind to the virus. A549 cells (20,000/well) were seeded in a 48-wells plate and cultured in DMEM supplemented with 10% FBS and 1% AA. They were grown until 30% confluency was reached and were starved for 24 hours. Prior to treatment, 8 μ g of 10mg/mL of polybrene was added to virus. The virus was incubated at 4°C for 10 minutes. Treatments, 12 μ M ELP, 12 μ M KGF-ELP, and 2 μ M LDLR3-ELP were added respectively to the virus and the micro-test tubes were incubated at 4°C for 15 minutes. Afterwards, all vials were incubated at 37°C for 30 minutes. Virus with the treatments were centrifuged at 10,000 G at 37°C for 1 minute. A negative control containing the virus was not centrifuged to determine if the centrifuge affected the virus throughout the protocol. Supernatant was collected and the pellet was re-suspended in an equal amount of 4°C heat inactive media. Both supernatant and the re-suspended pellet were incubated at 4°C for 15 minutes. Then all samples, supernatant and the re-suspended pellet were incubated for 30 minutes at 37°C. Treatments were added to cells. Then the cells were incubated for 24 hours with the virus containing NPs. After 24 hours, the old media was discarded, and fresh 37°C serum free media containing DMEM supplemented and 1% AA was added to each well. 72 hours later, Nuclei were counterstained with NucBlue and fluorescent pictures were taken (20X magnification; white bar is 200 μ m) using EVOS fluorescence microscope.

Figure 33: Quantification of GFP expression of ELP and KGF-ELP that do not bind to the virus. Average $\pm$ SD of the normalized GFP expression of supernatant. A549 cells (20,000/well) were seeded in a 48-wells plate and cultured in in DMEM supplemented with 10% FBS and 1% AA. They were grown until 30% confluency was reached and were starved for 24 hours. Prior to treatment, 8μg of 10mg/mL of polybrene was added to virus. The virus was incubated at 4°C for 10 minutes. Treatments, 12μM ELP, 12μM KGF-ELP, and 2μM LDLR3-ELP were added respectively to the virus and the micro-test tubes were incubated at 4°C for 15 minutes. Afterwards, all vials were incubated at 37°C for 30 minutes. Virus with the treatments were centrifuged at 10,000g at 37°C for 1 minute. A negative control containing the virus was not centrifuged to determine if the centrifuge affected the virus throughout the protocol. Supernatant was collected and the pellet was re-suspended in an equal amount of 4°C heat inactive media. Both supernatant and the re-suspended pellet were incubated at 4°C for 15 minutes. Then all samples, supernatant and re-suspended pellet were incubated for 30 minutes at 37°C. Treatments were added to cells. Then the cells were incubated for 24 hours with the virus containing NPs. After 24 hours, the old media was discarded, and fresh 37°C serum free media containing DMEM supplemented and 1% AA was added to each well. Then, 72 hours later, the transduction efficiency was quantified using flow cytometry. Treatments were normalized to control (virus). These experiments were repeated two more times with triplicates. ***indicates $P < 0.001$ when compared to control (virus). Error bars represent $\pm$ SD.

3.3.7 Statistical Analysis

For statistical significance, the p value was calculated for the indicated groups using ANOVA single factor. For the significance of the p values are: ***indicates $P < 0.001$, **indicates $P < 0.05$, and *indicates $P < 0.1$. The reported errors indicated the $\pm$ SD.

3.4 Conclusion

Targeted gene therapy is essential to lower the probability of side effects such as immunogenicity, toxicity, and insertional mutagenesis. Our work demonstrates an approach of using macropinocytosis pathway for selective uptake of the heterogeneous nanoparticles (NPs) with targeting cells that overexpress growth factor receptors. Specifically, the heterogeneous NPs consist of the low density lipoprotein receptor 3 (LDLR3)-ELP and the keratinocyte growth factor (KGF)-ELP. LDLR3-ELP is used because it was found that LDLR3, a section of the cell-surface glycoprotein LDLR, binds with VSV-G envelope in the presence of calcium [59]. The LDLR3 binding affinity to the VSV-G envelope allows LDLR3 to inhibit VSV infectivity [59]. Therefore, to prevent the virus from infecting undesired cells, we used the fusion protein LDLR3-ELP as our viral envelope binding domain. KGF-ELP is used as a targeted protein.

