Characterization and Optimization of Nanoparticles for Polynucleotide delivery

Bhavani Saranya Conjeevaram Nagarajan
University of North Texas Health Science Center at Fort Worth, saranya_sairajan@yahoo.co.in

Follow this and additional works at: http://digitalcommons.hsc.unt.edu/theses
Part of the Medical Sciences Commons

Recommended Citation
Conjeevaram Nagarajan, B. , "Characterization and Optimization of Nanoparticles for Polynucleotide delivery" Fort Worth, Tx: University of North Texas Health Science Center; (2015).
http://digitalcommons.hsc.unt.edu/theses/883

This Thesis is brought to you for free and open access by UNTHSC Scholarly Repository. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of UNTHSC Scholarly Repository. For more information, please contact Tom.Lyons@unthsc.edu.
CHARACTERIZATION AND OPTIMIZATION OF NANOPARTICLES FOR POLYNUCLEOTIDE DELIVERY

THESIS

Presented to the Graduate Council of the Graduate School of Biomedical Sciences
University of North Texas
Health Science Center at Fort Worth
in Partial Fulfillment of the Requirements
For the Degree of
MASTER OF SCIENCE

By

Bhavani Saranya Conjeevaram Nagarajan, B.Tech., M.Tech.

Fort Worth, Texas

April 2015
ACKNOWLEDGEMENTS

First and foremost, I would like to express my utmost gratitude to my mentor and advisor Dr. Andras Lacko, for his continued guidance which helped me accomplish the goals of my research. Dr. Lacko has been a great source of inspiration, for his patience and perseverance has helped instill confidence in myself to explore new opportunities. He has always been happy and willing to help me and support me during the course of research.

I am very grateful to my committee members Dr. David P. Cistola, Dr. Raghu Krishnamoorthy and Dr. Steve Mifflin for their valuable guidance and comments that have helped me in shaping the project. I would also like to thank Dr. Cistola for willing to process our lab samples in dynamic light scatterer and Dr. Vishwanatha for permitting the usage of zeta potential analyzer.

I am highly indebted to Dr. Nirupama Sabnis, senior research scientist of our lab who has almost been like a co-mentor and taught me all lab techniques. She is very humble, understanding and has been a great source of motivation for me. I am also very thankful to all my lab mates, Rebecca Johnson and Cheryl Hinze for teaching me a lot of lab techniques, Linda Mobberry (Post Doc), Marlyn Panchoo and Akshaya Pallod for all their kindness and help.

I am very thankful to Dr. Irina Akopova, Dr. Anne Marie and Dr. Larry Oakford for their patience and efforts in helping me with the AFM and TEM analysis. I am highly pleased to express my sincere thanks to Dr. Cistola’s lab members Michelle, Sneha and Ina for always been
willing to process the lab samples in DLS instrument. My sincere thanks to Dr. Katrina Gordon at the discovery centre lab for permitting the usage of the fluorimeter. I would also like to thank Dr. Amalendu Ranjan for helping me with the zeta potential measurements.

I am indeed very thankful to my parents, grandparents and in-laws for their moral support and encouragement. I am highly indebted to my parents who have been very caring, supportive and without whom my dreams and ambitions would have never come true. My special thanks to my husband Sundar, who has always been there for me when I needed him and always cheered me up and encouraged me throughout my coursework. I would have never been much happier without my friends who have encouraged me and given me wonderful moments to cherish in life. Last but not the least I would like to thank God for his bountiful blessings that has helped me achieve my goals.
TABLE OF CONTENTS

LIST OF TABLES ...................................................................................................................................... vii
LIST OF ILLUSTRATIONS ......................................................................................................................... viii

Chapter

I. INTRODUCTION ...................................................................................................................................... 1
   Gene Therapy ........................................................................................................................................... 2
   Rationale for Nucleic Acid Therapeutics ............................................................................................... 3
   Barriers for Systemic Gene Delivery ....................................................................................................... 4
   Overcoming the Challenges Facing Systemic Gene Delivery ................................................................. 5
   Non Viral Vectors for Gene Delivery ....................................................................................................... 6
   Nanoparticles for Nucleic Acid Delivery ................................................................................................. 7

II. BACKGROUND ..................................................................................................................................... 9
   Characterization and Optimization of STAT3 siRNA rHDL Nanoparticles ........................................... 9
   Antisense Nucleic Acids for Therapeutics ............................................................................................... 9
   Drug Delivery Systems for siRNA Therapeutics .................................................................................. 13
   Reconstituted High Density Lipoproteins(rHDL) ................................................................................. 14
   Rationale for STAT3 siRNA rHDL Nanoparticles ............................................................................... 17
   Characterization and Optimization of mRNA Entrapped Peptide Nanoparticles .............................. 19
   Plasmid DNA(pDNA) ......................................................................................................................... 19
   Messenger RNA(mRNA) ...................................................................................................................... 20
   Significance ............................................................................................................................................ 21

III. MATERIALS AND METHODS .......................................................................................................... 22
   Preparation of siRNA rHDL Nanoparticles(Cholate Dialysis Method (1)) ........................................ 22
LIST OF TABLES

Table 1: Description of the two methods for the preparation of siRNA rHDL nanoparticles..... 37

Table 2: Compositional analysis of siRNA rHDL nanoparticles prepared by two different methods cholate dialysis method (1) and (2). .......................................................... 40

Table 3: Comparison of zeta potential measurement of siRNA rHDL nanoparticles prepared with two varying siRNA: poly-L-lysine ratios (1:5) and (1:10)........................................ 47

Table 4: Size analysis of the mRNA-neutralizer complexes obtained using DLS. ............... 53

Table 5: Size analysis of mRNA peptide nanoparticles obtained using DLS....................... 53
LIST OF ILLUSTRATIONS

Page

Figure 1: Contribution of gene therapy to the treatment of specific disease categories .......... 3
Figure 2: The intracellular and extracellular barriers for systemic gene delivery ............... 5
Figure 3: Schematic representation depicting the advantage of nanoparticles ..................... 6
Figure 4: Properties of nanocarriers that can influence the uptake and the efficacy of the drug molecules .......................................................................................................................... 7
Figure 5: The mechanism of RNAi interference using siRNA .............................................. 13
Figure 6a and b: Schematic representation of HDL and rHDL ........................................... 15
Figure 7: A pictorial representation depicting the uptake mechanism of the rHDL nanoparticles ............................................................................................................................................. 16
Figure 8: A representation of the STAT3 signaling pathway ................................................. 18
Figure 9a and b: The secondary structure of the Apo-A1 peptide and the 5A peptide ........... 27
Figure 10: Structure of the neutralizers used in the preparation of mRNA peptide nanoparticles 28
Figure 11: Compositional analysis of siRNA rHDL nanoparticles prepared with or without lyophilization. ................................................................................................................. 34
Figure 12: Incorporation of siRNA in siRNA rHDL nanoparticles prepared with or without lyophilization. ......................................................................................................................... 35
Figure 13a and b: Particle size distribution analysis of siRNA rHDL nanoparticles prepared with or without lyophilization ............................................................................................................. 36
Figure 14: Compositional analysis of siRNA rHDL nanoparticles prepared by two different methods cholate dialysis method (1) and (2). ................................................................................. 38
Figure 15: Incorporation of siRNA in siRNA rHDL nanoparticles prepared by two different methods cholate dialysis method (1) and (2). ................................................................. 39

Figure 16: Particle size distribution analysis of siRNA rHDL nanoparticles prepared by cholate dialysis method (1) ................................................................. 41

Figure 17: Particle size distribution analysis of siRNA rHDL nanoparticles prepared by cholate dialysis method (2) ................................................................. 42

Figure 18: Compositional analysis of siRNA rHDL nanoparticles prepared using two different cholesteryl esters (cholesteryl oleate and cholesteryl linoleate) by cholate dialysis method (1). 43

Figure 19: Incorporation of siRNA in siRNA rHDL nanoparticles prepared using two different cholesteryl esters (cholesteryl oleate and cholesteryl linoleate) by cholate dialysis method (1). 44

Figure 20: Compositional analysis of siRNA rHDL nanoparticles prepared using two different cholesteryl esters (cholesteryl oleate and cholesteryl linoleate) by cholate dialysis method (2). 45

Figure 21: Incorporation of siRNA in siRNA rHDL nanoparticles prepared using two different cholesteryl esters (cholesteryl oleate and cholesteryl linoleate) by cholate dialysis method (2). 46

Figure 22: Atomic force Microscopic analysis (2D image) of siRNA rHDL nanoparticles. ....... 48

Figure 23: Atomic force Microscopic analysis (3D image) of siRNA rHDL nanoparticles. ....... 49

Figure 24: Transmission Electron microscopic analysis of siRNA rHDL nanoparticles. ........ 50

Figure 25: Stability studies: Incorporation of siRNA in siRNA rHDL nanoparticles stored up to 4 weeks at two different temperatures 4°C and -20°C prepared by cholate dialysis method (1). ... 51

Figure 26: Stability studies: Incorporation of siRNA in siRNA rHDL nanoparticles stored up to 4 weeks at two different temperatures 4°C and -20°C prepared by cholate dialysis method (2). ... 52

Figure 27: Particle size distribution analysis of mRNA peptide nanoparticles prepared using L-Myc mRNA, HTAB neutralizer and Myr5A peptide (100µg/ml). ................................. 54

Figure 28: Particle size distribution analysis of mRNA peptide nanoparticles prepared using L-Myc mRNA, DOTAP neutralizer and 5A peptide (100µg/ml). ................................. 55
CHAPTER I

INTRODUCTION

Recent advances in the field of molecular genomics have advanced the role of nucleic acids from drug targets to therapeutic agents with the potential to treat genetic and systemic disorders. Nucleic acids, especially polynucleotides, DNA and RNA, are the key molecules that participate in the coding and transmission of genetic information in living organisms. Accordingly, DNA molecules are the key components of genes (hereditary units) that contain the information for determining the amino acid sequence and the functional polypeptides and proteins. Genetic alterations, including mutations, in the structure of polynucleotides often lead to unregulated gene expression and defective protein production, the major cause of most genetic disorders.

