

**NANOTECHNOLOGY APPROACH FOR TREATMENT OF
CYSTIC FIBROSIS**

By

NOOR KBAH

A thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Pharmaceutical Science

written under the direction of

Professor Tamara Minko, PhD

and approved by

New Brunswick, New Jersey

Jan 2016

ABSTRACT OF THE THESIS
NANOTECHNOLOGY APPROACH FOR TREATMENT OF
CYSTIC FIBROSIS

BY

NOOR KBAH

Thesis Director: Tamara Minko, PhD.

Cystic fibrosis is a lethal, hereditary disease, caused by mutations in the gene cystic fibrosis transmembrane conductance regulator (CFTR) where it affects a cAMP-activated chloride (Cl⁻) channel and results in impaired ion and water transport, accumulation of viscous mucus in the airway, thereby leads to bacterial colonization. Frequent infections and inflammation result in an obstructive pulmonary disease. Similar conditions in the pancreas lead to pancreatic insufficiency. Several approaches have been designed to improve transepithelial ion transport in CF patients. These approaches involve correction of the CFTR mutation, potentiation of CFTR channel, and/or stimulation of alternative chloride channels. The purpose of this thesis involve using a nanotechnology to correct the defective ion transport in epithelial cells from CF patient. The first aim of the project is comparative study to evaluate the effect of using corrector and/or potentiator agent to restore the ion channel activity. The second aspect is nanotechnology-based delivery system that enhance drug delivery to the lung cells to treat pulmonary cystic fibrosis.

DEDICATION

I am thankful for God for giving me the good health and wellbeing that were important to
complete this goal

To my husband; Dhulfiqar

Who has been a constant source of support and encouragement during the challenges of
graduate

To my advisor, Professor Tamara Minko,

Who has helped and guided me and become an inspiration

To my wonderful parents;

Whose sacrifices, prayer, and support allowed me to succeed

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my gratitude to the people who were next to me to fulfill this goal. First, from the bottom of my heart I want to thank God for being with me all the way, for never leaving me, and for loving me. Thanks God for all I have!

My deepest gratitude is to my advisor, Professor Tamara Minko, for giving me the good opportunity to work in her lab and supporting me. I am so fortunate to have an advisor who gave me the freedom to explore my own idea, but still patient and supportive when my ideas faltered. I wish that one day I will be an advisor to my students like professor Tamara Minko has been to me.

My friends and colleagues, Andriy Kuzmov, Justin E. Sapiezynski, have been always there to listen and introduce advice. I am deeply grateful to them for the long discussions that helped me to do my work. Thanks for encouragement and guidance to finish this work. Further, my sincere gratitude for Dr. Xiaoping Zhang and Dr. Shike “Mary” Li (professor Sinko lab), Hetal, and Niket (professor Hatifi lab) for their help and kind treatment.

Special thanks to the members of my thesis committee professors Guofeng You and Leonid Kagan for taking time from their busy schedules to provide suggestions. I would like to further thank Dr. Olga Garbuzenko for her help and advice.

I would like to extend my gratitude to the staff in the Department of Pharmaceutics at Ernest Mario School of Pharmacy: Marianne Shen; Hui Pung; Fei Han; and Sharana Taylor for their efforts to ensure everything runs smoothly for everyone in the Department.

Finally, I would like to thank my family, Mom, Dad, my husband Dhulfiqar, my children Ghadeer and Muhammad Ridha, my sister Huda, and my brothers Baha and Ali. Thanks for supporting me during my studies and encouraging me on. Mom and Dad, you are my everything, I cannot imagine the life without you! Dhulfiqar, if I wrote down everything I ever wanted in a husband and best friend I would not have believed I could meet someone better! continued support and urging after moving to give up everything is deeply appreciated. Thanks for helping me to finish this thing. It's about time! Remember, "come on you are done". Thanks for being a great husband!

Ghadeer, and Muh.Ridha, you are my soul and, I never forget the difficulties that you had because of me, especially, the concomitant of my study with pregnancies of both of you. It was very hard to keep you without stress. If it wasn't for you, I wouldn't be here today! Sister and brothers, I could not ask for a better sibling and friends.

To all of you, thanks for always being there for me. Thank You, Thank You, Thank You!

TABLE OF CONTENTS

ABSTRACT OF THE THESIS.....	ii
DEDICATION.....	iii
ACKNOWLEDGEMENTS.....	iv
TABLE OF CONTENTS.....	vi
LIST OF TABLES.....	x
LIST OF FIGURES.....	x
1 INTRODUCTION.....	1
2 BACKGROUND AND SIGNIFICANCE.....	3
2.1 Signs and Symptoms of Cystic Fibrosis.....	3
2.1.1 Lung and Cardiorespiratory Systems.....	4
2.1.2 Gastrointestinal Tract.....	5
2.1.3 Endocrine System.....	7
2.1.4 Infertility.....	7
2.2 Causes of Cystic Fibrosis.....	8
2.3 Pathophysiology.....	9
2.4 Chronic Infection.....	11

2.5	Diagnosis.....	13
2.6	Management and Treatment.....	14
2.6.1	Antibiotics.....	15
2.6.2	Other Lung Diseases' Treatment.....	16
2.6.3	Small Molecules.....	17
2.6.4	Targeting-Receptors to the Respiratory Tract.....	19
2.6.5	Gene Therapy.....	20
2.6.6	CXCR2 Antagonist SB-656933 in Patients with Cystic Fibrosis.....	31
2.6.7	Targeting miRNA-Based Medicine to Airway Epithelial Cells using Nanotechnology.....	32
3	SPECIFIC AIMS.....	34
4	MATERIALS AND METHODS.....	35
4.1	Materials.....	35
4.2	Cell Culture.....	35
4.3	Cytotoxicity Assay.....	36
4.4	Chloride Efflux Measurements.....	37
4.5	RNA Extraction.....	37
4.6	Gene Expression (Real-Time Quantitative Polymerase Chain Reaction).....	38
4.7	Protein Extraction.....	38
4.8	Western Blotting.....	39

4.9 Nanostructured Lipid Carrier (NLC) Preparation and Characterization.....	40
4.9.1 NLC Preparation.....	40
4.9.2 Particle Size Distribution.....	41
4.9.3 Zeta Potential Measurement.....	41
4.9.4 Determination of entrapment efficiency and drug loading.....	41
4.10 Liposome Preparation and Characterization.....	43
4.10.1 Liposome Preparation.....	43
4.10.2 Liposome Particle Size measurement.....	43
4.10.3 Zeta Potential Measurement.....	43
4.11 Microscopic Technique.....	44
4.12 Statistical Analysis.....	44
5 RESULTS.....	44
5.1 <i>In Vitro</i> Cytotoxicity Assay.....	44
5.2 Fluorescence Measurements of Chloride Efflux.....	45
5.3 Analysis of CFTR mRNA Expression by Real-Time Quantitative Polymerase Chain Reaction (RT-QPCR).....	46
5.4 Expression of CFTR Protein by Western Blot.....	46
5.5 NLC Characterization.....	47
5.6 Liposome Characterization.....	47
5.7 Cellular Internalization of Liposomes.....	48

6 DISCUSSION.....	48
7 CONCLUSIONS.....	53
8 FUTURE DIRECTION.....	54
9 ILLUSTRATIONS.....	55
10 REFERENCES.....	70

LIST OF TABLES

Table 2.1 Different types of mutation with different frequencies among people wide world.

LIST OF FIGURES

Figure 2.1 Structure of CFTR.....	56
Figure 2.2 Behavior of mucoadhesive and muco-inert particles in mucus layers.	57
Figure 2.3 Penetration of polystyrene 200 nm nanoparticles through CF sputum.	58
Figure 2.4 An influence of mucolytic treatment on the mucus mesh.	59
Figure 2.5 Mean size (nm) of premiR-126-poly (ethyleneimine) (PEI) complexes.	60
Figure 2.6 Images for miRNA nanoparticles.....	61
Figure 5.1 <i>In vitro</i> cytotoxicity assay for CFBE41o- cells.	62
Figure 5.2 Chloride efflux measurements.	63
Figure 5.3 The relative quantity of CFTR gene expression.	64
Figure 5.4 Western blot of wild type CFTR and Δ F508-CFTR.	65
Figure 5.5 Illustration of PEG-NLC particles size, Zeta potential, and entrapment efficiency.....	66
Figure 5.6 Particle size distribution of liposome.	68
Figure 5.7 Representative images of human bronchial epithelial CFBE41o- cells.....	69

1 INTRODUCTION

Cystic fibrosis refers to an autosomal recessive disorder which is the most common fatal genetic disorder among the Caucasian population [1]. Although the life expectancy has been increased through the recent decades, it still only 40-50 years at most. It is caused by mutations in the gene of cystic fibrosis transmembrane receptor (CFTR) which coding for a cAMP-dependent chloride channel. This channel Transport negative charged ions (chloride and bicarbonate) through the apical membrane of epithelial cells thereby regulate the fluid and electrolyte balance in epithelial tissues of many organs such as airways, digestive system, reproductive system. There are about 1500 different type of CFTR mutations which have been described (www.genet.sickkids.on.ca/cftr). The most common type is delF508 mutation which results from deletion of 3 base pairs resulting in the loss of a phenylalanine at position 508 which is found in at least one allele in 75-90% of CF patients. Some of these mutations affect the quantity of CFTR at the cell membrane while others affect the function of these CFTR channels.

The main organ that is affected by cystic fibrosis is the lung where the mutations in the CFTR result in production of thick, viscus mucus that leads to airway obstruction, infection, inflammation and eventually end-stage lung disease. Pulmonary cystic fibrosis is considered as a primary cause of morbidity and mortality.

Different approaches have been investigated for treatment of cystic fibrosis. Some of these approaches were targeted the underlying cause of disease such as gene therapy. Others involved using of correctors that traffic the CFTR to the cell membrane. In order to enhance the CFTR function i.e. opening channel probability, potentiators have been used as another therapeutic approach.