Our work demonstrates that the fusion protein LDLR3-ELP binds to the virus, lentiviral particles pseudo typed with the VSV-G envelope containing the green fluorescent protein (GFP) gene, and does not induce cell death while the ELP and KGF does not bind to the virus. To add selectivity, we used the targeting protein, KGF-ELP. The presence of KGF-ELP allows cells to uptake LDLR3-ELP. Though the mechanism of the internalization of LDLR3-ELP in the presence of KGF-ELP was not addressed, it is possible that macropinocytosis may take part of the internalization due to the presence of the growth factor [65]. In the presence of the virus, our data shows that the heterogeneous NPs have a higher probability of getting internalized by cells that overexpress the KGF receptors compared to cells that have low KGFR expression.

CHAPTER 4: DISCUSSION

Gene therapy is a promising technique for the treatment of many debilitating genetic diseases, such as cystic fibrosis and cancer. Targeted gene therapy is essential because it may lower the probability of side effects including toxicity [35, 36], insertional mutagenesis [35, 36], and immunogenicity [35, 36]. Viruses, such as lentiviruses, are attractive options for gene therapy because they can integrate into the cells' genome [28] and infect both dividing and non-dividing cells [25-27]. Here, we described a novel approach that results in the selective internalization of the virus in cells that overexpress keratinocyte growth factor receptors, thereby delivering the gene to the targeted cells. Specifically, we created heterogeneous nanoparticles comprising of two chimeric fusion proteins namely low density lipoprotein receptor 3 (LDLR3)-ELP and keratinocyte growth factor (KGF)-ELP. We show that the heterogeneous nanoparticles deliver the virus selectively to high KGF receptor expressing cells resulting in selective delivery of the gene encoding green fluorescent protein to those cells.

Our experiments indicate that LDLR3-ELP inhibited transduction by a lentivirus pseudotyped with the vesicular stomatitis virus glycoprotein G (VSV-G) envelope. We further demonstrated that this inhibition was mediated by the binding of the LDLR3-ELP to the virus. This inhibition is similar to previous studies that have shown that recombinant soluble LDLR (sLDLR) inhibited transduction by a lentivirus pseudotyped with the VSV-G envelope [59]. This study further demonstrated that the inhibition was mediated by the binding of the sLDLR to the VSV-G envelope, thereby preventing the virus from binding to the LDL receptor on the target cells and denying its entry. We believe that LDLR3-ELP blocks viral transduction in a similar fashion. The binding of the LDLR3-ELP to the virus also indicates that the binding ability of LDLR3 remained unaffected because of the fusion. This observation is similar to previous studies by several others

and us for chimeric ELP fusion proteins where the biological activity of the functional domain remains unaffected by the ELP fusion [43, 63, 64]. Moreover, the inhibitory activity of LDLR3-ELP without KGF-ELP demonstrates that it can be a potential application as an agent in antiviral therapy.

Viruses can infect multiple cells causing undesired side effects. Thus, to minimize side effects, it is essential to selectively deliver the virus only to the target cells. We further show that heterogeneous nanoparticles comprising of LDLR3-ELP and KGF-ELP can induce selective transduction of VSV-G pseudotyped virus in high KGFR expressing cells. Though the experiments described here do not address the mechanism of the selective transduction, it is possible that the KGF-ELP in the heterogeneous nanoparticle enhances the internalization of the virus bound to the LDLR3-ELP via macropinocytosis; this has been shown previously for other heterogeneous ELP based nanoparticle systems in our lab [65]. While several nanoparticulate systems have been developed for the targeted delivery of plasmids [69] or siRNA [70], very few studies focus on the selective delivery of viruses for targeted gene therapy. For example, the use of magnetic nanoparticles loaded with adenoviral vectors for gene delivery to stented arteries has been described [71]. It is to be noted that a magnetic field was used to achieve site specific delivery in that approach. A similar approach involving magnetic nanoparticles and lentivirus has been described for the transduction of endothelial cells [72] and the delivery of oncolytic adenovirus [73]. Nonetheless, the targeted approach described in this work is different than these strategies as it focuses on the ligand receptor interactions as opposed to providing a magnetic field for site specific delivery. Moreover, applying site directed magnetic field in various parts of the body could be challenging as opposed to simply targeting the receptors for gene delivery.