Genetic diseases may be classified under three major categories [46]:

1. *Monogenetic disorders*: Disorders characterized by the mutation of a single gene, which include Cystic Fibrosis, Sickle Cell Anemia, Tay Sach’s syndrome, polycystic kidney disease.

2. *Chromosomal disorders*: Diseases caused by structural changes in the chromosome, or by an excess or deficiency of the genes located on chromosomes. Some of the chromosomal abnormalities include Down syndrome, Turner syndrome, and Klinefelter syndrome.
3. **Multifactorial inheritance disorder**: Disorders characterized by a combination of certain inherited variations in the gene and other environmental factors. These include cardiovascular diseases, diabetes and most types of cancers.

The established treatment for genetic disorders has traditionally been conventional drug therapy that can transiently reduce the accumulation of abnormal metabolites without necessarily addressing the underlying cause of the disease. Hence, there is an urgent need to develop new treatment strategies, including, gene therapy to efficiently address the root cause of genetic disorders.

**Gene Therapy**

Gene therapy can be broadly described as a process intended to treat or alleviate disease by modifying components of the “genetic machinery” (DNA synthesis, transcription or translation) of a patient [38]. This approach involves the use of genetic material (polynucleotides) intended to replace defective genes or block the expression of malfunctioning genes. With the technological advancement in genome mapping and the completion of the Human Genome Project, it has now become possible to identify the target sequences for designing therapeutic nucleic acid molecules for the treatment of several diseases. The theory that exogenous nucleic acids can be used to modulate gene expression was based upon the experiments conducted by Patterson et al., (1977) who used single stranded DNA to inhibit the translation of a complementary RNA in a cell free system [12]. Since then, a whole new field of biomedical investigations has developed adapting this strategy to produce synthetic polynucleotides, targeted to treat specific genetic disorders.
Figure 1: Contribution of gene therapy to the treatment of specific disease categories [43]

**Rationale for Nucleic Acid Therapeutics**

Ideally, one of the major goals of biomedical research is to discover and develop highly efficient therapeutic strategies that target specific diseases, including those due to a genetic abnormality. However, most of the currently approved therapeutic agents (especially in cancer therapeutics) have significant off target effects, resulting even prolonged post treatment toxicity. Safer outcomes will likely result from the therapeutic application of specifically targeted nucleic acids. Several nucleic acid based drug formulations have been evaluated as next-generation therapeutics, including aptamers, ribozymes, siRNA, miRNA, mRNA, cDNA etc. While these molecules share some physiochemical properties, including their highly negative charges, the mechanisms whereby they achieve their respective therapeutic impact varies widely. Therefore, based upon their functional classification, synthetic nucleotides may induce protein expression (cDNA, mRNA) or inhibit protein expression (siRNA, aptamers, and ribozymes).
Barriers for Systemic Gene Delivery

Although therapeutic nucleic acids have the potential for correcting genetic disorders, their instability in a biological environment limits their clinical applications for treating systemic diseases. The instability of therapeutic nucleic acids may be attributed to one or more of the following factors.

Nucleases: Naked nucleic acid (DNA/RNA) is extremely vulnerable to degradation by exo- and endo- nucleases and has a short half life upon injection into the blood, leading to rapid clearance. Accordingly, naked DNA has been shown to have a very limited half life of 1.2-21 minutes. Similarly the half life of naked siRNA is very short, less than five minutes, likely due to the presence of both exo and endo nucleases in the serum [14].

Plasma Membrane: The plasma membrane of living cells is another defensive barrier that prevents the entry of the naked nucleic acids. Due to their high anionic charge, oligo- and polynucleotides are electro statically repelled by the negative charges contributed by the head groups of phosphatidyl choline and other phospholipid constituents of the plasma membrane. In addition the large size of polynucleotides also hinders their entry to the target cells and tissues.

Immune System: Naked nucleic acids are significantly immunostimulatory and trigger an immune response, inducing the reticulo-endothelial system to remove them from the circulation, likely before they can exert their intended therapeutic effect. Consequently, there is a need to protect the nucleic acids from degradation and thus early removal from the circulation. Encapsulation into carriers, including into nanoparticles, has been extensively investigated as a protective mechanism for therapeutic nucleic acid formulations. [18].
Overcoming the Challenges Facing the Systemic Gene Delivery

Research on developing and evaluating \textit{in-vivo} delivery of nucleic acids has resulted in three different delivery strategies to enhance gene expression. The first strategy involved the use of viral vectors to deliver nucleic acids efficiently to the target cells [24]. Nevertheless, viral vectors, derived from the wild type pathogenic form may revert to their virulent state, thus leading to viral infections or insertional mutagenesis. Moreover viral vectors can only carry a limited amount of genetic material. The second strategy involves the synthesis of chemically modified naked siRNA which are more resistant towards nucleases, pose lesser immunological
problems and provide moderate transfection efficiency compared with the naked siRNA. However, the lack of target specificity of these siRNA derivatives could lead to undesirable side effects and early removal by the kidneys subsequent to systemic administration. The third and most frequently adopted gene delivery strategy involves the use of non-viral vectors that have been developed to efficiently entrap nucleic acids while ensuring safe and targeted delivery with the possibility of avoiding undesirable side effects [16].

**Non Viral Vectors for Gene Delivery**

Non-viral vectors, while preferred for being clinically safe provide moderate gene transfection efficiency compared to viral vectors. Nevertheless, non viral vectors provide an opportunity for the design of multivalent carriers which can incorporate several functional components that perform separate tasks. Non-viral vectors feature several additional favorable properties, including non infectivity, non immunogenicity, non oncogenicity, targeted delivery and lower cost/ease of production [1]. Among non-viral vectors, nanoparticles have been found to be particularly effective as delivery agents, owing to their small size and their ability to provide targeted delivery.

![Figure 3: Schematic representation depicting the advantage of nanoparticles [44]](image_url)
Nanoparticles for Nucleic Acid Delivery

The growth and the evolution of nanotechnology have contributed substantially to the development of nucleic acid delivery vehicles. Nanoparticles have been found to be particularly effective as systemic drug delivery agents because of their capacity to carry high drug payload, ability to accommodate a broad range of drug molecules, facilitate organ or tissue targeting [30], prevent adverse interactions with the biological surroundings [30], protection of drugs and thus providing increased stability and lower toxicity [26][8]. Moreover nanoparticles have a higher surface to volume ratio that improves their diffusion potential in biological fluids. In addition, the potential for functionalization of nanoparticles allows for addressing multiple ligands especially the surface receptors on target cells and tissues.

Figure 4: Properties of nanocarriers that can influence the uptake and the efficacy of the drug molecules [23]
Nanoparticles serve as ideal vectors for the transport and delivery of nucleic acids as they compress and entrap the nucleic acids consequently reducing their size, masking their negative charge and preventing them from enzymatic degradation. Additionally, the exceptionally small size of the nanoparticles is a major advantage that can be exploited for the delivery of nucleic acids to the leaky environment of malignant tissues (Enhanced permeation and Retention effect) and across the blood brain barrier.
CHAPTER II

BACKGROUND

Specific Aim 1: Characterization and Optimization of STAT3 siRNA rHDL Nanoparticles

This project is focused on developing a stable and homogenous nanoparticle delivery system that could efficiently entrap siRNA (or antisense polynucleotide). This was based on the hypothesis that rHDL nanoparticles that are efficient carriers of hydrophobic drugs can also entrap (neutralized) siRNA and generate stable and homogenous nanoparticles, ultimately for clinical applications.