Treatment of the pulmonary disease associated with CF is symptomatic, and involves using of physiotherapy to remove the obstructive mucus, aggressive treatment with antibiotics, bronchodilators, and recently, antibodies for treatment against *Pseudomonas* [2]. With the end stage of the disease, lung transplantation could be an option to extend the life of CF patients.

Over all, one or a combination of these therapeutic approaches of different mechanisms of action may be beneficial for patient with cystic fibrosis [3]. It is important to get these therapeutic agents in to the site of action to decrease its side effect and to enhance its therapeutics efficacy. Therefore, nanotechnology may play an important role in this field.

In the field of medical research, nanotechnology is utilized to overcome problems associated with delivery of lipophilic drugs, delivery of drugs across membranes, decreasing side effect of drugs, simultaneous delivery of multiple drugs, imaging, and real-time monitoring of in vivo effect [4].

Nanostructured lipid carrier (NLC) properties such as size, surface charge, and surface groups can be adjusted for the desired purpose. The aim of using of NLC gives many advantages to drug delivery [5]. Association of drug molecules with NLC via entrapment, conjugation, or adsorption, improves drug delivery. NLC can enhance drug transport across barriers and deliver drugs to target organ selectively. In addition, coating NLC with PEG may enhance the penetration in viscus mucous that associated with pulmonary cystic fibrosis. By using this technology, lower doses of drug can be administered to patients due to the minimization of the off-target deposition. An enhancement of drug stability in circulation, getting favorable pharmacokinetics, and overcoming drug resistance are other advantages associated with this type of technology.

Liposomes are another potential drug carrier system that enhance drug transport across barriers and selectively deliver drugs to the target organ [5]. Liposomes are suitable for delivery of hydrophobic, hydrophilic, and amphiphilic drugs. Liposomes further improve pharmacokinetic properties, increase efficacy, and reduce toxicity of the encapsulated agents. Consequently, Liposomes are considered as a potential option that opening up new avenues in formulation and delivery of therapeutic agents.

2 BACKGROUND AND SIGNIFICANCE

2.1 Signs and Symptoms of Cystic Fibrosis

Cystic fibrosis (also known as CF or mucoviscidosis) involves a wide range of symptoms and damages in different organs and systems of the body. The most common symptoms are salty tasting skin, poor growth, poor weight gain despite normal nutrition, accumulation of thick, purulent mucus, frequent chest infection, coughing or shortness of breath, and infertility in male. In childhood and infancy, bowel obstruction is considered the main manifestation. In addition, poor growth in children typically presents as an inability to gain weight and height at the same rate with their peers. This poor growth is caused by many factors such as chronic lung infection, poor absorption of nutrients from gastrointestinal tract (GIT) and increase metabolic demand due to chronic illness [6]

Rarely, cystic fibrosis can manifest as a coagulation disorder. Some CF patients may suffer from impaired vitamin K absorption. As a result, low level of vitamin K has an effect on clotting factor such as factors (II, VII, IX, and X) and lead to coagulation problems. The Young children are very sensitive to vitamin K malabsorption and this is

due to both very small amounts of vitamin K crosses the placenta and limited ability to absorb vitamin K from GIT after birth. Consequently, when a child presents with bruising, a coagulation evaluation may be warranted to determine whether there is an underlying disease [7].

2.1.1 Lung and Cardiorespiratory Systems

Most of the lung diseases result from airway obstruction. This obstruction is caused by an increase in the mucus production and decrease in the mucociliary clearance which result in an inflammation [8, 9]. Inflammation and infection affect lungs and cause structural changes which lead to a variety of symptoms. In the beginning, the bacteria grow out of control and cause pneumonia. Coughing and copious phlegm are the major symptoms in this period. Then, change in the lung structure like pathology in the airway followed by further difficulties in breathing. In addition to these symptoms, there are other health problems which include coughing up blood (hemoptysis) and heart failure (pulmonary hypertension), inadequacy in oxygen supply (hypoxia) and respiratory failure.

There are three types of bacteria [10] that are responsible for lung infection. They include *Staphylococcus aureus*, *Haemophilus influenza*, and *Pseudomonas aeruginosa* infection. Another type of lung disease is allergic bronchopulmonary aspergillosis. In this case, the body response to this fungus will cause worsening of the breathing problems. Moreover, *Mycobacterium avium* complex (MAC) is a group of bacteria involve in the tuberculosis [10]. It may cause a lot of damages and cannot be resolved by antibiotics. Furthermore, some lung infections are caused by thickening of mucus which leads to blockage of sinus passage. This type of lung infection is associated with facial pain, headache, fever, and nasal drainage. Sometime CF patients with chronic sinus infection may develop nasal

polyps. It was found that about 10-25 % [9] of CF patients have recurrent sinonasal polyps which block the nasal passage and lead to the increasing of breathing difficulty [11,12]. About 80% [9] of CF patients in the United States are dying because of cardiorespiratory complications.

2.1.2 Gastrointestinal Tract

About 5-10% of newborn infants have problems with passing feces (meconium) [9, 13]. This condition usually causes intestinal blockage and serious illness which are called meconium ileus. Another diagnostic way is the protrusion of the intestinal rectal membrane (rectal prolapse) which occurs in about 10% of CF children [9]. It is caused by an increase in the fecal volume, malabsorption, and increase intra-abdominal pressure due to coughing [14].

The second effect of cystic fibrosis on GI is through its effect on pancreas which involves in digesting of food. The thickened secretion from pancreas blocks the endocrine movement of digestive enzymes in to the duodenum which cause an irreversible damage to pancreas. Eventually, this damage will cause a painful inflammation known as pancreatitis [15]. In more severe cases, especially in older children and adult, the pancreas duct will be closed completely which results in atrophy of exocrine gland and progressive fibrosis [9].

In addition, malabsorption is a disorder that is common in most patients with cystic fibrosis [9]. It is caused by lack of digestive enzymes which lead to difficulty in absorbing nutrients consequently with increase their excretion in feces. Malabsorption may lead to malnutrition, poor growth, and loss of calories. In addition, it may cause

hypoproteinemia. This effect may be severe enough to develop generalized edema. Moreover, patients with cystic fibrosis cannot absorb fat-vitamins such as A, D, E, and K easily. Other GI symptoms involve heart burn, constipation [16], and intestinal blockage by intussusception [17]. Furthermore, older patients with CF may have distal intestinal obstruction syndrome due to intestinal blockage by a thickened feces.

Majority of CF patients 85-90% have exocrine pancreatic insufficiency [9]. This type of complication occurs in case of severe cystic fibrosis transmembrane conductance regulator - CFTR (ATP-binding cassette sub-family C, member 7, e.g. $\Delta F508/\Delta F508$). It may happen in 10-15% of CF patients with one severe and one mild CFTR mutation or in case of two CFTR mutations [9]. For these cases, there is no need for enzyme supplements because CFTR still has a little activity. In addition, there is sufficient exocrine activity. Pancreatic sufficient phenotype usually shows good growth and development and there is no GI complication. Although, patients with this type of CF have high survival rate, a subset of pancreas-sufficient individuals with cystic fibrosis may develop idiopathic chronic pancreatitis [9] that can be associated with recurrent abdominal pain and life-threatening complications. Finally, CF patients may develop several liver problems as a result of thickened secretion. For example, blocking the bile duct due to thickened bile secretion may cause liver damage. Eventually, it can lead to scarring and nodulating (cirrhosis). It may cause another problem such as blood clotting in which the liver will not be able to remove toxins from the blood and produce some important proteins [18, 19].

2.1.3 Endocrine System

2.1.3.1 Diabetes

The islets of Langerhans are presented in the pancreas. They maintain blood glucose level by insulin secretion. Any damage to pancreas will affect them and result in a specific type of diabetes which is unique for patients with CF [20]. This type of diabetes is called cystic fibrosis–related diabetes (CFRD) and has the same characteristic of type 1 and type 2 diabetes. It is also considered as one of the major non pulmonary complications in CF patients [21].

2.1.3.2 Bone Disease

CF is usually associated with malabsorption. It affects the absorption of fat-soluble vitamins, especially; vitamin D. Vitamin D plays an important role in calcium and phosphate regulation. Disturbances in the regulation of calcium and phosphate may result in poor absorption that, in turn, can lead to the development of bone disease and osteoporosis. Bones of patients with osteoporosis are usually weak and fracture easily [22]. In addition, most patients with CF show clubbing of their fingers and toes. These symptoms are caused by chronic illness and low oxygen amount in the tissues [23, 24].

2.1.4 Infertility

Studies found that about 97% of CF patients are infertile. Fortunately, they are not sterile and they can get children with help of reproductive techniques [25]. Congenital absence of Vas deferens, which is responsible for connecting the testes to the ejaculatory ducts of the penis, is the main cause of infertility in men with CF. Other causes involve azoospermia, teratospermia, and oligoasthenospermia [26]. Some women with CF may

develop infertility which is resulted either from thickened cervical mucus or malnutrition. In case of malnutrition, it may affect ovulation and cause amenorrhea.