Interestingly, we observed a 5.5 fold increase of the GFP gene expression in high KGFR expressing cells when treated by the virus in the presence of heterogeneous nanoparticles. We believe that this can further be improved upon by creating a single homogeneous nanoparticle comprising of KGF-ELP-LDLR3 fusion protein. This will prevent the formation of nanoparticles

that solely contain either LDLR3-ELP or KGF-ELP; both of which are a possibility in the case of heterogeneous nanoparticles. These studies are currently underway in our lab to further increase the selective transduction efficiency.

Important characteristics of ELP include: they can undergo self-assembly at a transition temperature; they are genetically encodable; they are immunogenic; and they are biocompatible. We demonstrated that the fusion protein, LDLR3-ELP, maintained the phase transition property and self-assembled into nanoparticles above transition temperature. These fusion proteins retained the biological activity and phase transitioning properties of ELPs, as shown in previous studies [43, 63, 64]. This allowed recombinant expression and simple purification of the fusion proteins by a series of hot and cold spins. Self-assembly is important because this quality allows for the creation of chimeric nanoparticles comprising of different chimeric fusion proteins such as KGF-ELP and LDLR3-ELP, as described here. Additionally, synthetic genes encoding any ELP fusion proteins can easily be synthesized using standard molecular biology approaches. Thus, this approach can easily be broadened to include other growth factors to target other cells expressing different growth factor receptors such as epidermal growth factor receptor (EGFR) or nerve growth factor receptor (NGFR).

In summary, we have developed a novel nanoparticle based delivery system for targeted delivery of the virus to cells. Delivering the virus to the target cells selectively has been a major challenge. We demonstrated that the chimeric ELP fusion protein heterogeneous NPs consisting of LDLR3-ELP as a viral binding domain and KGF-ELP as a targeting protein, can overcome this challenge. This novel design may help target specific cells and can be used as an application for treatment of genetic diseases while limiting side effects of non-targeted gene delivery.

CHAPTER 5: FUTURE DIRECTIONS

5.1 Creating a Fusion Protein Consisting of LDLR3 Fused With KGF-ELP

To improve the fold increase, we would like to eliminate the possibilities of having empty particles (Figure 34). We have a 5.5 fold increase in cells that have high expression of KGF receptor and we can always improve this fold by creating a peptide consisting of LDLR3 fused with KGF-ELP.

Figure 34: Schematic of undesired nanoparticles combinations.

We would like to create the desired combinations of having KGF-ELP and LDLR3-ELP (Figure 35). This should prevent the formation of empty nanoparticles; solely LDLR3-ELP without the

KGF-ELP, or solely KGF-ELP without the LDLR3-ELP. Future studies are to focus on creating a single peptide KGF-ELP-LDR3. This consists of both LDLR3 and KGF-ELP where the LDLR3 acts as the viral binding domain and the KGF acts as the targeting protein. To do this we will perform a recursive ligation transformation process (Figures 36 and 37). Once the peptide is transformed into the BLRD cells, we can purify it using inverse temperature cycling (ITC) and test for its purification.

Desired combinations

● LDLR3

● KGF

⌘ ELP

● Virus

Figure 35: Schematic of desired nanoparticles combinations.

*Gel extraction was performed to extract peptide

Figures are not drawn to scale

Figure 36: Schematic of recursive ligation and transformation process of KGF-ELP-LDLR3.

Figure 37: KGF-ELP-LDLR3 design. The top figure represents the sequence of KGF-ELP-LDLR3 gene inserted into the expression vector. The bottom figure represents KGF-ELP-LDLR3 fusion protein holding the characteristics of ELPs where below transition temperature it is soluble and above transition temperature it is insoluble.

Once we obtain the desired protein, LDLR3-KGF-ELP, we will first incubate it with the virus at 4°C so that the virus can bind to the LDLR3 then we will incubate the solution at 37°C (Figure 38).

Figure 38: Schematic of desired nanoparticle, KGF-ELP-LDLR3, at 4°C and 37°C.

If we incubated the virus and the protein together at 37°C instead of 4°C, there could be a possibility that the nanostructures without the virus could be formed (Figure 39).