Antisense Nucleic Acids for Therapeutics

Antisense nucleotides are designed to selectively inhibit or block the expression of proteins which are implicated in the pathogenesis of genetic diseases. There are four major classes of oligonucleotides that are used in antisense therapeutics to inhibit gene expression in cells. These include antisense oligodeoxynucleotides, aptamers, ribozymes and siRNA.

Antisense Oligodeoxynucleotides

These are short synthetic strands of DNA or analogs (typically 12-25 bps) that inhibit the translation of a specific gene by hybridizing to the complementary mRNA through Watson Crick base pairing. Though their mechanism of action is not well understood, it is hypothesized that
they sterically interfere with the translation process or promote the mRNA transcript degradation by RNAses H activation [12]. In spite of their therapeutic potential in treating diseases, they are subjected to several disadvantages that inhibit them from being adopted widely as therapeutics. The major drawback being the intricate complexities involved in the design as it includes the consideration of factors affecting its activity and stability [39].

**Aptamers**

Aptamers are single stranded oligonucleotides (DNA or RNA) that can bind to proteins and peptides with high affinity and specificity. In contrast to the other oligonucleotide therapeutics, the aptamers are unique as they disrupt the expression of the target molecule at the protein level rather than at the mRNA level. Additionally, aptamers can assume a variety of shape owing to the formation of helices and loops. Due to this inherent property, its specificity and characteristics are attributed to its tertiary structure rather than their primary sequence which subsequently increases their targeting specificity. These aptamers have been found to be very good alternatives for small molecules and antibodies owing to their small size, stability, low toxicity and low immunogenicity. They are currently emerging in the field of therapeutics, drug development and target validation. In spite of these advantages the aptamers are not cost effective as the technology involved in the large scale production of aptamers is still under development and the chemical modifications that are required to protect them from nuclease degradation involve additional costs.

**Ribozymes**

Ribozymes are nucleic acid enzymes with catalytic motif that can hybridize and cleave the mRNA in a sequence specific manner. Upon causing the cleavage of mRNA strand and
inhibiting the translation, they are instantaneously prepared for knocking another complementary mRNA molecule, leading to highly specific knockdown of the targeted protein [35]. Although ribozymes are found to be effective *in vitro* and *in vivo* suppression of the gene expression, their effect is short lived due to their high susceptibility to endonucleases. Moreover, as the natural repertoire of ribozyme molecules is quite limited, it necessitates the design of newly modified ribozymes that could provide catalytic activity against larger number of RNA substrates [10].

**Small interfering RNA (siRNA)**

Post-transcriptional gene silencing via siRNA is a powerful approach achieved through a mechanism called RNA interference (RNAi). The RNAi is a natural cellular process in eukaryotic cells essential for gene regulation, although it is primarily used by the innate immune system as a defense mechanism against invading viruses. RNAi takes advantage of the cell’s natural machinery, to route the exogenously applied siRNA to the complementary mRNA thereby inhibiting the translation and suppressing the target gene expression. siRNAs are synthetic double stranded oligonucleotides (19-23bp), consisting of a sense strand (passenger strand) and an antisense strand (guide strand) that can function as critical regulators of gene expression. Transfection of siRNAs across the cell membrane primes the loading of siRNA onto the RNA induced silencing complex (RISC). RISC is a complex of proteins amongst which the Argonaute protein , AGO2 is the major player that facilitates the loading of the siRNA duplex, followed by unwinding which causes the attachment of the guide strand and the removal of the passenger strand from the AGO2. The activation of the RISC complex causes the guide strand to direct the AGO2 protein to specifically bind to the complementary mRNA and degrade the mRNA thereby suppressing the expression of the target gene.
RNAi based therapeutics have produced promising data from ongoing clinical trials for the treatment of several systemic diseases including ocular and retinal disorders, dominantly inherited brain and skin disease, viral infections, cancer and several metabolic disorders [3]. There are two major advantages of using siRNA over other antisense therapeutics. The major benefit is the rapidity in designing siRNA with high specific inhibition for the target, thereby increasing the efficiency of the therapy. Additionally the synthesis of siRNA unlike other conventional drug molecules is relatively simple and does not require a cellular expression system, complex protein purification, or refolding schemes and is relatively less expensive. The major drawback with the naked siRNA molecules is their unsuitability for systemic delivery because of their immense negative charge, large molecular weight (~13kDa.) and lack of serum stability. These limits can be overcome by using a vector (nanoparticle) that can mask the negative charge, augment their serum half life and help them cross the cell membrane thereby enabling it to be suitable for clinical therapy. Additionally, the impregnation of nanoparticles with high affinity ligands specific for the targeted cell surface receptor will enable the nucleic acid march successfully towards efficient and targeted gene delivery.
Drug Delivery Systems for siRNA Therapeutics

Despite the extensive research conducted in designing nano-delivery systems for siRNA therapeutics, currently, the most widely used and clinically approved nanodelivery systems for siRNA therapeutics are the lipid and polymer based nanoparticles [17]. It has been observed that lipid mediated siRNA transfection is thousand times more efficient than that of naked siRNA entity [46]. Liposomes, one of the most commonly used and extensively studied lipid based drug delivery vehicles, have provided an effective delivery system for several hydrophobic, hydrophilic and nucleic acid based drug molecules. They have also been found to be safe, nontoxic, and non-immunogenic. Although the chemical composition of the liposomes may be altered using cationic and neutral lipids to increase the entrapment efficiency, they suffer from a lack of stability which results in the leakage of entrapped drug molecules [15].
Most currently investigated siRNA-therapeutics use chitosan and PLGA polymers as drug carriers. Although chitosan has been studied as a siRNA delivery vehicle both for \textit{in vivo} and \textit{in vitro} purposes, its use is being limited due to cytotoxicity issues [2]. PLGA siRNA conjugates although stable in nature, are facing significant challenges with regard to the low electrostatic interactions between PLGA and siRNA, inefficient endosomal escape of siRNA and non-specific cell delivery [36]. Consequently, there continues to be an acute need to develop a stable, non-toxic, biodegradable drug carrier for the delivery of siRNA for therapeutic applications.

**Reconstituted High Density Lipoprotein (rHDL)**

Researchers at UNTHSC and MD Anderson Cancer Center developed a novel drug delivery system using rHDL as a carrier primarily for hydrophobic drugs that specifically targets cancer cells and tumors via the Scavenger receptor type B 1(SR-B1) [25][33]. Reconstituted HDLs (rHDL) are synthetically formulated nanoparticles, compositionally mimicking High Density Lipoproteins (HDL) a natural component of human blood plasma. rHDL nanoparticles are composed of an outer layer of phospholipids, apolipoprotein A-1(a apo A-1), and unesterified cholesterol protecting an inner core region, composed of cholesteryl ester and the drug payload. HDL, an important component of cholesterol transport and homeostasis, is a major rate limiting component of the reverse cholesterol transport pathway, facilitating the delivery of the excess cholesterol from the peripheral tissues to the liver. Previous studies have indicated the high expression of SR-B1 receptors in cancer cells, including ovarian, breast and prostate [20]. Proof-of-concept studies have shown that the over expression of the SR-B1 receptor is likely to be required by the cancerous tissues to meet their excessive demands of cholesterol to maintain their
high proliferation rate [5][20]. The Apo A-1 protein acts as a high affinity ligand for the SR-B1 receptor facilitating the targeted delivery of the core (drug) component of the rHDL to the tissues. The over expression of the SR-B1 receptor is exploited for a Trojan horse delivery strategy that enables rHDL nanoparticles to deliver anti-cancer agents selectively to the cancer cells and tumors [21].

![Diagram of HDL and rHDL](image)

**Figure 6a and b: Schematic representation of HDL [42] and rHDL [33]**

**Advantages of rHDL as drug delivery system**

The size of the rHDL nanoparticles (12-18nm) offers a major advantage, as it facilitates their extravasation to the target tissues and their penetration through interfibrillar openings in the tumor environment [7]. They are also biodegradable as they resemble the human HDL. They are non-immunogenic and circumvent the detection by the mono nuclear phagocytic cells. rHDL nanoparticles have been observed to have prolonged circulation in the blood which eliminates the need for PEGylation. The basic structure of lipoproteins (hydrophilic surface and inner hydrophobic compartment) that facilitates the delivery of hydrophobic cholesteryl esters suggest that they can also deliver hydrophobic bioactive compounds [11][31]. Additionally, the Apo-A1
protein on the surface of the rHDL nanoparticles serves as a targeting moiety for the SR-B1 receptor that is highly over expressed in cancer cells and tumors, thereby making rHDL nanoparticles an ideal delivery vehicle for cancer therapy.

In contrast to the native HDL, reconstituted HDL nanoparticles offer several advantages as a drug delivery vehicle. Having a comprehensive knowledge of the components (lipid / protein / cholesterol) of the human HDL, aids in manipulating factors such as the lipid/protein stoichiometry and the choice of lipid, to control the physiochemical properties of the nanoparticles, which includes the size, charge, zeta potential. Hence this project is focused on leveraging the characteristics of rHDL to improve the size, stability, homogeneity and the entrapment efficiency of siRNA-rHDL formulations for large scale production and clinical studies.