2.2 Causes of Cystic Fibrosis

CF is caused by mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [9, 29]. There are different types of mutations with different frequencies (**Table 2.1**) [27]. Mutations of CFTR have been divided in six major classes 4: (Class I) mutation where there is a defective synthesis of full-length CFTR protein. Premature stop codon prevents full translation of mRNA, resulting in truncated CFTR protein. Few to no mature CFTR proteins are formed. (Class II) mutation is characterized by defective CFTR protein processing and trafficking thereby defective post-translational processing and transport reduce quantity of CFTR protein delivered to cell surface. (Class III) causes a defective CFTR channel gating where CFTR is at the cell surface but has reduction in channel-open probability. (Class IV) mutation has defective CFTR channel conductance in which CFTR is at the cell surface but has impaired movement of ions through channel. (Class V) reduced synthesis of CFTR protein. A splicing defect reduces quantity of properly processed CFTR mRNA transcripts, decreasing quantity of CFTR protein at the cell surface. Finally, (Class VI) mutation is associated with reduced stability of CFTR protein. Accelerated turnover of CFTR protein at the cell surface reduces quantity

The most prevalent type is (Class II) mutation with a deletion of three nucleotides [28] which accounts for two third of CF patients wide world and 99% in the United States. This mutation involves a loss of amino acid phenylalanine in the position of 508th on the protein ($\Delta F508$). CF is an autosomal recessive pattern of inheritance. In normal person,

both copies (alleles) of CFTR are work. One working copy (allele) is enough to prevent CF. CF occurs when allele cannot produce functional CFTR protein.

CFTR is a gene with 20,000 bp. It makes a protein that is 1480 amino acid long. It was found between base pairs 117,120,016 to 117,308,718 on the long arm of chromosome 7, band 1, region 3 sub-band 2, represented as 7931.2 [29]. CFTR is a member of ATP-binding cassette (ABS) family. It produces ion chloride channel. This channel is essential for producing sweat, mucus, and digestive juice. It consists of four domains, two ATP hydrolyzing which provides protein with energy and two helical domains (each consists of 6 alpha helices helps the protein to cross cell membrane) **Figure 2.1** . In addition, this protein has a regulatory binding site which is important in activation. The activation occurs by phosphorylation mainly via cAMP -dependent protein kinase [29]. cAMP - Cyclic adenosine monophosphate (cyclic AMP or 3'-5'-cyclic adenosine monophosphate). Moreover, this protein has a terminal carboxyl group which is responsible for protein binding to the cytoskeleton via a PZD domain interaction [30]. It was found that any genetic modification in patients with CF will change the severity and frequency of the disease. For instance, CF patients with a polymorphism in one or two mannan-binding lectin alleles, which is the gene essential in innate immunity and phagocytosis of microorganism, will decrease the circulatory level of the protein and result in three fold higher risk of end stage lung disease. Further risk may involve chronic bacterial infection which worse patients with CF [9].

2.3 Pathophysiology

Cystic fibrosis is usually range from mild to severe. Its severity depends on the type of defects in the CFTR protein. Different defects in CFTR protein result from different

mutations in CFTR gene. Many approaches are directed to target drugs to these defective proteins in order to improve their function.

The most common mutation is $\Delta F508$ -CFTR. It produces protein that does not fold normally; also it may degrade by the cell. Too short protein is another type of mutation. This type of mutation is caused by a premature ending of protein production. Other type of mutation produces protein that cannot use energy normally, prevent some elements like chloride, iodide, and thiocyanate from crossing cell membrane [31]. It is usually destroyed within short period in contrast to the normal proteins which stay stable for longer time. Moreover, some mutations cause production of small amount of CFTR copies [29].

CFTR gene produces protein that anchored to the membrane of different cells for example, lung sweat glands and all exocrine glands. It helps to connect the intracellular compartments to the outside environment. In addition, it controls the movement of halogens from inside to the outside of the cell; while in sweat glands, the movement of chloride from sweat gland to the cytoplasm is controlled by CFTR protein. The chloride and thiocyanate are trapped inside the airway cells or out of skin if there is a defect in CFTR protein. As a result, the immune defense system will not be able to synthesize hypothiocyanate (OSCN) [32, 33]. Another problem is the differences in the electrical potential inside and outside the cell. This is due to the negatively charge chloride which give rise to cations to cross and trap inside the cell. One of the important extracellular cations is sodium. It combines with chloride to make the salt form. This salt will be lost in the sweat of the CF patients and it is considered as a marker for the sweat test [20]. The blockage of the narrow path of the organs with thickened mucus is the major cause

of damage in CF patients. It will lead to several problems such as changing in the lung structure and infection, pancreas damage due to accumulation of digestive enzymes, and intestinal blockage by thick feces.

There are different concepts why the defect in the CFTR protein causes these problems. The first idea suggests that losing of halogens and pseudo halogen (especially, chloride, iodide, and thiocyanate) will cause accumulation of more viscous mucus (nutrient-rich) in the lung and consequently blot out the microorganism from immune system. The second theory suggested that the paradoxical increase in the uptake of sodium and chloride due to the defect in CFTR protein causes an increase in the water reabsorption which consequently produces thick and dehydrated mucus. The third idea proposed that the dehydration of mucus and other secretion are caused by the abnormal movement of chloride out of the cell [20].

2.4 Chronic Infection

The early stage the lung of patients with cystic fibrosis starts to be filled infected with bacteria. These bacteria which are usually common among patients with CF grow in the mucus of the small airway of the lung following by biofilm formation. This biofilm is considered as bacterial microenvironment. Most immune cells and antibiotics fail to penetrate this biofilm layer. It was found that repeatedly persistence of respiratory infection together with viscous secretion may cause gradual structural changes in the lung and worse the eradication process [34]. After period of time, patients with CF will develop different types of bacteria with different properties. For example, in an early stage the most prevalent bacteria are *Staphylococcus* and *Haemophilus influenza* [9]. Later, *Pseudomonas Aeruginosa* or sometime *Burkholderia Cepacia* will invade the CF

patients. These types of bacteria have ability to adapt the environment and resist most common used antibiotics. Many CF patients with pseudomonas have special characteristic which is called "mucoid" Pseudomonas. It is resulted in formation of large colonies. There are different ways through which the infection transfers among CF patients [35]. One of these ways is gathering in closed places such as recreation centers, summer CF camps and other [28]. Hospitals play role in transfer infection between patients. Most hospitals group CF patients in one place and use the same, non-sterilized equipment [36] which facilitate the spread of dangerous strain of bacteria [37]. It is important to reduce these problems to limit the transmission of strong resistant bacteria strains.

In addition to bacterial infection, CF patients may develop chronic fungal infection such as *Aspergillus fumigatus*, *Scedosporium apiospermum*, *Aspergillus Terreus* and /or yeasts (such as *Candida Albicans*). *Aspergillus Flavus* and *Aspergillus Nidulans* (occur transiently in CF respiratory secretion) filamentous fungi are less prevalent. Patients, who are in an exclusive state of CF, show some filamentous fungi like *Penicillium Emersonii* and *Acrophialophora Fusispora*. There are several factors that trigger fungal growth. The first one is defective mucociliary clearance. This factor will cause local immunological problems. Another factor involves prolong administration of therapy such as antibiotics and corticosteroid. This will increase the incidence of fungal growth. Even though, the clinical relevance is not confirmed yet, filamentous fungi may cause a local inflammation response which lead to progressive disturbance in the lung function. For example, allergic bronchopulmonary aspergillosis (ABPA), which is considered the most

common disease in the CF history, associates with a TH2-driven immune response to *Aspergillus* [38, 39].

2.5 Diagnosis

There are many different methods to diagnose cystic fibrosis. Newborn screening is one of these methods [40]. It involves measuring an increase in the blood concentration of immune reactive trypsinogen [41]. To confirm the abnormal results of newborn screen in infants, sweat screen is used. This test is usually performed when an infant taste salty [9]. In general, CF screening in newborn is controversial because in some cases it gives false positive results [42, 43]. These cases involve individuals with single mutated copy of CFTR gene or in rare case with two normal copies of CFTR gene. Therefore, most countries don't screen for CF at birth. Many people are diagnosed for CF after several symptoms that need for evaluation such as GI complications and Sino pulmonary disease.

One of the most usable tests is sweat test. It involves delivery of medications that stimulate sweating (such as pilocarpine) through the skin by using iontophoresis. In this method, one electrode is placed on the medication and another electrode is placed on the skin through which an electric current is passed. After that they collect sweat by using filter paper or capillary tube then they measure the amount of sodium and chloride in their sweat. In contrast, they show fewer amounts of thiocyanate and hypothiocyanate in their mucus and saliva [44]. Moreover, the identification of mutation in the CFTR gene may also be used to diagnose patients with CF [45].

Before pregnancy, measurement of CFTR gene on one or both parents might be used to determine whether newborn child will have cystic fibrosis or not. In case of higher risk of

cystic fibrosis in newborn, a test on fetus will be done. The reason behind doing this test on one parent in the beginning is the high cost of test. In addition, for child to have CF he/she needs to get mutant copy from both parents. If they diagnosed the first parent with cystic fibrosis, the second parent will diagnose to determine the risk that their child will have. There are thousand variant mutations that cause cystic fibrosis. The most common commercially available test is looking for 32 or less different mutations. This means that not all mutation types can be determined by this test. Therefore, negatively screen result does not usually mean that the child does not have CF [46].

In order to diagnose the CF during pregnancy, CF test is usually performed either by amniocentesis (which involves drawing fluid around the fetus) or by chorionic villus sampling (which is done on placenta). Studies found that the second way has death risk for fetus which is about 1 per 100, while amniocentesis of 1 per 200 [47]. However, the recent studies demonstrated amniocentesis as 1 per 1600 [48].

2.6 Management and Treatment

About 70 years ago, there were no significant treatment for cystic fibrosis and most patients die after first year of birth. Nowadays, treatment of CF has been improved and become possible for infant with CF to live in to adulthood. The best way to treat CF is introducing a protective management for airway infection, good nutrition, and active life style. In addition, it is important to continue with pulmonary rehabilitation as a management for CF for entire life. It helps to improve organ function and quality of life. Therefore, all the directions are to slow down the decline in the organ function. Because of the large differences in the disease symptoms, the treatment is usually done at specialist multidiscipline centers and it is fit according to the patient's circumstance.