Figure 39: Possible combinations of nanoparticles, KGF-ELP-LDLR3 formation at 37°C in the presence of virus.

We believe that this particle can enhance the fold increase of selectively targeted high growth factor receptors cells (eg. KGFR) (Figure 40).

Figure 40: Schematic of heterogeneous nanoparticles of KGF-ELP-LDR3 with the virus that have a higher probability of being internalized by high growth factor receptor expressing cells (A549 cells) than low growth factor receptor expressing cells (H293 cells).

We would also like to create the homogeneous peptide KGF-ELP-LDLR3 so that another peptide that can only fused to ELP can be inserted. To do this we will perform a recursive ligation transformation process (Figure 41). Once the peptide (Figure 42) is transformed into the BLRD cells, we can purify it using inverse temperature cycling (ITC) and test for its purification.

*Gel extraction was performed to extract peptide

Figures are not drawn to scale

Figure 41: Schematic of recursive ligation and transformation process of KGF-ELP-LDLR3.

Figure 42: LDLR3-KGF-ELP design. The top figure represent the sequence of LDLR3-KGF-ELP gene inserted into the expression vector. The bottom figure represents the LDLR3-KGF-ELP fusion protein holding the characteristics of ELPs where below transition temperature it is soluble and above transition temperature it is insoluble.

Once we obtain the desired protein, LDLR3-KGF-ELP, we will first incubate it with the virus at 4°C so that the virus can bind to the LDLR3 then we will incubate the solution at 37°C (Figure 43).

Figure 43: Schematic of desired nanoparticle, LDLR3-KGF-ELP, at 4°C and 37°C.

If we incubated the virus and the protein together at 37°C instead of 4°C, there could be a possibility that the nanostructures without the virus could be formed (Figure 44).

Figure 44: Possible combinations of nanoparticles, LDLR3-KGF-ELP at 37°C in the presence of virus.

We do not want empty particles. Once the protein is incubated at 4°C then we will incubate that solution at 37° so the heterogeneous nanoparticles with the virus can be formed. The final product we would like is either KGF-ELP-LDLR3 or LDLR3-KGF-ELP to improve the fold increase.

5.2 Testing Longevity of Transduction Efficiency

We would like to test the longevity of the transduction efficiency by checking when the cell divides if the new daughter cell obtains the desired gene that was delivered to the parent cell. We would study the geometric mean and the transduction efficiency. Geometric mean (GM) tells you the intensity of the cells. This means that if there are multiple viral particles that one cell

expresses, it will fluoresce a brighter green than a cell that has fewer viral particles. There can be multiple possibilities that can happen, where all the viral particles can be expressed in one cell or can be distributed among other cells. This distribution allows for a higher transduction efficiency (expression of green fluorescent protein (GFP) gene) (Figure 45).

Figures are not drawn to scale

Figure 45: Comparison of high geometric mean and high expression GFP.

5.3 Testing the Internalization Pathway of LDLR3-ELP in the Presence of KGF-ELP

Since we do not want toxicity, immunogenicity, or insertional mutagenesis to occur we added a targeted protein to add selectivity. The targeting protein that we used is KGF-ELP. The presence of KGF-ELP allows cells to uptake LDLR3-ELP. Though the mechanism of the internalization of LDLR3-ELP in the presence of KGF-ELP was not addressed, it is possible that macropinocytosis may take part of the internalization due to the presence of the growth factor [65]. Macropinocytosis has been well studied by others and they have shown that growth factors can enhance it [65]. Cells can uptake particles through different pathways such as receptor-mediated endocytosis and

macropinocytosis [74-76]. The macropinocytosis pathway is beneficial in comparison to the receptor-mediated endocytosis. In the macropinocytosis pathway, large vacuoles called macropinosomes, greater than 1 μm , allow uptake of large particles [77-79]. However, the receptor-mediated endocytosis allows uptake of particles that are less than 200 nm [78-80].

Since macropinosomes are naturally leaky, the particles internalized by cells in the macropinocytosis pathway escape the lysosomal degradation [76], but in receptor-mediated endocytosis particles are trapped in the endosomes followed by enzymatic degradation in lysosomes [76]. Finally, during a macropinocytosis pathway multiple nanoparticles can be internalized by cells due to the cell surface ruffling induced upon stimulation by the growth factor [76, 79] while in receptor-mediated endocytosis cells can uptake one particle at a time [76]. We would like to perform an experiment to test the pathway of LDLR3-ELP in the presence of KGF-ELP and to confirm if it gets internalized via macropinocytosis.