Figure 7: A pictorial representation depicting the uptake mechanism of the rHDL nanoparticles [37]
Rationale for STAT3 siRNA rHDL Nanoparticles

This project is aimed at entrapping Signal Transducer and Activator of Transcription 3 (STAT3) siRNA in the rHDL drug delivery carrier. STAT proteins are cell signaling molecules (transcription factors) that are crucial for maintaining several important cellular processes, including cell proliferation, survival and differentiation [33]. Among the STAT proteins, constitutive activation of STAT3 protein is being widely reported to play a significant role in carcinogenesis, including for ovarian tumors [19]. The STAT3 cell signaling molecules are activated by the interaction of cytokines and growth factors (IL-6, EGF), with the kinase linked receptors. These activated STAT3 molecules undergo phosphorylation and dimerization before translocating to the nucleus where they act as transcription factors. Consequently, they activate the transcription of specific genes that play a crucial role in regulating cell growth (Myc), apoptosis (survivin, Bcl-xl) and cell survival and carcinogenesis. Aberrant expression of STAT3 molecules in cancer cells has been found to elevate the expression of anti-apoptotic and cell cycle regulating proteins. Thus cancer cells expressing constitutively activated STAT3 molecules are highly resistant to apoptosis, promoting their survival even during therapy.

Failure to knock down the STAT3 protein by naked siRNA triggered the need to develop alternative strategies. Although STAT3 is activated in solid tumors, it is also responsible for important cellular functions in normal tissues. Hence knocking down STAT3 may elicit unwanted side effects, thereby necessitating the need for targeted delivery.

Although there are extensive studies being conducted with STAT3 siRNA loaded - PLGA nanoparticles, their clinical applications are limited due to inefficient and non-specific delivery issues. Moreover, in therapeutic proof-of-concept studies, rHDL nanoparticles were found to be effective in silencing the expression of two proteins that are vital for cancer growth
and metastasis, namely STAT3 and focal adhesion kinase (FAK) in orthotopic mouse models of ovarian and colorectal cancer [33]. This indicates that rHDL nanoparticles may be an appropriate candidate for the delivery of siRNA. However, large scale manufacturing of nanoparticles for therapeutic and clinical application necessitates the need for developing homogenous and significantly efficient nanocarriers. Hence this project is aimed at developing highly homogenous and stable rHDL nanoparticles that can facilitate the delivery of STAT3 siRNA eventually for therapeutic applications.

Figure 8: A representation of the STAT3 signaling pathway (Courtesy: Journal of Clinical Investigation)
Specific Aim 2: Characterization and Optimization of mRNA peptide nanoparticles

This project is aimed at developing novel homogenous peptide nanoparticles which could efficiently entrap mRNA. This was based on the hypothesis that mRNA besides being large in size and negatively charged, can be compressed using neutralizer and entrapped in a peptide nanocarrier for efficient in vivo delivery and subsequent protein expression.

Nucleic acid therapeutics besides being used to inhibit gene expression are also extensively used in gene replacement therapy to replenish somatic cells with healthy genes. Currently there are several ongoing clinical trials that are experimenting gene replacement therapy as a sole intervention or in combination with other treatments for several diseases including cardiovascular diseases, cancer, viral diseases and other genetic diseases [44]. The most commonly used nucleic acids for gene therapy include the mRNA and the plasmid DNA.

Plasmid DNA (pDNA)

Cells encoding mutant or aberrant protein can be replaced by plasmid DNA (pDNA) constructs containing the transgenes encoding for specific protein (up regulation or gain of function). The mechanism of action of the pDNA molecules demands the delivery of the plasmid across the nuclear membrane and its integration with the host genome. But the major drawback is the efficiency of the gene expression being challenged by the ability of the plasmid to traverse the nuclear membrane that acts as a physical barrier. Another major risk is the probability of insertional mutagenesis which might occur due to incorrect integration of the plasmid with the host genome. Besides requiring access to the nuclear membrane pDNA also
requires promoter and terminator sequences to functionally activate the target gene expression. These disadvantages make plasmidDNA relatively less preferred for gene therapy.

**Messenger RNA (mRNA)**

Gene replacement therapy using mRNA is currently evolving and rapidly progressing as it has more advantages than pDNA based gene expression. mRNA lacks the need for nuclear transport and is devoid of the risks caused by insertional mutagenesis as seen with the pDNA. Moreover, employment of mRNA over pDNA is a more effective gene delivery strategy when we consider the real state of the majority of cells *in vivo* which are mostly non-dividing or slowly dividing [9]. Hence, the success of mRNA transfection stems from its superior cytoplasmic transfection efficiency, simplicity over viral transduction protocols and its clinical safety profile (strictly transient gene expression and absence of insertional mutagenesis) [40].

Following the evolution of non-viral gene therapy, mRNA based gene transfer technology has gained more attention since the last decade. MessengerRNA based therapeutics are emerging as effective strategies in the field of cancer therapy, immunotherapy (for the production of prophylactic vaccines for infectious diseases), and protein replacement therapies. Although these novel therapeutics have a great potential in gene therapy, their success in clinical trials is based, to a great extent, on the development of novel delivery vectors. Due to their excessive negative charges, large size and instability (due to nuclease sensitivity) the delivery of mRNA to target cells and tissues is a daunting task. Consequently, entrapping mRNA into a nanoparticle should facilitate further functionalization via linking of a targeting ligand, thus further enabling the targeted delivery of the mRNA. Studies conducted to analyze the *in vitro* transfection efficiency of GFP mRNA in naked and encapsulated form (nanoparticles) in primary human and mouse
dendritic cells have shown that only the encapsulated mRNA species was successful in expressing the transgene[27]. Recent observations indicated that the mRNA coding for thymidine kinase gene, entrapped in liposomal formulation was successful in suppressing tumor growth in lung Cancer mice model [47]. Nevertheless issues including the toxicity of the polymer and off-target effects on healthy cells remain unresolved. Consequently, this study is focused on developing and optimizing a novel drug carrier that is stable, biodegradable and able to facilitate the targeted delivery of the L-Myc mRNA . Based on the size and the commercial availability, this mRNA was chosen for our preliminary studies to investigate the possibility of entrapping mRNA in peptide nanoparticles.

**Significance**

Nucleic acid delivery has the potential to revolutionize medicine by providing an opportunity to develop pre-clinical trials targeting previously untreatable diseases. They have immense potential to treat some of the most chronic diseases like cancer, diabetes, and other genetic disorders that affect the quality of lives of billions of people. Though siRNA and mRNA molecules have found to be successful in treating genetic diseases, the ultimate success of these molecules depends upon the non-viral vectors that would facilitate the expression of the specific gene at therapeutic levels in the desirable cell populations. Our study aims to develop and improvise a drug delivery system that utilizes reconstituted high density lipoproteins (rHDL) and peptide nanoparticles to facilitate the entrapment of nucleic acid and enable the targeted delivery of the nanoparticles to the desirable cell population. Clinical applications of polynucleotide based nanoparticles would improve the efficiency of the treatment and eliminate the undesirable side effects, thereby enhancing patient compliance.
CHAPTER III

MATERIALS AND METHODS

Preparation of siRNA rHDL Nanoparticles (Cholate Dialysis Method (1))

Briefly, a mixture of lipids including Free cholesterol [FC]/cholesteryl oleate [CE]/egg yolk phosphatidylcholine [EYPC] in chloroform, was prepared with Apo-A1 (MC Labs, California) at a molar ratio of Apo-A1:FC:CE:EYPC- 1:5:1.3:1.15M. The lipid mixture (FC,CE,EYPC) was dried under Nitrogen to form a thin film. siRNA (Sigma-Aldrich, U.S.A) was boiled at 60°C for one minute and then incubated at 37°C for one hour. After incubation, the siRNA was combined with poly-L-lysine (mean M.wt 500-2000Da.Sigma-Aldrich, U.S.A) at a 1:5 ratio incubated at 37°C for 30 minutes. The poly-L-lysine /siRNA mixture was then combined with lipids film and Apo-A1 (2.5 mg/mL) solution. This was then treated with sodium cholate, 70 µl (100 mg/ml stock in 0.15 M NaCl, 0.003 M KCl, 0.15 M KH2PO4, pH 7.4 [designated as PBS]) was added to produce a final PC-to-cholate molar ratio of ~1:1.6. The final volume of the sample was adjusted to 1 ml with the Tris-EDTA buffer (10 mMTris, 0.1 M KCl, 1 mM EDTA pH 8.0). The lipid-protein-cholate mixture was then lyophilized (Labconco freeze
drier, Labconco Corp, Kansas, U.S.A), followed by dialysis against PBS, for 2 days, with three buffer changes. The dialysis procedure (sodium cholate dialysis) was adapted from the method described for the synthesis of rHDL particles [20].