There are many targets to therapy such as lungs, reproductive organs, and gastrointestinal tract [41]. The cornerstone in CF treatment is to limit and manage the damage caused by thick and infected mucus, especially for the lung, to maintain the quality of life. For acute and chronic infection, they can be treated by different dosage forms and routes of administration of antibiotics such as oral, intravenous injection (IV), and inhalation. In order to clear out the thick mucus or even to alter its characteristics, mechanical devices and inhalation rehabilitation are used. They are extremely consuming time even though there are effective. Furthermore, an obstacle with CF patient is to find time to respond to treatment while balancing a normal life.

2.6.1 Antibiotics

Patients with cystic fibrosis are usually used one or more types antibiotics for many reasons. One of them is to prevent any infection that may happen (prophylaxis). Other conditions are significant disturbances in the lung function or expected pneumonia. There are several antibiotics that are used for treatment of patients with CF. Both sputum results and patient's previous response are put in consideration to select the type of antibiotics. These antibiotics are usually used for a long period of time. Therefore, when hospitalization is needed, some insertion techniques for prolong administration of drug(s) are used, for example, port-a-cath or peripherally inserted center catheter (PICC Line).

To maintain lung function and decrease the growth of colonized bacteria [49, 50, 51], it is important to use inhaled antibiotics therapy such as tobramycin, aztreonam, and colistin. However, there are some limitations such as resistance to an antibiotic, tinnitus, and voice change [52]. In addition, long term using of aminoglycoside, for example tobramycin, has side effects on the body and causes inner ear damage, loss of hearing, and kidney

problems [54]. Most these side effects can be prevented by continuously measuring and adjusting the blood concentration of these antibiotics. Moreover, antibiotics such as ciprofloxacin and azithromycin [53] are used orally as prophylaxis to prevent infection or to limit the existing infection.

2.6.2 Other Lung Diseases' Treatment

In order to displace sputum and urge its expectoration, several mechanical techniques are used. One of them is chest physiotherapy (CPT). It involves percussing the patient's chest by hand several times a day to clear up secretion. Therapy vest and intra pulmonary percussive ventilators (IPV) are usually used in percussive therapy. Several newly discovered methods that are designed for home use [55] such as Biphase Cuirass ventilation and the clearance device that is present in such device integrate a cough assistance phase as well as a vibration phase for removing secretion. Dornase alpha and hypertonic saline are aerosolized drugs that enhance loosen secretion [56]. Dornase is a recombinant human deoxyribonuclease. It decreases the sputum viscosity [57] through destroying its DNA. Another investigated drug that used to liquefy secretion is Denufosol [58]. It works to open an alternative chloride channels.

Moreover, bilateral positive airway pressure ventilator is a special machine that has many advantages such as pushing air to the lung in case of severe lung disease, helping maintain enough blood oxygen level during sleep, and improving sputum clearance during physical therapy [59]. Sometime, it might be important to place tube in to the throat to enable breathing that is supported by ventilator. This method is called tracheostomy and used in severe illness. For children with CF, pediatric massage can improve the quality of life [60].

2.6.3 Small Molecules

There are several approaches to develop small molecules which may help to correct the CFTR gene mutations. Three groups of small molecules compounds have been approved [65]. The first one is CFTR correctors. They enhance the transport of CFTR to the cell surface. There are three types of CFTR correctors. They include chemical chaperones (which resemble molecular chaperones), compounds that affect the level of molecular chaperones by interacting with it or binding to the defective protein (such as curcumin), and target-specific pharmacochaperones. Chemical chaperones are required in large amount because they are non-specific such as diethyl sulfate, and glycerol. VX-809 is an example of CFTR correctors. It targets the $\Delta F508$ mutation, which exists in the majority of CF patients' population [61]. A misfolding is a result of this mutation which leads to a quantitative reduction in the CFTR function due to protein destroying prior to reach cell surface. As a result, this type of small molecules is designed to enhance the CFTR correction and chloride transport. VX-661 is another CFTR corrector. It is an investigational agent which used alone or in combination with VX770 in homozygous for the $\Delta F508$ mutation. The mean reactive improvement of about 9% in FEV1 is only seen with combination (VX-661 and VX770) and they need to be in large doses verses placebo at day 28, then it will back to the baseline during a 28 days post treatment. Further clinical trials are awaited [62].

The second group is CFTR potentiator. It acts to activate the mutant CFTR by prolonging the time that CFTR channel being open. Ivacaftor is an example of CFTR potentiator. It is the first small molecule compound that was approved to target CFTR defects directly. It improves lung function, enhances weight gain, shows decline in the sweat chloride, and

improves the overall quality of life. This type is usually used for CF patients with at least one G551D mutation. Unfortunately, this type of mutation is limited and found only in 5% of cystic fibrosis population. In addition, it helps to increase the apical height of fluid and the frequency of ciliary beat. It potentiates the mutant CFTR through a phosphate dependent and not ATP-dependent manner. Moreover, most clinical studies show a significant change in FEV1 and sweat chloride one second after treatment with Ivacaftor [63].

The third group of small molecules is called CFTR suppressor. It acts through promoting the ribosomal read through of non-sense mutation (premature stop codon) [63]. Non-sense mutation is usually resulted from a premature stop codon which leads to formation of truncated CFTR protein which has no function. About 10% of have this type of mutation. In contrast, it does not work on mutation of terminal stop codons. Ataluren (formerly PTC124) is a CFTR suppressor [64]. It has finished phase 3 clinical trial. It decreases the decline in the lung function and pulmonary exacerbation. Treatment with Ataluren results in formation of full length CFTR and enhancement of chloride transport in the airway. Duchenne muscular dystrophy is another indication of Ataluren. Even though it did not show a statically significant improvement in pulmonary function, it was found to increase both the CFTR expression in respiratory epithelium and potential differences in the nasal trans-epithelium. Furthermore, gentamicin, which is an aminoglycoside, shows a promotion effect on read through effect of premature stop codons. However, it has adverse effects such as ototoxicity and nephrotoxicity when administered parenterally.

All of these groups are either used alone or in combination for treatment of CF specific patients. High throughput screening is used to recognize most of these small molecules and the one that sounds active will be further investigated.

2.6.4 Targeting-Receptors to the Respiratory Tract

There are several receptors in the respiratory system that might be used to target drug(s) to them in order to treat some respiratory diseases [66]. They include $\beta 1$ and $\beta 2$ -adrenergic, muscarinic, and leukotriene receptors.

Inhalable drug delivery systems have a high potential for targeting $\beta 1$, $\beta 2$ -adrenergic and muscarinic receptors. This type of delivery may decrease the side effects and increase efficacy of the treatment since these receptors are presented throughout the body. For leukotriene receptors, most drugs are given systemically because these receptors are mainly located in the respiratory tract so they show little side effects. $\beta 2$ adrenergic receptors are presented in airway smooth muscle, vascular smooth muscle, and sub mucosal glands [67]. In addition, both $\beta 1$, $\beta 2$ receptors are found in the alveoli walls and have different effects such as anti-inflammatory, antiedema, enhance the mucociliary clearance and lower the exudation of plasma. Muscarinic receptors are presented in the smooth muscle of airway [66]. It is used as target for inhaled anticholinergic medication to treat COPD. Epithelial cells and sub mucosal glands also possess these receptors. They produce mucus in response to acetylcholine. Airways have four types of muscarinic receptors M1, M2, M3, and M4. Mucus secretion is mediated by M1 and M3 muscarinic receptors. While M3 receptors are also responsible for bronchoconstriction and release of nitric oxide which lead to vasodilation.

The third receptor is glucocorticoid receptor. It is cytoplasmic receptor and found in all types of airway cells. In addition, it may be present in immune cells such as macrophage, eosinophil, T- cells, and dendritic cells [67]. Moreover, systemic administration of drugs targeting these receptors will lead to systemic effects. This is due to present of these receptors in many organs in the body.

2.6.5 Gene Therapy

Since cystic fibrosis is caused by CFTR gene mutation, there has been significant attempt to use gene therapy to correct the mutation at a cellular level. In this approach, a normal copy of CFTR gene will be placed in the affected epithelial cells. Regardless of the genotype, this type of therapy can be considered as treatment for all CF patients. Although theoretically it has good characteristics, it still has some challenges in the clinical application. One of these challenges is difficulties in identification of the amount of normal CFTR gene which is required to prevent lung problems in CF patients. Some studies have shown that 5% of normal CFTR expression restores approximately 50% of normal chloride channel. This means that there is no linear relationship between phenotype and genotype [68]. While for sodium transport correction, it needs about 100% of affected cells to be corrected. For other function of CFTR gene, it is less distinct how much the percentage of cells with normal CFTR function is needed. Some studies show that when CFTR is delivered to 25% of the patient cells, it will restore the normal transport of mucus. Another challenge is the technical difficulties to deliver gene therapy to the target site. This is attributed to many reasons. The innate and acquired protective mechanism in the lung affect the delivery of these therapies as a result, direct application

of vector to the target cells is required. This can be achieved by nasal infusion, endobronchial instillation, and aerosolization.

Another reason is the structural nature of the respiratory tract. The respiratory tract is provided well to prevent the drug delivery accomplished by heavy clearance by mucociliary [69]. This obstacle occurs even in healthy persons but it is more aggressive in CF patients due to thicker mucus secretion which retard the delivery of the vector to the distal airway in advance stage of cystic fibrosis. When this problem has been overcome, there are still immune barrier such as innate immunity which is accomplished by macrophages [68] and acquired hormonal immunity which is very considerable for viral vectors.

Even with these challenges, there are still efforts to develop gene therapy. Many studies have come up with preclinical and early stage of human clinical trial. These studies involved local and systemic administration of viral and non-viral vectors with both *in vitro* and *in vivo* applications. In addition, there were several studies on CF patients like phase 1 and phase 2. These approaches involve using viral and non-viral gene therapy.

2.6.5.1 Viral Gene Therapy

It has been proven that cystic fibrosis conditions [70] introduce a suitable environment for viral infection. This idea paved the way to discover more about viral gene therapy. A misfolded CFTR protein, which is caused by $\Delta F508$ mutation, will go through proteosomal degradation. Subcellular stress, inflammation, and endoplasmic reticulum expansion are combined with this degradation. Eventually, impairment of the cellular

defense mechanism against viral infection is resulted and leads to production of normal CFTR in all target cells.