5.4 Using Different Growth Factor Receptors

There are multiple routes to deliver drugs to the body. The routes of administration include: oral, parenteral, inhalation, intrathecal, rectal, and topical. Based on the type of cell that needs to be targeted, the type of administration varies. There are multiple types of growth factor receptors; epidermal growth factor receptors, fibroblast growth factor receptors, platelet-derived growth factor receptors, nerve growth factor receptors, insulin growth factor receptors, and neurotrophic growth factor receptors. A way that we can deliver the heterogeneous nanoparticles is based on the cells that have high expression of the growth factor (Figure 46). This will help to selectively target cells in the gene therapy field.

Figures are not drawn to scale

Figure 46: Schematic of selectivity of nanoparticles to high expression growth factor cells.

Our work demonstrates an approach of for selective uptake of the heterogeneous nanoparticles (NPs) with targeting cells that overexpress growth factor receptors. Specifically, the heterogeneous NPs consist of the low density lipoprotein receptor 3 (LDLR3)-ELP and the keratinocyte growth factor (KGF)-ELP. The chimeric ELP fusion protein system ensures that it is easily fused to any growth factor receptor to broaden the application for selective uptake of the heterogeneous nanoparticles (NPs) to multiple cell types, depending on growth factor expression.

This approach allows for delivery of the heterogeneous NPs in different parts of the body based on the overexpression of growth factor in the targeted cells. Growth factors such as epidermal growth factor (EGF) and nerve growth factor (NGF) can be fused to ELP (Figures 47 and 48).

Figure 47: EGF-ELP design. The top figure represents the sequence of EGF-ELP gene inserted into the expression vector. The bottom figure represents the EGF-ELP fusion protein holding the characteristics of ELPs where below transition temperature it is soluble and above transition temperature it is insoluble.

Overexpression of the EGF receptor gene leads to rapid growth of cancer cells [81]. Studies have shown potential therapies for glioblastoma, a tumor developed in the brain due to the amplification and mutation of the EGFR gene, are limited by acquired drug resistance [81]. We can use our approach of including LDLR3 and EGF-ELP in the heterogeneous NPs to deliver a gene that can deactivate the mutated EGFR gene in order to stop the cells from growing rapidly. Studies have shown that NGFR is produced abnormally in high amounts of cancer cells, leading to inactivation of the tumor suppressor, p53, causing the cancer cells to grow rapidly [82]. We can

use our approach of including LDLR3 and NGF-ELP to deliver a gene that can activate the tumor suppressor, p53, so that it can activate NGFR transcription to suppress tumor growth.

Figure 48: NGF-ELP design. The top figure represents the sequence of NGF-ELP gene inserted into the expression vector. The bottom figure represents the LDLR3-ELP fusion protein holding the characteristics of ELPs where below transition temperature it is soluble and above transition temperature it is insoluble.

Currently, scientists are using the overexpression of EGFR in glioblastoma multiforme (GBM) tumor cells to deliver the therapeutic gene (cetuximab), an anti-EGFR antibody, directly to the central nervous system using an adeno-associated virus serotype rh.10 gene transfer vector (AAVrh.10CetMab Vector) to reduce GBM tumor growth [83]. Though this study showed reduction in tumor growth, however, it did not eliminate these cells. That could lead to the possibility that the tumor cells will grow again; therefore a higher dosage maybe needed to reduce the GBM tumor growth. We could create nanoparticles consisting of EGF-ELP-LDLR3 (Figure 49).

Figure 49: EGF-ELP-LDLR3 design. The top figure represents the sequence of EGF-ELP-LDLR3 gene inserted into the expression vector. The bottom figure represents the EGF-ELP-LDLR3 fusion protein holding the characteristics of ELPs where below transition temperature it is soluble and above transition temperature it is insoluble.

To do this we will perform a recursive ligation transformation process (Figure 50). Once the peptide is transformed into the BLRD cells, we can purify it using inverse temperature cycling (ITC) and test for its purification. Also we would like to add a peptide that can kill the cancer cells. Therefore we would have heterogeneous nanoparticles consisting of EGF-ELP-LDLR3 and P-ELP (Figures 51 and 52). Then we will perform a recursive ligation transformation process of P-ELP (Figure 53) and purify it using ITC.