**Preparation of siRNA rHDL Nanoparticles (Cholate Dialysis Method (2))**

The procedure for the preparation of siRNA rHDL nanoparticles by cholate dialysis method 2 was adapted from Dr.Shin JY’s protocol [34]. This involved the preparation of the lipid film containing EYPC, free cholesterol and cholesteryl ester as mentioned in the above method. (The siRNA rHDL nanoparticles were also prepared with the addition of EYPC alone in the absence of cholesterol components). The lipid film was combined with sodium cholate 70 µl (100 mg/ml stock in 0.15 M NaCl, 0.003 M KCl, 0.15 M KH2PO4, pH 7.4 [designated as PBS]) and the pre-calculated amount of 1x Tris EDTA buffer (10 mMTris, 0.1 M KCl, 1 mM EDTA pH 8.0) required for the preparation of 1ml sample and incubated at 37º- 44ºC (depending on the transition temperature of the cholesteryl ester) for 2 hrs. Simultaneously, the siRNA poly-L-lysine mixture was prepared according to the method mentioned in the original procedure (cholate dialysis method 1). Finally, the siRNA- poly-L-lysine mixture and Apo-A1 protein (2.5mg/ml) was added to the incubated lipid mixture and the sample was lyophilized, reconstituted in diethyl pyrocarbonate (DEPC) treated water and dialyzed against 1xPBS.(DEPC treatment is followed to make the water RNAse free)

**Characterization of siRNA rHDL Nanoparticles**

Protein content of the rHDL nanoparticle was determined using BCA Reagent Kit (Thermo Scientific). Phospholipid and cholesterol contents were determined using Phospholipid C and Cholesterol E kits respectively (Wako chemicals, U.S.A). The Spectrophotometric
analysis for protein, phospholipid and, cholesterol assays were carried out using Biotek Microplate Reader. The incorporation of the siRNA in the rHDL particles was determined using RiboGreen assay (Quant-iT Ribogreen Kit, Invitrogen, CA). The fluorimetric analysis for estimating siRNA incorporation was performed using Gemini XS Fluorescence plate reader. The entrapment efficiency of siRNA was calculated using the formula mentioned below.

\[
\text{Entrapment efficiency} = \frac{\text{Final siRNA content}}{\text{Initial siRNA content}} \times 100 \%
\]

**Size and Zeta Potential Analysis**

The Dynamic Light Scattering (DLS) technology is used to determine the size of the particles by measuring the intensity of the light scattered by the molecules present in the sample as a function of time. The size analysis for the siRNA-rHDL was carried out using Dynamic Light scatterer (Wyatt 122 MOBIUS). The nanoparticles were diluted at a ratio of 1:20 and analyzed in the DLS at the detector angle of 171°C. The measurements were repeated thrice with 1024 scans.

The zeta potential measurements of the nanoparticles indicate the magnitude of the electrostatic repulsion/attraction between the particles. These measurements were made using Zeta potential analyzer (Malvern Zetasizer). The sample was diluted at 1:20 in 1xPBS buffer and analyzed as per the manufacturer’s instructions.

**Atomic Force Microscopic (AFM) Analysis**
The sample for AFM analysis was dialyzed against sterilized deionized water at 4°C for 12-18 hrs to remove the excess salt from the sample. The sample was further diluted at a ratio of 1:5 with sterile distilled water and 10µl of the sample was spin coated on a polylsine coverslip in order to enhance the attachment of the nanoparticles. The sample was air dried and processed with scanning atomic microscopy on the NTEGRA Prima scanning probe microscope manufactured by NT-MDT (Moscow, Russia). Closed-loop feedback semi-contact mode has been used at rate 0.5 Hz. Scanning was controlled and images were analyzed with NOVA software by NT-MDT.

**Transmission Electron Microscopic (TEM) Analysis**

The rHDL samples were dialyzed against a buffer containing 0.125 M ammonium acetate, 2.6 mM ammonium carbonate and 0.26 mM EDTA (pH 7.4). The isolated rHDL samples were then negatively stained with 2% Uranyl formate and 5 µl of the sample (1:1000) was placed on the carbon-coated 300-mesh nickel grid support films (Electron microscope sciences, USA). The particles were visualized (under a magnification of 50,000 and accelerating voltage of 100kv) using a Zeiss 910 (Carl Zeiss SMT Inc., Peabody, Massachusetts, USA) transmission electron microscope. Images were recorded as 16bit TIFF files using an advanced microscopy technique XR401 CMOS digital camera.

**Stability Studies**

The stability study for siRNA rHDL nanoparticles was conducted to analyze the leakage of free siRNA at the time of storage. This was performed by storing 1 mL aliquots of the sample at 4°C and -20°C (in order to determine the appropriate temperature for the nanoparticles) for two
weeks and four weeks. Nanoparticles in -20°C were lyophilized before storage (in order to avoid any structural damages during freezing) and re-suspended in 1 mL of DEPC (RNAsent inhibitor) treated water during the respective time points. Samples were finally redialyzed against 1xPBS before analyzing the chemical composition of the nanoparticles in order to remove any unincorporated siRNA.

**Preparation of mRNA-peptide Nanoparticles**

2.5 µg of L-Myc mRNA (Stemgent Inc, U.S.A) was boiled at 65°C for 10 minutes and then cooled immediately for 15 minutes. This was then incubated with 10µg of poly-L-lysine (cationic neutralizer) and 100µl of 1x Tris EDTA buffer (pH 7.0) at room temperature for 15 minutes. The mRNA neutralizer complex was then combined with the 20µg of the peptide Apo–A1 (MC labs California) and 70µl of sodium cholate (100 mg/ml stock in 0.15 M NaCl, 0.003 M KCl, 0.15 M KH2PO4, pH 7.4 [designated as PBS]). The final volume of the sample was adjusted to 1 ml with the Tris-EDTA buffer (10 mMTris, 0.1 M KCl, 1 mM EDTA pH 8.0). The sample was then lyophilized, followed by dialysis against PBS, for 2 days, with three buffer changes. Experiments were also conducted with the following neutralizers including Tetrabutyl Ammonium Hydroxide (TBAH), Hexadecyltrimethyl ammonium bromide (HTAB), 1,2-dioleoyl-3-trimethylammonium-propane ((DOTAP) Avanti Polar Lipids) and Protamine sulphate (Sigma-Aldrich). Peptides including Myristoylated 5A peptide (Myr-5A) and 5A peptide were also used in the synthesis of peptide nanoparticles and they were obtained from Dr. Alan Ramaley, NIH, Bethesda.
Determination of the Entrapment Efficiency of mRNA

The following procedure was used to estimate the entrapment efficiency of the mRNA in the peptide nanoparticles. Initially, the unentrapped mRNA from the sample was removed by the addition of OligodT beads (Invitrogen) that can bind to the polyA tail of the mRNA. The supernatant was subsequently treated with Trizol reagent to lyse the mRNA entrapped nanoparticles for the release of entrapped mRNA. Quantification of mRNA was performed using Ribogreen assay and the fluorescence was measured at excitation 485nm and emission 525nm using Gemini XS Fluorescence plate reader. The entrapment efficiency was calculated using the formula mentioned below.

\[
\text{Entrapment efficiency} = \frac{\text{Final mRNA content}}{\text{Initial mRNA content}} \times 100 \%
\]
Size Analysis

The size analysis for the mRNA-rHDL was carried out using Dynamic Light scatterer (Wyatt 122 MOBIUS). This was carried out using the same procedure described earlier.

Figure 10: Structure of the neutralizers used in the preparation of mRNA peptide nanoparticles
CHAPTER IV

RESULTS

Characterization of siRNA rHDL Nanoparticles

Heterogeneity in therapeutic nanoparticle formulations is a huge barrier which is preventing their entry in the clinical trials. Heterogeneity of therapeutic nanoparticles is postulated to negatively interfere with the efficiency of the treatment. Hence, the aim of the study was projected to increase the homogeneity and the encapsulation efficiency of the siRNA rHDL nanoparticles.

As a first step, the effect of lyophilization (freeze drying) on siRNA rHDL nanoparticles was analyzed to observe the improvement in the nanoparticle homogeneity. Prior studies (unpublished data) conducted in our lab have established that the introduction of lyophilization process to the preparation of valrubicin rHDL nanoparticles increased the homogeneity and the encapsulation efficiency of the drug. Hence this lyophilization procedure (0.3Mbar, -55°C) was applied to the siRNA rHDL nanoparticles frozen at -80°C (overnight), in order to determine its effect on encapsulation efficiency and homogeneity of the nanoparticles. Chemical composition analysis revealed a minor decrease in the protein content and the encapsulation efficiency of siRNA of the lyophilized nanoparticles in comparison to the non lyophilized nanoparticles (Fig. 11 and 12). Results pertaining to the size analysis conducted by DLS showed that there was a
very marginal increase in the homogeneity of the nanoparticles as they shifted from 90\% to 94\% of 15nm particles upon lyophilization (Fig.13).