There are several challenges with delivery of these vectors to the pulmonary system. The presence of vector receptors such as Cox-sackie-AVR on the basolateral side of the epithelial cell is considered the major problem [71, 72]. In addition, these receptors are found in many species but they are usually absent in human airway cells. This problem will cause lower delivery of vectors to the target cells compared with animals. Adenovirus is an example of gene delivery vectors in CF. It has some limitation such as weak transduction capacity in airway cells, promotion of a strong immunogenic response, and low penetration in the mucus tissues of lower lung when compared with nasal epithelium, and low, transient expression of the CFTR [70, 73]. Several trials by repetitive administration will only lead to increase viral antibody without increasing in the CFTR expression.

All these limitations have led to search for another viral vector. Adeno-associated virus (AAV) was the next vector which associates with less immune response. It consists of single stranded linear DNA virus which is identical to adenovirus but shows longer duration of exogenous gene expression with no pathological effect on human. Therefore, there is no need for repetitive dose administration. Multiple clinical studies in the late of 1990 to the early 2000s were used adeno-associated virus (AAV2) [74, 75]. They involve 209 patients which make these studies the best gene therapy vector studies for CF patients yet. The first study involved phase I safety studies which tested single inoculation in patient with cystic fibrosis. The results investigated that (AAV2) vectors

were generally well tolerated with consistent deletion of transfected CFTR expression and ambiguous clinical implications.

From phase I clinical trials, only two trials led to phase II studies. In phase II studies, they used repetitive administration of AAV-CFTR. There are other viruses that have been discovered as CF gene therapy vectors. They include simian vacuolating virus 40, RNA virus, and lentivirus. Simian vacuolating virus 40 is a circular DNA virus. It has ability to integrate in to the genome of the host cell. Although, it was considered a better replacement to AV, it had some limitations. The first limitation is its small size. This may cause difficulty in packaging of large CFTR transcript.

Some trials have been done in CF mouse models showed modest improvement in the weight and inflammatory biomarker. However, such studies were limited and not applied on human [76].

Another vector is RNA virus. It is negative stranded which stays in the cytoplasm without incorporating in to the host cell genome. Parainflamatory virus, respiratory syncytial virus, and sendia virus are examples on RNA vectors. Sendia virus is the only vector that has been used in animal models. It has some limitation such as short time (days) for expression and hard to repeat administration owing to neutralizing antibody response in animal models [77].

The third vector is lentivirus. It is being assessed currently as gene therapy vector in treatment patients with cystic fibrosis. It is linear RNA virus with ability to integrate in to the host cell genome without need for cell division. In addition, it shows the potential sustained CFTR expression. The problems with this virus are related to its common

pseudotype with somatovirus which has low uptake in to the apical epithelium of human airway. This problem can be solved by using an adjuvant tight junction modulator such as lysophosphatidyl chlorine. These modulators enhance the transfer in to the host progenitor cells in the deep epithelium [78, 79]. Partial_correction of chloride transport has been shown with nasal expression of the CF knockout mice. It was sustained for up to 12 months after a single dose of CFTR [80].

The following studies have demonstrated persistent luciferase receptor expression up to 18 months after transduction of sendia virus -F/HN pseudotype lentivirus following pretreatment with lysophosphatidylcholine [81]. After that some follow up studies reported that a single dose with the above regimen in mice with CF resulted in sustained luciferase activity for the life time of the mice [82]. These studies were also demonstrated that CFTR expression was increased with monthly repeated inoculation [82]. Even though there is no human trial yet, all these characteristics make lentivirus the choice for ongoing viral dependent studies in CF.

2.6.5.2 Non-Viral Gene Delivery

Non-viral gene delivery is considered as an alternative to the viruses. It consists of plasmid or carrier molecules which are (most often) designed to have a positive charge. The positive charge tends to form an electrostatic bond with a negatively charged nucleic acid. The most important advantage of non-viral vectors is the reduction in the immunomodulatory response with better tolerance for repetitive administration when compared to the viral vectors.

The delivery of particles to the nasal epithelium is the major part in most clinical and preclinical trials because they possess similar cell composition to the lower respiratory tract. In addition, they provide an easier access and safer administration through lung route [71]. However, the effective clinical achievement of therapy requires delivery to the lung which introduces additional barriers to a successful transfection.

The complex airway architecture is considered one of these barriers. Moreover, the structural difference between animals and human models in preclinical studies represents another problem. Some high order animals such as chimpanzees represent the human airway better, but these experiments will be more expensive. Most often, such studies are performed on rodent (mice and rats).

Another major barrier is the mucus nature in patients with CF. It is highly viscous and purulent. Some studies on mucus penetration demonstrated that the charged and hydrophobic molecules and nanoparticles cannot diffuse through the mucus of CF patients which result in ineffective therapy in many cases [83, 84].

Matsui et al [85] tried to detect the mechanism of internalization of liposome-DNA complexes in both differentiated and non-differentiated airway epithelial cells. This study has shown that the uptake of liposome-DNA complexes from non-differentiated cells was done via phagocyte while it was absent in differentiated cells. The possible explanation for this situation is that the non- differentiated cells tend to be more negatively charge than the differentiated cells. Furthermore, it was suggested that the entry of liposome-DNA complexes into the differentiated cells involves receptor-mediated endocytosis.

The UK cystic fibrosis Gene Therapy consortium is responsible for the biggest trial of non-viral gene therapy for patient with CF. The cationic liposomes are used to deliver a plasmid encoding CFTR protein (PGM169) [86, 87]. This plasmid is controlled by the hybrid elongation factor 1 α promoter. It also contains no CpG islands (or CG islands) so it does not cause inflammation [88]. Pringle et al [89] illustrated that the higher transfection efficacy can be achieved by incorporating of murine cytomegalovirus (CMV) enhancer in the plasmid compared with human cytomegalovirus. The CFTR gene expression was noted in a mouse model four weeks after a single dose administration.

Another study involved a pre-clinical evaluation of aerosol delivery to sheep lung. This study has shown that GL67A liposomes triggers the highest gene expression when compared with 25KDa branched polyethylene imine (PEI) and compacted DNA nanoparticle with (PEG)-substituted lysine 30-mer (90). The human clinical trials have tested PGM169/GL67A delivery system (91). Despite this system was delivered to the CF patient intra-nasally which is considered easier to administer and evaluate, it does not result in an effective treatment of cystic fibrosis. These trials have shown that CFTR protein was not express in the lung tissues which mean that there is not promising to targeted CFTR in order to improve the respiratory symptoms. In addition, such penetrate the purulent mucus to go to epithelial cells. targeted system does not have to pass the complicated respiratory architecture and

One application for such system is nanoparticle delivery system. It is designed by Mannuta et al [92]. It consists of cationic liposomes DHDTMA/DOPE, plasmid DNA, and targeted peptide. The sequence of the targeted peptide was K16GACSERSMNFCG. It had similar receptor binding activity as rhinovirus and Listeria monocyte genes. The

K16 helps DNA packaging and the SERSMNF motif is similar to the intracellular adhesion molecule 1 sequence of rhinovirus. This nanoparticle delivery complex was 150 nm in size and had zeta potential of +50 mV. Different nebulizers were used to deliver nanocomplex with no effect on physico-chemical properties of the system. β -Galactosidase was used as a gene reporter and there was no statically significant difference between control group and treatment.

Another approach used compacted plasmid DNA nanoparticles with PEG-substituted lysine 30-mer peptide to act as a vector. One of three doses (0.8mg, 2.67mg or 8mg of DNA) was administered by twelve patients to the nasal epithelium. CFTR restoration was evaluated by measuring the potential difference in the nasal epithelium. The results showed that eight out of twelve patients with CF were evaluated with partial or complete restoration; while the effect proceeded for only six days [93].

Through the past couple decades; the area of drug delivery has been developed with discovery of nanoparticles. They are an inert carrier for drug and gene to the site of action such as cells or tissues [94]. Although many research groups have studied the properties of nanoparticles for inhalation, they are still not being applied for clinical use for CF treatment. The main reason is lack of an effective treatment for CF.

Once gene therapy has been discovered, most application focused on viral vectors as gene delivery carriers. Based on *in vitro* experiments, most of non-viral vectors exhibited lower transfection efficacy compared with viral vectors.

Moreover, the tendency of conventional nanoparticles to be trapped in the mucus and rapidly cleared from it by mucociliary action affects their efficacy [95]. For this reason,

mucoadhesion is important in numerous applications of drug delivery. It can be achieved by different methods. Various non-specific forces such as hydrogen bond, van der Waals forces, and hydrophobic or electrostatic interaction forces produce the adhesion between mucus and particles. Experiments have shown that the retention time of nanoparticles in the mucus may be increased by using cationic surface charge of polymer [96, 97].

Targeted mucoadhesive drug delivery can be accomplished by using tomato lectin which specifically binds the N-acetylglucosamine on the cell surface [98]. For patients with CF, it is still unknown whether these mucoadhesive particles can improve therapy. This is due to inability of nanoparticles to penetrate mucus and enter the underlying the epithelium **Figure 2.2**. Therefore, they are considered unsuitable for drug or gene delivery into the lung of patients with CF [95]. As a result, mucoadhesive properties may be unhelpful in case of CF. An increasing in the viscosity of mucus is one of the main characteristics of lung disease. Besides that, there is a gradual accumulation of purulent sputum accompanied with obstruction due to decrease clearance.