*Gel extraction was performed to extract peptide

Figures are not drawn to scale

Figure 50: Schematic of recursive ligation and transformation process of EGF-ELP-LDLR3.

Figure 51: P-ELP design. The top figure represents the sequence of P-ELP gene inserted into the expression vector. The bottom figure represents the P-ELP fusion protein holding the characteristics of ELPs where below transition temperature it is soluble and above transition temperature it is insoluble. P is the peptide that is toxic to cancer cells.

Figure 52: Schematic of heterogeneous nanoparticle comprising of EGF-ELP-LDLR3 and P-ELP. P is the peptide that is toxic to cancer cells.

*Gel extraction was performed to extract peptide

Figures are not drawn to scale

Figure 53: Schematic of recursive ligation and transformation process of P-ELP. P is the peptide that is toxic to cancer cells.

Due to the benefits of the chimeric ELP fusion protein system of fusing to any growth factor receptors or peptides, our approach can have the LDLR3-ELP binding to the virus which preventing the virus from infecting cells; have EGFR as a targeting protein; have a knock out gene that can prevent the glioblastoma cells from proliferating and also; have another peptide that can kill cancer cells fused with the ELP. We can try to create the peptide LDLR3-EGF-ELP-P (Figure

54) or EGF-LDLR3-ELP-P (Figure 55). We can do this by recursive ligation and transformation process (Figure 56) and purify it using ITC.

Figure 54: LDLR3-EGF-ELP-P design. The top figure represents the sequence of LDLR3-EGF-ELP-P design gene inserted into the expression vector. The bottom figure represents the LDLR3-EGF-ELP-P design fusion protein holding the characteristics of ELPs where below transition temperature it is soluble and above transition temperature it is insoluble.

Figure 55: EGF-LDLR3-ELP-P design. The top figure represents the sequence of EGF-LDLR3-ELP-P design gene inserted into the expression vector. The bottom figure represents the EGF-LDLR3-ELP-P design fusion protein holding the characteristics of ELPs where below transition temperature it is soluble and above transition temperature it is insoluble.

*Gel extraction was performed to extract peptide

Figures are not drawn to scale

Figure 56: Schematic of recursive ligation and transformation process of LDLR3-EGF-ELP-P. P represents the peptide that is toxic to cancer cells.

For selectivity, when using the EGF-ELP-LDLR3, the nanoparticle will most likely get internalized by cells with high EGFR than cells with low EGFR (Figure 57).

Figure 57: Schematic of heterogeneous nanoparticles of EGF-ELP-LDLR3 with virus that have a higher probability of being internalized by high growth factor receptor expressing cells preventing cells from proliferating.

If the nanoparticle is solely EGF-ELP-LDLR3 and P-ELP we can test the ratio of EGF-ELP-LDLR3 to P-ELP to assess the concentration of each particle and also perform a proliferation

assay and a cytotoxicity assay. We believe the addition of the P-ELP with EGF-ELP-LDLR3 means that the cancer cells will not proliferate and they will die (Figure 58).

Figure 58: Schematic of heterogeneous nanoparticles of EGF-ELP-LDR3-P with virus that have a higher probability of being internalized by high growth factor receptor expressing cells preventing cells from proliferating.

5.5 Using the Heterogeneous Nanoparticles for Lung Injuries

A lung injury can be caused by inhalation of foreign particles such as those from cigarette smoke, dysfunctional autoimmune system, and many more. If the foreign particles are not

removed by the mechanism of the mucociliary clearance, they can be engulfed by the macrophages [84]. However, viruses can surpass the lung defense and prolong in the macrophages. When foreign particulates surpass the lung defense and stay in the lung, this causes the body to send signals such as chemoattractants through the process called chemotaxis [85]. This results in an increase in pro-inflammatory cytokines, chemokines and effector cells such as macrophages and neutrophils, leading to an increase in inflammation [86, 87]. Figure 59 below summarizes one way that elastin degradation occurs from the formation of lung injury.

Figure 59: Schematic of lung injury to elastin degradation.