Continued efforts to enhance the homogeneity and the incorporation efficiency of the nanoparticles lead us to a second set of experiments which involved a change in the procedure for preparing siRNA rHDL nanoparticles. The modified procedure (cholate dialysis method (2)) and the contrasting difference with the original procedure (cholate dialysis method (1)) are outlined in table 1. Comparison of the chemical compositional analysis of the two samples revealed a relative decrease in the incorporation of protein and phospholipid components in the sample prepared by cholate dialysis method (2) (Fig.14). Nevertheless, based on the overall composition analysis, the incorporation of siRNA in siRNA rHDL nanoparticle was observed to increase from 1.2 \% (method 1) to 2 \% (method 2) (factor of 1.6) as shown in table 2. Comparison of the size analysis between the two samples did not reveal a significant change in homogeneity (Fig.16 and 17).

Experiments were conducted to determine the role of the cholesteryl ester, the core component of the rHDL. Hence, studies were undertaken to compare the siRNA rHDL nanoparticles prepared by different procedures cholate dialysis methods (1) and (2) using two different cholesteryl esters (cholesteryl oleate and cholesteryl linoleate). There were no significant changes observed in the compositional analysis and the incorporation efficiency of siRNA (Fig.18-21). There were no significant changes in the size analysis of the nanoparticles.

Our preliminary studies regarding the zeta potential measurements indicated an average overall surface charge of -12mV for the siRNA rHDL nanoparticles (Table 3). This finding suggested the possibility of negatively charged particles getting repelled by the negative surface
charge on the cell membrane. Hence, the preparation of siRNA rHDL nanoparticles was modified by increasing the ratio of the cationic neutralizer, poly lysine to the siRNA in order to decrease the negative charge. As a result the siRNA: polylysine ratio was changed from 1:5 to 1:10. The zeta potential of the siRNA: poly lysine 1:10 nanoparticles were found to have null charge (0mv).

The 2D AFM image (Fig.22) obtained using the NOVA imaging software(NT MDT) revealed the presence of spherical particles in the range of 8-20nm in diameter, with the mean diameter being 16nm. The 3D image (Fig.23) generated (using the NOVA imaging software) to reveal the topological features confirmed the spherical morphology of the nanoparticles. The TEM image (Fig.24) also revealed the spherical nature of siRNA rHDL nanoparticles ranging from 5nm - 20nm particles, with majority of the particles ranging from 10-16nm.

The stability of nanoparticles is a major issue to be considered during large scale preparation of the drug formulations. Hence we conducted the stability studies for siRNA rHDL nanoparticles by storing them at two different temperatures 4ºC and -20ºC (to determine the optimum temperature) for a month, and analyzed them during the second week and fourth week. Compositional analysis revealed no significant loss of siRNA prepared by the two methods indicating the stability of the nanoparticles under both the temperatures over the time period of 1 month (Fig.25 and 26).

Characterization of mRNA Peptide Nanoparticles

The objective of this project was to develop stable and homogenous mRNA peptide nanoparticles with high incorporation efficiency of mRNA.
Preliminary studies were aimed at optimizing the protocol for the preparation of mRNA entrapped peptide nanoparticles which could generate smaller and homogenous nanoparticles. As the experiments were conducted with L-Myc mRNA (1800bp), the large size and the highly negative charge of the mRNA necessitated the need for a neutralizer to compress the molecule and mask the negative charge. Hence the first set of experiments was directed towards selecting a neutralizer which could neutralize the mRNA and generate optimally sized mRNA neutralizer complex. Preliminary choice of neutralizers included poly-L-lysine, hexadecyl trimethyl ammonium butyride (HTAB) and tetrabutyl ammonium hydroxide (TBAH). The structure of the neutralizers can be observed in Fig.10. The size determination analysis of the mRNA neutralizer complexes indicated the presence of nanoparticles in the range of 1000-2800nm in diameter (Table 4). The preparation of mRNA peptide nanoparticles was started with the HTAB neutralizer for preliminary trials.

The mRNA-HTAB complex was incubated with two different concentrations (20µg/ml and 100µg/ml) of Myr5A peptide to determine the ideal concentration required for generating optimally sized nanoparticles. The size analysis of the mRNA nanoparticles formed using Myr5A (20µg/ml) revealed that 35% of the particles had a diameter of 270nm (Table 5). However, increasing the concentration of Myr5A peptide from 20µg/ml to 100 µg/ml resulted in 75% of the sample population with a diameter of 556nm (Fig.27). This evidence seems to indicate a direct correlation between the concentration of Myr-5A and the size of the nanoparticles generated during the process.

Extensive studies were also carried out using several different neutralizers and peptides (mentioned previously) at varied concentrations in order to achieve the optimal size of mRNA nanoparticles. Finally, the size analysis of mRNA nanoparticles prepared with the neutralizer
DOTAP (10µg) and 5A peptide (20µg) resulted in 50% of the particles with diameter of 268nm (Fig.28).

Among the several methods explored to determine the incorporation efficiency of mRNA, it was observed that removal of the unentrapped mRNA using OligodT beads, followed by the addition of TRIZOL reagent to lyse the nanoparticles will result in the detection of the entrapped mRNA.
Figure 11: Compositional analysis of siRNA rHDL nanoparticles prepared with or without lyophilization. Bars represent Standard Error of Mean (S. E. M.), n=2.
Figure 12: Incorporation of siRNA in siRNA rHDL nanoparticles prepared with or without lyophilization. Bars represent Standard Error of Mean (S.E.M.), n=2.
(a) siRNA rHDL nanoparticles prepared without lyophilization

(b) siRNA rHDL nanoparticles prepared with lyophilization

Figure 13a and b: Particle size distribution analysis of siRNA rHDL nanoparticles prepared with or without lyophilization. The measurements were performed using MOBIUS DLS instrument.
<table>
<thead>
<tr>
<th><strong>CHOLATE DIALYSIS METHOD(1)</strong></th>
<th><strong>CHOLATE DIALYSIS METHOD (2)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Preparation of siRNA -poly-L- lysine complex</strong></td>
<td><strong>1. Preparation of rHDL nanoparticles</strong></td>
</tr>
<tr>
<td>a. Addition of siRNA and poly-L-lysine(1:5)</td>
<td>a. Lipid film- Addition of EYPC alone or combination of EYPC, free cholesterol and cholesteryl ester</td>
</tr>
<tr>
<td></td>
<td>b. Incubation of the lipid film with sodium cholate and Tris-EDTA buffer at 37°C</td>
</tr>
<tr>
<td><strong>2. Preparation of rHDL nanoparticles</strong></td>
<td><strong>2. Preparation of siRNA-poly-L- lysine complex</strong></td>
</tr>
<tr>
<td>a. Lipid film-Mixture of EYPC, free cholesterol and cholesteryl ester</td>
<td>a. Addition of siRNA and polylysine(1:5)</td>
</tr>
<tr>
<td><strong>3. Preparation of siRNA-rHDL nanoparticles</strong></td>
<td><strong>3. Preparation of siRNA-rHDL nanoparticles</strong></td>
</tr>
<tr>
<td>a. Addition of siRNA and poly -L-lysine complex to the lipid film</td>
<td>a. Addition of siRNA and poly -L-lysine complex to the incubated lipid –detergent complex.</td>
</tr>
<tr>
<td>c. Lyophilization and dialysis</td>
<td>c. Lyophilization and dialysis</td>
</tr>
</tbody>
</table>

Table 1: Description of the two methods for the preparation of siRNA rHDL nanoparticles.
Figure 14: Compositional analysis of siRNA rHDL nanoparticles prepared by two different methods cholate dialysis method (1) and (2). Bars represent Standard Error of Mean (S. E. M.), n=2.
Figure 15: Incorporation of siRNA in siRNA rHDL nanoparticles prepared by two different methods: cholate dialysis method (1) and (2). Bars represent Standard Error of Mean (S.E.M), n=2.
<table>
<thead>
<tr>
<th>COMPOSITION ANALYSIS</th>
<th>PROTEIN (%)</th>
<th>PHOSPHOLIPID (%)</th>
<th>CHOLESTEROL (%)</th>
<th>SIRNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholate dialysis method (1)</td>
<td>18.2</td>
<td>77.1</td>
<td>3.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Cholate dialysis method (2)</td>
<td>25</td>
<td>73</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2: Compositional analysis of siRNA rHDL nanoparticles prepared by two different methods cholate dialysis method (1) and (2).
Figure 16: Particle size distribution analysis of siRNA rHDL nanoparticles prepared by cholate dialysis method (1).
The measurements were performed using MOBIUS DLS instrument.
Figure 17: Particle size distribution analysis of siRNA rHDL nanoparticles prepared by cholate dialysis method (2).