Some researchers have found the correlation between physical properties of particles and the ability to penetrate sputum of CF patients. It was found that neutrally charged polystyrene particles with diameter less than 200 nm have ability to transport faster in the sputum of CF patients compared with charged particle [99]. Recombinant human DNase (pulmozyme[®]) is used to reduce the macroscopic elasticity and viscosity of sputum by 50% and 40 %, respectively. It resulted in more homogeneous diffusion of particles with no effect on the mean diffusion rate of the particles. This is probably due to DNA fragments which may have increase micro viscosity of the sputum.

Development of mucoinert particles seems to be more beneficial to penetrate CF sputum and enter the target epithelial cells [100]. The average mesh spacing in CF sputum was designed by Suk et al [100] to be 140 ± 50 nm with a range of 60- 300 nm. They also established that particles diameter up to 200 nm, which densely coated with low molecular weight PEG, can pass the CF sputum up to 90-fold faster than the uncoated one with same the size **Figure 2.3** [101].

Another study has examined the transportation of nanoparticles after treating the sputum of CF patients with N-acetyl cysteine (NAC) [101]. The authors found that the NAC resulted in increasing the average spacing between the sputum mesh elements from 145 ± 50 nm to 230 ± 50 nm **Figure 2.4**. As a result, the transport of densely coated particles with a diameter of 200 nm in CF sputum became near their theoretic speed in water. When NAC was combined with mucoadhesive cationic liposome and polymers for gene therapy, there was no obvious correlation of nasal transepithial potential differences in CF-null mice [95].

Typically, nanoparticles are coated with PEG in order to increase the biocompatibility and hydrophilicity. In addition, the transport of nanoparticles through the mucus is largely affected by the PEG coating. Lai et al [102] evaluated the coating of polystyrene nanoparticles with either 2 kDa PEG or 10 kDa PEG. It was found that the neutral charge of particles with relatively fast transport through mucus was resulted from high density coating with 2 kDa PEG. While the higher density coating with 10 kDa PEG caused an increase in the adhesion of nanoparticles to the mucus. Further study was done on low density coating with 2 kDa PEG to test the role of coating density. This type of coating produced mucoadhesive particles with a cationic zeta potential (-10 ± 3 mV).

Small interfering RNA (siRNA) may offer an option to protect the mutant CFTR from proteosomal degradation [103]. Because of both delivery of siRNA to the target cells has many barriers and the proteosomal degradation is one of the normal cellular functions, local delivery or use of target moiety is required to reduce the off-target effect of siRNA therapy.

Some investigations performed to use small molecules to protect the mutant CFTR from proteosomal degradation. One of these small molecules is curcumin [104]. It acts to inhibit the sarco (endo) plasmic reticulum calcium ATPase (SERCA) pump. SERCA inhibition will cause mutant CFTR to escape from endoplasmic reticulum and possibly reach the membrane.

PLGA nanoparticles are designed to deliver curcumin, enhance its absorption and reduce metabolism. They were designed with a diameter of 77 ± 16 nm and drug loading capacity of 7.6 % w/w. It was shown that after an oral administration the curcumin nanoparticles conjugate had higher efficacy than free curcumin.

Two-fold decrease in proteosomal activity following by control of inflammation caused by pseudomonas lipopolysaccharides were resulted after intranasal administration of encapsulated Bortezumab in PLGA/ PEG matrix by mice with CF [105]. Bortezumab (ps-341; Velcade[®]) is a proteosomal inhibitor approved by FDA to treat refractory multiple myeloma. Regarding the treatment with Bortezumab, normal cells overcome proteosomal inhibition while cancer cells will be subjected to apoptosis.

Another technique is using stem cells. Stem cells act as an option to restore CFTR function. This approach involves the use of early progenitor or bone marrow derived cells

in which the CFTR function has been restored and replaced to the host either by localized intratracheal or systemic delivery. It is only starting to emerge. However, there are some challenges that have recently been identified [106,107]. The low efficiency of uptake raises a question whether there is significant correction after treatment with this technique.

2.6.6 CXCR2 Antagonist SB-656933 in Patients with Cystic Fibrosis

Moss et al [108] hypothesized that a specific inhibition of recruitment of neutrophils and other inflammatory cells can be achieved by using CXCR2 receptor antagonist. CXCR2 receptor is located on neutrophils, subset of T-cell, dendritic cells, macrophage, and mast cells. SB-656933 is a selective CXCR2 antagonist. It inhibits the neutrophils recruitment but not microbial killing [109] and this will potentially restore the balance between potent defense and tissue damage mediated by neutrophil products.

SB-656933 was well tolerated after 28 days dosing with 20mg and 50mg. Active treatments did not show specific adverse effect such as changing sputum biology, exacerbation, or peripheral blood neutropenia. Although the drug concentration in serum was lower than that expected for patients receiving higher dose (50 mg), the extensive biomarker sampling showed reduction in sputum neutrophil activation. However, lower dose (20 mg) showed the opposite effect. In addition, a more consistent reduction in sputum neutrophil elastase and myeloperoxidase was seen in patient taking 50 mg of SB-656933.

2.6.7 Targeting miRNA-Based Medicine to Airway Epithelial Cells using Nanotechnology

Micro RNA (miRNAs) are endogenous small molecules. They act on messenger (m) RNA in the level of post transcription. There is a thought that an alteration in the expression of miRNA is associated with cystic fibrosis phenotype. McKiernan et al [110] demonstrated that modulation of miRNA by replacement with miRNA mimics is considered a new therapy for patients with cystic fibrosis. However, there are some obstacles such as difficulty of safe and effective delivery to the epithelium of CF patients.

Polymeric nanoparticles such as Herein were used for delivery of miRNA mimics to the CF airway cells. Polymers such as Polyethylenimine (PEI) and chitosan were used to prepare nanoparticles [111]. Studies have shown that the size of PEI-miRNA complex is buffer-dependent and it can be significantly reduced by preparing the complex in 5% w/v glucose solution rather than PBS **Figure 2.5** [110].

Like other nucleic acid such as plasmid DNA and siRNA, PEI polymer appears to be effective as a complexing agent for miRNA mimics. It makes complex with miRNA mimics at low N/P ratio to produce small, cationic nanoparticle. While for Herein nanoparticles prepared from chitosan-miRNA, it was found that they have slightly larger size than chitosan-siRNA polyplexes [112]. Therefore, a cross linker is utilized to reduce the size of miRNA nanoparticles and modify the intracellular distribution of miRNA in Cystic Fibrosis bronchial Epithelial (CFBE) cells **Figure 2.6** [110].

High content analysis indicated that the delivery of miRNA mimics-PEI nanoparticles to CFBE cells was twice more effective than transfection by RiboJuice (it is a transfection agent usually use in molecular biology applications).

Additionally, PEI-miRNA nanomedicine was found to be more efficient than chitosan-miRNA formulation for two reasons. The first one is the higher binding capacity of miRNA mimics to PEI than chitosan. Another reason is the highly cationic nature that helps to bind with cell membrane and consequently improves transfection. Local delivery of miRNA to the lung of patients with CF is considered one of the successful future approaches to introduce miRNA to the clinic.

Although inhalation route provides a specific tissue targeting for miRNA and reduces the systemic exposure, both anatomic and pathologic barriers limit inhaled nanomedicine therapy. For example, obstructed airway covered with thickened secretion and mucous plug prevent the delivery of these formulation to the target cells. In order to develop local aerosolized delivery to lung with CF, researchers should evaluate the efficacy in mucus-producing interface culture, CF airway epithelial culture, and then in animal models [110].

3 SPECIFIC AIMS

Specific Aim 1: To compare the activity of drugs lumacaftor, ivacaftor and their combination in the correction of the defect in the CFTR protein

The goal of this investigation is to reveal the role of lumacaftor and/or ivacaftor in the delF508 CFTR repairing process. Lumacaftor is supposed to correct the defect in the delF508 CFTR folding thereby enhance the trafficking of them to the cell membrane and eventually improve chloride efflux throughout the cell membrane of CFBE41o- cells. Since the CFBE41o- cells have a homozygous delF508/ delF508 mutation, they characterize by little to no CFTR on cell membrane. Therefore, this specific aim is designed to test the activity of ivacaftor as potentiator. We expect that this drug should work on improving CFTR channels opening probability when they are present on the cell membrane. Further, this study is aimed at comparing the activity of each drug separately and in combination.

Specific Aim 2: To investigate the efficacy of PEGylated nanostructured lipid carrier and PEGylated liposomes as a nanocarrier for treatment of pulmonary cystic fibrosis

Nanostructured lipid carrier drug delivery system will be developed and loaded with both lumacaftor and ivacaftor. The investigations related to this specific aim are designed to test the possibility of delivering these drugs *in vitro*. The advantage of using PEG-NLC is to increase the biocompatibility and hydrophilicity. In addition, the transport of nanoparticles through the mucus is largely affected by the PEG coating. Further, *in vitro* test of activity will be done to assure that there is no NLC interference with drugs

activity. Another form of nanocarrier is liposome which will be examined for particle size, zeta potential, and cellular internalization.

4 MATERIALS AND METHODS

4.1 Materials

Lumacaftor (VX-809) and Ivacaftor (VX-770) were purchased from selleckchem®, collagen, bovine type1 (corning®, Bedford, MA), Fibronectin were obtained from Life Technology). MQAE dye was purchased from Life technology (Eugene, OR, USA). SYBR® green PCR master mix was ordered from (Applied Biosystems, Warrington, UK). PCR custom primers hQCF3 GACAGTTGTTGGCGGTTGCT (sense) and hQCF4 ACCCTCTGAAGGCTCCAGTTC (Antisense) were synthesized by Invitrogen (Carlsbad, CA). Procirol AT05 (glycerol distearate) GATTEFOSSE®, sequalene (Sigma, Louis, MO), span 85 (sigma, Louis, MO), tween 80 and polyethylene glycol (PEG 2000) (Avanti Polar Lipid, Inc., Alabaster USA). Egg phosphatidylcholine (EPC), Cholesterol and 1,2,-distearoyl-sn-glycero-3-phosphoethanolamine-N-aminopolyethelenglycol – Mw - 2000 ammonium salt (DSPE-PEG, and Egg phosphatidylcholine labeled with Rhodamine were purchased from Avanti Polar Lipids, Alabaster, AL.