In lung injuries, cytokines, chemokines, and effector cells are overexpressed. Normally alveoli type two cells (AECII) differentiate to alveoli type I cells (AECI) since the AECI cannot proliferate and they die during an injury [88]. Since the rate of elastase activity is higher than the rate of AECII differentiation, the amount of AECII in the body starts decreasing resulting in elastin degradation leading to emphysema [89]. Mediators that cause elastin degradation are targeted to decrease the elastin degradation.

The imbalance of oxidant and antioxidant occurs because of high concentrations of oxidants and free radicals that are coming in the body; the oxidants that are produced and released in the body; and the inactivation of the antiproteases. This imbalance leads to oxidative stress creating an antiprotease/protease imbalance, inflammation, and cell apoptosis resulting in the inactivation of antiproteases, and degradation of alveoli elasticity by the increased amount of the elastase activity [90, 91]. While the activity of elastase increases, the amount of inflammation also

increases, and the occurrence of apoptosis also increases leading to an imbalance of protease and antiprotease.

Creating gene delivery vectors has been challenging because these vectors have to be able to surpass the body's natural immune system to reach the targeted site and also incorporate the gene that can get activated and stay stable [92]. This paper will focus on two lung injuries, emphysema and cystic fibrosis (CF). Emphysema is a type of chronic obstructive pulmonary diseases that is characterized by airflow limitation [90, 93] and the degradation in the elasticity of the alveoli and increased in collagen [94]. Emphysema can be caused by the cigarette smoke damaging alveoli walls or by naturally having alpha-1 antitrypsin (AAT) deficiency. There are 3.4 million of adults diagnosed with emphysema in the United States [95]. Currently there is no cure for emphysema.

We propose the development of recombinant elastin like peptides (ELPs) composing of LDLR3-ELP and growth factor (GF)-ELP that can be used as a delivery system to incorporate a persistent gene. Specifically in this work, we propose the development of nanoparticles that can be used as a delivery system that can incorporate the gene SERPINA-1 or pro-inflammatory inhibitor to repair the elasticity of the alveoli. We chose the SERPINA-1 gene because it encodes the normal form of AAT. Due to the imbalance between the antioxidants and oxidants that can cause emphysema from cigarette smoke, delivering a pro-inflammatory inhibitor is essential [91].

5.6 Testing for Immunogenicity

The immune system protects the body from infections and malignancies [96, 97]. When delivering viruses to the body, there is a likely chance of immunogenicity to occur. Immunogenicity is the process of inducing the immune response to foreign antigens in the body. Immunogenicity limits the use of treatment because it can lead to production of antidrug antibodies [98] and result in loss of efficacy [99]. Since lentivectors are intrinsically immunogenic [100], we would like to perform an immunogenic test. We will evaluate pro-inflammatory signaling pathways and the activation of TLR3 and TLR7 as previously described in previous studies [97].

5.7 Using LDLR3-ELP as an Antiviral Drug for Respiratory Syncytial Virus

Viruses are small infectious agents that use the cell mechanism and that can infect all life forms such as animals and plants. Viruses can infect cells in the blood [101], liver [102], and the respiratory system [103]. When viruses infect and reproduce in the host cells they can cause diseases such as influenza [104], shingles [105], herpes [106], and human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) [107]. Vaccines have been used to reduce the number of viral diseases such as chickenpox (varicella) [108], polio [109], and measles [110]. However, there are challenges that are faced to create vaccines or drugs that can prevent viral infections for diseases like HIV/AIDS and shingles while being safe and effective for the human being. Strategies to prevent and treat viral infections are challenging because viruses adapt the cell mechanism, live in the cells, and may lead to morbidity and mortality. While LDLR3-ELP can be used for targeted gene therapy with the presence of KGF-ELP, it can also be used as an antiviral drug.