The measurements were performed using MOBIUS DLS instrument.
Figure 18: Compositional analysis of siRNA rHDL nanoparticles prepared using two different cholesteryl esters (cholesteryl oleate and cholesteryl linoleate) by cholate dialysis method (1). Bars represent Standard Error of Mean (S. E. M.), n=2.
Figure 19: Incorporation of siRNA in siRNA rHDL nanoparticles prepared using two different cholesteryl esters (cholesteryl oleate and cholesteryl linoleate) by cholate dialysis method (1). Bars represent Standard Error of Mean (S. E. M.), n=2.
Figure 20: Compositional analysis of siRNA rHDL nanoparticles prepared using two different cholesteryl esters (cholesteryl oleate and cholesteryl linoleate) by cholate dialysis method (2). Bars represent Standard Error of Mean (S. E. M.), n=2.
Figure 21: Incorporation of siRNA in siRNA rHDL nanoparticles prepared using two different cholesteryl esters (cholesteryl oleate and cholesteryl linoleate) by cholate dialysis method (2). Bars represent Standard Error of Mean (S. E. M.), n=2.
<table>
<thead>
<tr>
<th>SAMPLES PREPARED USING VARIOUS SIRNA: POLY-L-LYSINE RATIO</th>
<th>ZETA POTENTIAL MEASUREMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. 1:5</td>
<td>-12mV</td>
</tr>
<tr>
<td>b. 1:10</td>
<td>0mV</td>
</tr>
</tbody>
</table>

Table 3: Comparison of zeta potential measurement of siRNA rHDL nanoparticles prepared with two varying siRNA: poly-L-lysine ratios (1:5) and (1:10)
Figure 22: Atomic force Microscopic analysis (2D image) of siRNA rHDL nanoparticles. The AFM 2D image reveals the presence of nanoparticles in the range of 8-20nm particles with the mean diameter being 16nm.
Figure 23: Atomic force Microscopic analysis (3D image) of siRNA rHDL nanoparticles. The AFM 3D image reveals the presence of spherical nanoparticles.
Figure 24: Transmission Electron microscopic analysis of siRNA rHDL nanoparticles. The image reveals the presence of spherical nanoparticles in the range of 5-20nm particles with majority of the particles ranging from 10-16nm.
Figure 25: Stability studies: Incorporation of siRNA in siRNA rHDL nanoparticles stored up to 4 weeks at two different temperatures 4°C and -20°C prepared by cholate dialysis method (1). Bars represent Standard Error of Mean (S. E. M.), n=2.
Figure 26: Stability studies: Incorporation of siRNA in siRNA rHDL nanoparticles stored up to 4 weeks at two different temperatures 4°C and -20°C prepared by cholate dialysis method (2). Bars represent Standard Error of Mean (S. E. M.), n=2.
Table 4: Size analysis of the mRNA-neutralizer complexes obtained using DLS. The complexes were prepared as three different preparations with the L-Myc mRNA and the neutralizers, Poly-L-lysine, TBAH and HTAB. The results obtained from the MOBIUS DLS instrument were compiled to generate the table.

<table>
<thead>
<tr>
<th>mRNA+NEUTRALIZER COMPLEX</th>
<th>SIZE (nm)</th>
<th>MASS %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.mRNA+ Poly-L-lysine</td>
<td>1755</td>
<td>98.6</td>
</tr>
<tr>
<td>2.mRNA+ TBAH</td>
<td>774.6</td>
<td>67</td>
</tr>
<tr>
<td>3.mRNA+ HTAB</td>
<td>2865</td>
<td>94</td>
</tr>
</tbody>
</table>

Table 5: Size analysis of mRNA peptide nanoparticles obtained using DLS. The table shows the size and mass % of the mRNA peptide nanoparticles prepared using L-Myc mRNA, HTAB neutralizer and Myr-5A peptide at two different concentrations 20µg/ml and 100µg/ml. The results obtained from the MOBIUS DLS instrument were compiled to generate the table.

<table>
<thead>
<tr>
<th>SAMPLE PREPARATION</th>
<th>SIZE (nm)</th>
<th>MASS %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.Myr 5A(20µg/ml) +mRNA-HTAB</td>
<td>269</td>
<td>35.5</td>
</tr>
<tr>
<td>2.Myr 5A(100µg/ml) +mRNA-HTAB</td>
<td>556.9</td>
<td>76.7</td>
</tr>
</tbody>
</table>
Figure 27: Particle size distribution analysis of mRNA peptide nanoparticles prepared using L-Myc mRNA, HTAB neutralizer and Myr5A peptide (100µg/ml). The measurements were made using MOBIUS DLS instrument.
Figure 28: Particle size distribution analysis of mRNA peptide nanoparticles prepared using L-Myc mRNA, DOTAP neutralizer and 5A peptide (100µg/ml). The measurements were made using MOBIUS DLS instrument.
CHAPTER V

DISCUSSION

Genetic diseases are disorders caused solely or partially due to the abnormal mutations in the DNA sequence of specific genes. According to the most recent publications, human genome is roughly estimated to contain 21,000 coding genes [45]. Mutations in any of the coding genes can lead to deleterious effects in humans including the onset of genetic diseases, physical disability and shortened life span. Some of the mutations are inherited from parents and passed onto several generations, affecting the health and lives of many individuals harboring the mutations. These include Hemophilia, cystic fibrosis, sickle cell anemia and several thousands of disorders. Some of the disorders are acquired due to random mutations in the gene caused due to lifestyle changes and environmental factors. Extensive research on several commonly acquired disorders has lead to the conclusion that the onset of diabetes, obesity and certain types of cancer is closely associated with genetic mutations.

Gene therapy is no longer limited to rare genetic diseases. Instead, it is now being developed as a key therapeutic strategy for the treatment of several major disorders, including cancer, cardiovascular diseases, diabetes and others, where the underlying root cause stems from genetic abnormalities. Gene therapy is an approach that involves the therapeutic delivery of nucleic acids into human cells to restore a defective protein, or inhibit the expression of an
aberrant protein in order to correct genetic disorders. But, the large size, negative charge and the sensitivity of the nucleic acids to the endonucleases prevent them from being successfully used in the naked form for the systemic delivery. However, these challenges can be easily overcome with the help of nanoparticles (drug carriers) that provides an opportunity for the nucleic acids to permeabilize the cell membrane, while protecting them from serum nucleases.

**Characterization of siRNA rHDL Nanoparticles**

There are several different types of nanoparticles that are currently being employed for the therapeutic delivery of nucleic acids. However, the most commonly used drug carriers for nucleic acids including liposomes and PLGA polymer nanoparticles suffer from instability and toxicity issues. Moreover, stability and homogeneity are two important criteria for nanoparticle formulations to be approved for therapeutic purposes in clinical trials. Hence this study is focused on developing and characterizing stable, biodegradable and homogenous nanoparticles for the therapeutic delivery of polynucleotides. rHDL nanoparticles are novel, biodegradable, non immunogenic nanoparticles well established for the targeted delivery of anti cancer drugs. Additionally based on the therapeutic proof-of-concept studies siRNA rHDL nanoparticles were found to be effective in silencing the expression of the STAT3 and the FAK genes in mice models of ovarian cancer [33]. Hence we postulated that rHDL can be suitable carriers for the entrapment of STAT3 siRNA and conducted experimental studies to improve the homogeneity of the nanoparticles for clinical studies. Having comprehensive knowledge of the human HDL proved to be a great advantage in modulating the chemical constituents of the rHDL nanoparticles to improve the homogeneity.
Preliminary experiments involving siRNA entrapped rHDL nanoparticle formulations were conducted to investigate the effect of lyophilization in improving the particle homogeneity. Previous experimental studies on valrubicin rHDL nanoparticles conducted in our laboratory have indicated that the inclusion of lyophilization as a prefinal step before the dialysis of the sample significantly improved the homogeneity of the formulation. Lyophilization (freeze drying) is a dehydration process which converts frozen sample to powder upon being subjected to high vacuum. This process is used by many pharmaceutical companies to increase the shelf life and maintain the stability of their drug products. Hence this procedure was adapted to determine its role in improving the homogeneity of siRNA entrapped rHDL formulation.

Chemical compositional analysis did not reveal any significant changes in the incorporation efficiency of siRNA in lyophilized sample (Fig.11 and 12). However, dynamic light scattering analysis indicated a marginal increase in the particle homogeneity upon lyophilization (Fig.13). This increase might be due to the incorporation of lyophilization to the protocol. It is possible that the freezing process may have played a role in limiting the increase in the homogeneity of the sample. However, this could be mitigated with the addition of cryoprotectant to the formulations before freezing. Certain experimental studies have shown that addition of cryoprotectant like sucrose can help protect the structural integrity of the nanoparticles thus preventing any sample leak from the formulations [22].