4.2 Cell Culture

16HBE41o- and CFBE41o- cells were kindly provided by Dr. Dieter C. Gruenert (California Pacific Medical Center Research Institute, San Francisco, CA) CFBE41o- cells were produced by transformation of bronchial epithelial cells, obtained from a CF patient, with SV40 large T antigen via the replication defective pSVori- plasmid. Cells

were grown in fibronectin-coated flasks (25 cm² and 75 cm² in surface area (Coaster®, Corning, Germany) at a density of 2×10^5 cells per cm² with Minimum Essential Medium w/ Earle's salt (EMEM supplemented with 10% Fetal bovine serum FBS, 2 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin G), at 37 °C in a 5% CO₂ incubator. Flasks were coated with the fibronectin solution that contains Bovine serum albumin, Collagen I, bovine) and Human fibronectin. The culture medium was changed every other day. The passage percentage was 1:10 for one-week passage. 16HBE14o- cells (used as control for CFBE41o- cells) were cultured as previously reported [113]. Briefly, cells were seeded in coated flasks with condition similar to CFBE41o- cells. However, passages of 2.56 to 2.63 were used in this study and the culture medium was changed every 2 days.

4.3 Cytotoxicity Assay

The effect of drugs on the viability of CFBE41o- cells was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay [114]. The cells were plated at 3×10^3 cells per well in 200 µL complete medium containing different concentrations of (vx-809, vx-770, combination, or combination PEG-NLC) in a 96-well plate, and each concentration of drug (24, 12, 6, 3, 1.5, 0.75, 0.375, 0.1875, 0.09375, 0.046875, 0.0234375, and 0.01171875) was repeated in 3 wells. The cells were incubated for 48 hours at 37 °C in an incubator. After that, MTT reagent (20 µL, 5 mg/mL in PBS) was added to each well and incubated for 3 hours. Then, the solubilization solution was added and incubated overnight. An equivalent volume of fresh medium was added to the control cells. Absorbance was recorded on a microplate reader at 540 nm wavelength.

The relative cell viability (%) was expressed as a percentage relative to the untreated control cells.

4.4 Chloride Efflux Measurements

CFBE41o⁻ cells were plated with density of 25×10^3 cells/well in a 96 well plate with clear bottom (Coaster®, Corning, Germany) for 24 hours [115,116,117]. Cells were then treated with different concentrations of lumacaftor alone (0.1 μ M, 3 μ M, 8 μ M, 10 μ M), ivacaftor alone (0.1 μ M, 3 μ M, 8 μ M, 10 μ M), a combination of both (0.1 μ M, 3 μ M, 8 μ M, 10 μ M), and drugs-PEG-NLC (3 μ M). After 48 hours, all cells were loaded with 10 mM MQAE for 2 h, then cells were washed with a chloride buffer containing of 140 mM NaCl, 5 mM HEPES, 5 mM KCl, 1 mM MgCl₂, 5 mM glucose, and 1 mM MgCl₂ pH 7.4, incubated with this buffer for 1 hour at 37 °C with 5% CO₂ followed by exposure to a chloride-free buffer of similar composition (NO₃ as the substituting anion). After that, cells were exposed for 10 minutes to chloride-free buffer containing 100 mM IBMX and 5 mM forskolin (Sigma, Louis, MO). Fluorescence was recorded with a Cary Eclipse Varia spectrofluorometer using 360 nm (bandwidth 10 nm) as excitation wavelength and 450 nm (bandwidth 10 nm) as emission wavelength. All measurements were done in triplicate using the untreated CFBE41o⁻ cells as a control.

4.5 RNA Extraction

CFBE41o⁻ and HBE41o⁻ cell lines were grown on fibronectin-coated flasks. RNA was extracted from confluent cells using the RNeasy mini kit (Qiagen, Valencia, CA). The concentration and quantity of RNA were assessed using absorbance multi-mode microplate reader (Tecan Trading AG, Switzerland) with the absorbance at 260 nm. After

that, the RNA was reverse-transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK). Three cDNA samples from separate RNA extractions and reverse transcription reactions were used for each cell line.

4.6 Gene Expression (Real-Time Quantitative Polymerase Chain Reaction)

Total RNA was extracted with the RNeasy Mini Kit. The mRNA was reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK). Quantitative RT-PCR was performed with an Applied Biosystems StepOne™ Real-Time PCR System and SYBR Green PCR master mix (Applied Biosystems, Warrington, UK). All samples were run in triplicate. The amplification was done as follows: an initial step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec. and 60 °C for 60 sec. The $2^{-\Delta\Delta CT}$ method was used to calculate the amount of gene expression [118]. CFTR mRNA expression was normalized to the parallel measured endogenous controls GAPDH in each cell line. CFTR expression value of 16HBE14o-cells was defined as 1 and CFTR expression of CFBE41o- cells were normalized to this value. The sequences of primers were: GAPDH ATCGAAATCCCATCACCATCTT (sense), CGCCCCACTTGATTTTGG (antisense) and hQCF3 GACAGTTGTTGGCGGTTGCT (sense), ACCCTCTGAAGGCTCCAGTTC (antisense) [119]. The comparative Ct method was used to calculate the relative amounts of mRNA.

4.7 Protein Extraction

16HBE14o- and CFBE41o- cells grown in fibronectin-coated flasks until confluent, then trypsinized and after centrifugation (500 g for 5 min), cells were lysed in 55 µL lysis buffer (TRIS 1 mM, NaCl 15 mM, EDTA 0.2 mM, 2% Triton X-100) and protease inhibitor cocktail 1% (Sigma, Louis, MO) then placed on ice for 30 min [120]. After that,

the contents were centrifuged for 5 min ($14,000 \times g$) at 4 °C to remove nonsoluble material, and then the pellet was discarded and the supernatant were transferred to other tubes. The protein concentration was quantified by the Bradford method.

4.8 Western Blotting

For the detection of protein, an aliquot of 25 µg of protein was diluted in Laemmli buffer (Sigma, Louis, MO) and separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% acryl amide) mini-protean® TGX™ (Bio-Rad, Hercules, CA) [120,121,122]. The separated proteins were transferred to polyvinylidene membrane (0.45µm pore size). A tank blot aperture (Mini Trans-Blot® Electrophoretic Transfer Cell; Bio-Rad, Hercules, CA) was used to transfer proteins to a PVD membrane at 25 V on ice overnight. Next day, blocking of nonspecific binding sites were done for 2 hours with 5% nonfat dry milk in Trisbuffered saline-Tween (TBST: 10 mM Tris HCl, pH 7.4; 0.1% Tween 20; 140 mM NaCl) at room temperature. A monoclonal mouse anti-human and murine CFTR primary antibody Pierce™ (CF3) (Thermoscientific Fischer, IL, USA), diluted in 5% nonfat dry milk/TBST was used to detect the CFTR at room temperature for 1 h. After washing with TBST 2-3 times, the membrane was incubated with the affinity purified goat anti-mouse immunoglobulin (Ig)G conjugated with alkaline phosphatase (Dianova, Germany) diluted in 5% nonfat dry milk/TBST 1:10 000 for 1 h at room temperature. After washing the membrane again with SigmaFast NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Sigma, Chemical Co, St. Louis, MO) were used for detection. ImageJ, version 1.41 software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) was applied for densitometric evaluation of the intensity of the CFTR bands. Further, protein extract from nontreated

CFBE41o– cells and 16HBE14o– cells were used as control in these experiments. To assure comparable protein amount and expression, anti- α -tubulin mouse IgG1 monoclonal (Tubulin alpha, Life Technologies, Eugene, OR) use for normalization of the Western blot data.

4.9 Nanostructured Lipid Carrier (NLC) Preparation and Characterization

4.9.1 NLC Preparation

In this study, the drugs–PEG–NLC was prepared by emulsion-evaporation and low temperature-solidification method [123]. Two phases the lipid and aqueous phases were prepared separately. The aqueous phase consists of 65mg tween 80, 150mg span 85, 30mg DSPE PEG, 75 mg span 80 and 7ml of deionized water. The lipid phase consists of 50 mg squalene, 50mg Procirol, 100ul of lumacaftor (25mg/ml), 100ul of ivacaftor (25mg/ml) and 800ul ethanol. After that, both aqueous and lipid phase were allowed to warm up at 67°C for 30 minutes on dry bath get the homogeneous emulsion. The homogenization of particles has be done using Bio-Gen pro200 (Proscientific, USA). During this process, the lipid phase has been added to the aqueous phase. This homogenization step continued for 5 minutes at 9000rpm followed by 5 minutes at 12000 rpm. After that, the hot pre-emulsion was pulse sonicated for 10 minutes with 50% amplitude using Fisher Scientific™ Model 120 Sonic Dismembrator. Then the emulsion was cooled on ice bath for 90 minutes, and then the dispersion of drugs–PEG–NLC is obtained.

4.9.2 Particle Size Distribution

Particle size was determined at room temperature by dynamic light-scattering (DLS) using 90 Plus Particle Sizer Analyzer (Brookhaven Instruments Corp., New York, NY) [123]. Aliquot of 40 μL of each sample was diluted in 2 mL of its external buffer.

4.9.3 Zeta Potential Measurement

Zeta potential was determined on PALS Zeta Potential Analyzer (Brookhaven Instruments Corp, New York, NY) [123]. It measures the electrophoretic mobility that reflects the electric charge on the particle surface. The strength of the applied field was 20 V cm^{-1} and zeta potential values were recorded automatically by Zetasizer. All measurements were done in triplicate, and average values were calculated.