One of the virus infection that is being focused is the respiratory syncytial virus (RSV) infection. RSV can enter the body through coughs, sneeze, and droplets. It causes moderate-to-severe acute and lower respiratory infection [111] in children and is estimated that 66,000-199,000 children younger than 5 years died from RSV-associated acute lower respiratory infection in 2005 [112]. This leads to a majority of children's hospitalization for bronchiolitis [113], pneumococcal pneumonia and Haemophilus influenza type b [114]. RSV not only affects children, but it also affects the elderly from age 65 and older [115] which can cause them to have pneumonia [113], exacerbation of chronic obstructive pulmonary disease (COPD) [113, 116] and acute deterioration [113]. RSV, a member of the paramyxovirus family [117], entry requires an attachment protein (HN, H, or G) that binds to a cellular receptor and a fusion protein (F) that drives membrane merger [118, 119]. Though the mechanism of RSV entry to a cell is not fully elucidated, it is thought that when RSV infects by binding the fusion (F) protein to TLR4 receptor. Due to this binding, the G protein acts as an antigen signaling immune cells [113, 120]. Therefore,

most researchers focus on inhibiting the binding of fusion F to prevent RSV infection. The scientific rationale to inhibit RSV infection is to develop drugs that can interfere with virus attachment, fusion, or intracellular replication [113]. Some of the drugs that have been used for preventing or inhibiting RSV infection are: antibodies [121]; fusion inhibitors [122]; and nucleoside analogs. Drugs such as palivizumab, a humanized monocle antibody, is used against RSV F protein by preventing the spread of the virus to the lower respiratory tract [113, 123, 124]; ribavirin, a nucleoside analog [125], is used to inhibit inosine monophosphate dehydrogenase (IMPDH) reducing formation of mRNA and viral polymerase [113, 126]; and BMS-433771, VP-14637, RFI-641, and JNJ-2408068 are small molecules that inhibits RSV fusion [113, 127, 128]. However, these drugs have efficacy and toxicity issues [117]. Recently SAH-RSVF peptides, a stabilized α -helices of RSV-F, has been developed and has shown to prevent nasopulmonary RSV infection [129]. Though this peptide is a combination of peptide stapling and nanoparticles, it still has to expand in the clinical route. Currently a nucleoside analog, ALS-008176 has shown to significantly reduce RSV viral load and it is ongoing in clinical trials [125]. With the knowledge that is obtained from current research to inhibit RSV infection, the combination biomaterials that bind with the F fusion protein and deal with cholesterol is essential.

This study will focus on using LDLR3-ELP with the heptad repeat 1 (HR1) analog (5-Helix) fused with ELP, 5-Helix-ELP; this combination inhibits respiratory syncytial virus (RSV) infectivity. 5-Helix was found to interact with the heptad repeat of the virus and this interaction inhibits RSV viral entry. Many therapeutic strategies are being developed to create antiviral drugs that can prevent and treat viral infection, especially RSV. This study will focus on the targeted and antiviral activities of LDLR3-ELP with the presence of recombinant ELPs.

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APPENDIX A: AMINO ACID SEQUENCE FOR KGF, LDLR3, 5-HELIX, AND ELP

Table A Sequence and molecular weight information of KGF, LDLR3, 5-HELIX, and ELP

Protein	Sequence	Molecular weight
ELP	VPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVP GVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGV GVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVP PGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGV VGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVP VPGVGVPGVGVPGVGVPGVGVPGVGVPGC GVPGVGVP GVGVPGVGVPGVGVPGC GVPGVGVPGVG	20500 Da
KGF	MHKWILTWILPTLLYRSCFHIICLVGTISLACNDMTPEQMAT NVNCSSPERHTRS DYMEGGDIRVRRLFCRTQWYLRIK RGKVKG TQEMKN NYNIMEIRTVAVGIVA IKGVESEFYLAM NKEGKLYAKKECNEDCNFKELILENH YNTYASAKWTHNG GEMFVALNQKGIPVRGKKTK KEQKTAHF LPMAT	22509 Da
LDRL3	PPKTCSQDEF RCHDGKCISRQFVCDSDRDCLDGSDEASC	4355 Da
5-HELIX	KVLHLEGEVN KIKSALLSTNKAVVSLSNGVSVLT SKVLHLK NYIDKQFLGSSGGDEF DASISQVNEKINQSLAFIRKSDELL HN VNAGKSTINGGSGGKVLHLEGEVN KIKSALLSTNKAVV SLSNGVSVLT SKVLHLK NYIDKQFLGSSGG SDEF DASISQ VNEKINQSLAFIRKSDELL HN VNAGKSTINGGSGGKVLHLE GEVN KIKSALLSTNKAVVSLSNGVSVLT SKVLHLK NYIDKQ FL	26162 Da