Determinations to increase the homogeneity of the siRNA rHDL nanoparticle formulations lead to the incorporation of minor changes in the preparation of the nanoparticles. These changes were adapted from another experimental study [34] that indicated the presence of homogenous rHDL paclitaxel nanoparticles as a result of the selectively designed protocol. The modified procedure (cholate dialysis method (2)) and the contrasting difference with the original
procedure (cholate dialysis method (1)) is outlined in table 1. The major difference in the cholate dialysis method 2 involved the incubation of the EYPC phospholipid film, sodium cholate and the Tris EDTA buffer at 37°C for 2 hrs and the absence of free cholesterol and cholesteryl ester. Comparison of the chemical compositional analysis revealed a relative decrease in the incorporation of protein and phospholipid components in the sample prepared by cholate dialysis method (2) (Fig.14). However, the incorporation of siRNA in siRNA rHDL nanoparticles prepared by cholate dialysis method 2 seemed to increase by a factor of 1.6 when compared to the siRNA incorporation of nanoparticles prepared by the original method (Table 2). Comparison of the size analysis between the two samples did not reveal a significant change in homogeneity (Fig. 16 and 17). Although there is a need for repetition of the experiments to establish statistical significance, the relative decrease of the protein and phospholipid incorporation might be due to the lack of unesterified cholesterol, that is usually interspersed at smaller concentration between the protein and the phospholipid components of the rHDL. The increase in the incorporation efficiency of siRNA might have possibly been achieved due to the addition of the siRNA - polylysine complex (containing hydrophilic amine groups) to the aqueous solvent mixture (EYPC, sodium cholate and Tris EDTA buffer).

The physical characteristic of cholesteryl linoleate ester with a melting point of 42°C, fairly close to the human body temperature (37°C), redirected our focus to replace the cholesteryl oleate ester (M.P-44-47°C), with cholesteryl linoleate ester. It was postulated that the replacement of cholesteryl oleate with linoleate might help in increasing the solubility of the core component of HDL, thereby allowing the formation of more homogenous and smaller particles, with an enhanced ability to incorporate higher drug load in the core region of rHDL. Experiments were designed to prepare siRNA rHDL nanoparticles using the two different
cholesteryl esters by the two different methods, cholate dialysis method (1) and (2). However, there were no significant changes observed in neither the compositional analysis (Fig.18 -21) nor the size analysis by DLS.

Goals to reduce the net negative surface charge (-12mV) of the siRNA rHDL nanoparticles prepared by the cholate dialysis method 1 lead to modifications in the protocol to overcome the problem. We hypothesized that increasing the siRNA-polylysine ratio from 1:5 to 1:10 would reduce the overall negative charge of the nanoparticles. This hypothesis was pursued based on the fact that increasing the concentration of the cationic neutralizer (poly-L-lysine) would further reduce the negative charge of the siRNA and hence the net charge of the nanoparticles. The ratio modifications lead to a significant change as the net charge of the nanoparticles was reduced to almost 0mV or null (Table 3). Reduction of negative charge was undertaken to increase the transfection efficiency of the nanoparticles and prevent its repulsion from the negative charge of the cell membrane.

The physical characterization of the siRNA rHDL nanoparticles was quite challenging due to the overall net negative charge of the nanoparticles. The negative charge of the nanoparticles demanded the use of a poly-L-lysine cover slip for the sample attachment for the characterization of the nanoparticles using AFM. Similarly, the high concentration and the negative charge of the nanoparticles lead to several trial and error regarding the choice of the stain and dilution of the sample for physical characterization under the electron microscopy. Characterization using AFM (Fig.22 and 23) and electron microscopy (Fig.24) revealed the presence of majority of the nanoparticles in the range of 10-16nm which coincides with the measurements conducted using DLS.
Stability studies for siRNA rHDL nanoparticles were undertaken to determine the shelf life of the formulation. Compositional analyses conducted during the second and fourth week showed absence of leakage of siRNA thereby indicating the stability of the sample (Fig.25 and 26).

**Characterization of mRNA Peptide Nanoparticles**

mRNA entrapped nanoparticles are an emerging class of nucleic acid therapeutics being used in cancer immunotherapy, and treatment of neurological disorders. The synthesis of mRNA entrapped peptide nanoparticles was undertaken to develop an efficient *in vivo* delivery model for mRNA.

The preliminary objective of the project was to compress the immense mRNA (1800bp) and mask its negative charge using a cationic neutralizer which could produce optimally sized mRNA neutralizer complexes. Hence the preliminary experiments were targeted to select a cationic neutralizer that could achieve the above mentioned objective. Initial trial experiments involved the interaction of L-Myc mRNA with poly- L- lysine, HTAB and TBAH. These neutralizers were chosen based on their cationic functional groups and previous experimental studies conducted in lab. The dynamic light scattering analysis for the mRNA-neutralizer complex revealed the presence of nanoparticles in the range of 1000-2800nm in diameter (Table 4). Among the three different mRNA neutralizer complexes, HTAB-mRNA complex was chosen for preliminary investigation. The mRNA HTAB complex was further added to the Myristoylated 5A peptide to generate mRNA peptide nanoparticles. Myristoylated 5A peptide is a synthetically designed peptide that contains 5A peptide (Apo-A1 mimetic peptide) attached to the myristoyl fatty acid. The size analysis of the mRNA nanoparticles prepared using Myr 5A
(20µg/ml) revealed 35% of the nanoparticle population with a diameter of 270nm (Table 5). Whereas, increasing the concentration of Myr 5A to 100µg/ml resulted in 75% of the sample population with a diameter of 556nm (Fig.27). This evidence indicates a direct correlation between the concentration of Myr-5A and the size of the nanoparticles. This might have possibly been achieved due to the increased concentration of the protein component (Apo-A1 mimetic peptide) that could have aided the compaction of the mRNA neutralizer complex. This statement was based on the previous experimental observations which indicated the negative correlation between the Apo-A1 peptide concentration and the size of the siRNA rHDL nanoparticles [4].

Several experimental changes were made to the protocol to improve the homogeneity and the size of the nanoparticles. The changes involved the elimination of the detergent (sodium cholate) and extending the duration of dialysis from 48 to 72 hours. Size analysis (DLS) of the mRNA peptide nanoparticles synthesized devoid of the sodium cholate detergent resulted in aggregation of the nanoparticles. However, extending the duration of dialysis (72hrs) against 1xPBS upon the synthesis of the nanoparticles indicated the presence of more heterogeneous population (based on DLS analysis), probably caused due to the excessive removal of the detergent.

The task of synthesizing smaller sized mRNA peptide nanoparticles seemed quite challenging even upon using several other cationic neutralizers including protamine sulphate and DOTAP in combination with Apo- A1 and 5A peptide (Apo- A1 mimetic peptide). Protamine sulphate is a cationic peptide used in gene transfer, protein purification and gene therapy for increasing the transduction efficiency of viral and nonviral carriers. DOTAP, a cationic
phospholipid is very often used as a liposomal transfection reagent for the delivery of nucleic acids and proteins in eukaryotic cells.

Finally, the size analysis of mRNA nanoparticles prepared with the neutralizer DOTAP (10µg) and 5A peptide (20µg) resulted in 50% of the particles with diameter of 268nm (Fig.28). This might have been possible due to the contribution of the cationic phospholipid which could have efficiently compressed the nucleic acid and entrapped the nucleic acid–neutralizer complex. Although repetition of the above experiment is necessary to establish statistical significance, this indicated that further optimization can be achieved with the help of DOTAP neutralizer and 5A peptide.

**Future Directions**

The first and foremost goal is to repeat the significant experiments pertaining to siRNA rHDL nanoparticles and establish consistent results for determining statistical significance. We also plan to vary the N/P ratio (number of polyamine groups in the polymer/number of phosphate groups in the polynucleotide) in the siRNA rHDL nanoparticle preparation to determine the optimum N/P ratio for higher encapsulation efficiency of the siRNA and homogeneity. Upon obtaining stable and homogenous siRNA rHDL formulations, we intend to test the *in vitro* gene silencing efficiency of the formulation in ovarian cancer cell line SKOV3, experimentally proven to contain high expression of SRB1 receptors and STAT3 protein. These studies would be further extended to test the *in vivo* gene silencing efficiency of the STAT3 siRNA rHDL nanoparticles in mice models and ultimately in humans.

Experiments would be planned to optimize the mRNA peptide nanoparticle preparation using DOTAP and 5A peptide to obtain more homogenous nanoparticles. We are also planning
to use Time domain (TD) NMR to understand and compare the characteristics of mRNA peptide nanoparticles with well established Valrubicin (hydrophobic drug) rHDL nanoparticles. Upon obtaining optimal mRNA peptide formulations, the ultimate goal would be to investigate the *in vitro* efficiency of the eGFP mRNA nanoparticles and analyze the expression of eGFP protein using confocal microscope.
REFERENCES


42. WWW.wiley.co.uk/genmed/clinical

43. www.nih.gov/science/colorectalcancer/nanotech.htm

44. https://clinicaltrials.gov/ct2/results?term=gene+therapy

45. www.genome.gov/