4.9.4 Determination of entrapment efficiency and drug loading

The drug loading capacity and entrapment efficiency of the NLC formulation was performed by measuring the amount of the un-entrapped drug by using Amicon ultra centrifugal filter units (Sigma–Aldrich Ultra-15, MWCO 10 KDa) then was quantified using High performance liquid chromatography (HPLC) [124]. For this, 1 mL of the NLC formulation was diluted with 2 mL of diluents to allow the un-loaded drug particles to dissolve. The diluted sample was kept in the upper compartment of the ultra-centrifuge tube and centrifuged at 8,000 rpm for 20 min. The free drug in the aqueous phase moves through the semi permeable filter membrane to the lower chamber, whereas the drugs entrapped within the nanoparticle retained in the upper chamber. The flow thorough in the lower chamber was measured by HPLC with an autosampler, dual wavelength absorbance detector with wavelength set to 229 nm and controller, all of these parts were

obtained from Waters Co. A column with 125 mm 4 mm Lichrosphere 100 RP-18 was obtained from Merck, used to identify and quantify the concentration of lumacaftor and ivacaftor. The mobile phase was composed of 50% acetonitrile and 50% D.I. water eluted isocratically throughout the measurement. A sample was dissolved in acetonitrile before injection into a 20 L sample loop. Drug incorporation efficiency in this study were representing the retention of both drugs in the filtered NLC with respect to the originally added drugs. The retention time of lumacaftor and ivacaftor are 4.43 and 4.56 minutes respectively. The flow rate was kept at 0.5 mL/min. The NLC were lyophilized and stored at 4°C for further shelf stability test. The entrapment efficiency and drug loading was calculated by the following equations.

$EE\% = \frac{w_1 - w_2}{w_2} * 100\%$
$DL\% = \frac{w_1 - w_2}{w_3} * 100\%$

Where the EE%: entrapment efficiency, DL%: drug loading, w_1 = amount of drug added in the NLC, w_2 = amount of un-entrapped drug, w_3 = amount of the lipids added.

4.10 Liposome Preparation and Characterization

4.10.1 Liposome Preparation

PEGylated liposomes were prepared using the procedure published in [125, 126]. Briefly, lipids: EPC, Cholesterol and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-aminopolyethelenglycol – Mw - 2000 ammonium salt (DSPE-PEG) were dissolved in chloroform, evaporated to a thin film layer using rotary evaporator Rotavapor® R-210/R-215 (BUCHI Corp., New Castle, DE, USA) and rehydrated with 0.9 % NaCl to final lipid concentration 20 mM. In order to prepare fluorescently labeled liposomes, 5 mg of EPC labeled with Rhodamine were added to the lipids mixture. The lipid mole ratio for this formulation was 51:44:5 EPC: Chol: DSPE-PEG respectively. Liposomes were stored at room temperature for an hour followed by extrusion through polycarbonate membranes 200 nm and 100 nm using the extruder device (Northern Lipids Inc., Vancouver, BC, Canada).

4.10.2 Liposome Particle Size measurement

Particle size distribution was determined by dynamic light-scattering (DLS) at room temperature) using 90 Plus Particle Sizer Analyzer (Brookhaven Instruments Corp., New York, NY).

4.10.3 Zeta Potential Measurement

Zeta potential was determined by Folded Capillary Zeta Cellon at PALS Zeta Potential Analyzer (Brookhaven Instruments Corp, New York, NY).

4.11 Microscopic Technique

CFBE41o⁻ cell lines were plated with density of 2×10^3 cell/ well in 6 wells plate for 24 hours. After that, media were removed and replace by fresh media with 100 μ l PE-Lissamine-Rhodamine B dye (red fluorescence)-labeled liposome and incubated for 24 hours. cells were then washed with PBS buffer 2-3 times and incubated for 20 minutes with fluorescent stain DAPI (4',6-diamidino-2-phenylindole) 1:10000 (Invitrogen, Carlsbad, CA). After washing with PBS buffer, imaging was captured using an Olympus IX170 inverted epifluorescence microscope equipped with step motor, 83000 filter set (Olympus America Inc., Melville, NY), filter wheel assembly (Ludl Electronics Products, Hawthorne, NY), and SenSys-cooled charge-coupled high-resolution camera (Photometrics, Tucson, AZ). Partial deconvolution of images was performed using DP Controller software (Scanalytics, Fairfax, VA).

4.12 Statistical Analysis

Data were analyzed using appropriate a paired Student's t test or single-factor analysis of variance (ANOVA), and shown as mean values \pm standard deviation (SD). The difference between variants was considered significant if $P < 0.05$.

5 RESULTS

5.1 *In Vitro* Cytotoxicity Assay

In vitro Cytotoxicity of drugs (Lumacaftor alone, Ivacaftor alone, combination, and drugs-PEG-NLC) was selected in the current study to evaluate the influence of these drugs (whether alone, in combination, or drugs-PEG-NLC) on CFBE41o- human lung cystic fibrosis cell viability [63]. **Figure 5.1** shows the average data obtained in three

independent experiments with an increasing concentration of drugs. For VX-809 alone, there was (65.9, 67.2, and 70.9) % cellular viability with drug concentrations of (24, 12, and 6) μM respectively. Lesser cytotoxicity was registered under lower concentrations ($\leq 3 \mu\text{M}$) where 80% or more cells were viable. VX-770 alone showed slightly lower cytotoxicity when compared with VX-809 alone for most measured concentrations. It should be stressed that a combination of both drugs (VX-809 and VX-770) demonstrated slightly high cytotoxicity when compared with each drug applied alone. On other hand, drugs-PEG-NLC demonstrated a slight decrease in cellular viability in compare with plain combination of both drugs.

5.2 Fluorescence Measurements of Chloride Efflux

After incubation of CFBE41o- cells, which are homozygous for ΔF508 mutation, with lumacaftor alone, ivacaftor alone, combination of both, or drugs-PEG-NLC for 48hrs, the chloride efflux was measured as a relative change in fluorescence ($F_{\text{Relative Fluorescence}}$) of the chloride-sensitive dye MQAE N-(ethoxycarbonylmethyl)-6-methoxyquino-linium bromide:

$$F_{\text{Relative Fluorescence}} = \Delta \left(\frac{F}{F_0} \right)$$

where F denotes measured fluorescence value and F_0 – a level of fluorescence in the untreated cells(control).

Cells treated with lumacaftor alone (3 μM) showed an increase in chloride efflux (2.97 ± 0.3) comparing to the control **Figure 5.2A**. Further increase in drug concentration (8 μM) resulted in a higher chloride efflux (3.27 ± 0.28 , $P < 0.05$ when compared with control).

The cells that treated with ivacaftor alone (3 μ M, and 8 μ M) showed a slight increase in chloride transport throughout the cell membrane when compared with lumacaftor alone (1.29 ± 0.19), and (1.38 ± 0.22) respectively ($P < 0.05$). The highest chloride efflux has been found in cells treated with both drugs at concentrations 3 μ M, and 8 μ M (3.85 ± 0.34 and 4.16 ± 0.35) respectively. Further, all measurements with drug concentrations ≥ 10 μ M did not show a superior effect in chloride transport activity, instead it showed a state of little or no change (plateau). Regarding cells treated with drugs-PEG-NLC (3 μ M), there is a slight improvement (4.25 ± 0.342) in chloride efflux comparing to the drugs combination (3.87 ± 0.323) **Figure 5.2B**.

5.3 Analysis of CFTR mRNA Expression by Real-Time Quantitative Polymerase Chain Reaction (RT-QPCR)

Real-time PCR was used to analyze the expression of mRNA in both 16HBE14o- and CFBE41o- cell lines. The level of CFTR mRNA in all cell lines was quantified. The 16HBE14o- CFTR expression value was defined as 1 and the CFBE41o- CFTR expression then was normalized to this value. CFTR mRNA levels of the CFBE41o- cell lines were expressed as a fold change (-5.9 ± 0.51) relative to native CFTR mRNA 16HBE14o- cells (1 ± 0.19) **Figure 5.3**. Lower CFTR mRNA expression was observed in CFBE41o- cells when compared with normal cells.

5.4 Expression of CFTR Protein by Western Blot

Results from immunoblot of normal cells (16HBE41o-) showed an intense band with molecular weight 180 KDa which is denoted as “Band C” **Fig 5.4A**. The immunoblot for CFBE41o- cells expressing F508del-CFTR showed less intense band with a molecular weight 150 KDa which is denoted as “Band B”. Regarding CFBE41o- cells treated with

VX-809 alone, a mature form of CFTR protein (Band C) could be detected with ratio of 1:1 compared to untreated CF cells **Fig 5.4B**. In contrast, CFBE41o- cells treated with VX-770 alone showed only the immature form of protein (Band B). Immunoblot for CFBE41o- cells treated with both drugs appeared to have similar outcomes to that treated with VX-809 alone with 1:1 ratio compared to untreated CF cells.

5.5 NLC Characterization

The average particle size of drugs-PEG-NLC was 128.04 ± 1.58 (mean \pm SD) nm **Figure 5.5A**. The zeta potential was -46.5 ± 8.05 (mean \pm SD) mV **Figure 5.5B**. The entrapment of lumacaftor and ivacaftor within the PEG-NLC was quantified. From the HPLC calibration curve, our formulation was able to entrap 10 mg of drugs used in the synthesis yielding an entrapment efficiency of 100% **Figure 5.5C**. The drug loading percentage was 2%.

The main advantage of entrapping drugs-PEG-NLC is that muco-inert nanocarrier will be more beneficial to penetrate CF sputum and enter the target epithelial cells. In addition, the coating of NLC particles with PEG has an important role in increasing the biocompatibility and hydrophilicity of this formulation. Together with inhalation, they may enhance the accumulation of drugs in the lung tissue with cystic fibrosis.

5.6 Liposome Characterization

The average size of liposomes after extrusion was $(153 \pm 36.9 \text{ nm})$ **Figure 5.6**. Liposomes had a slight negative Zeta potential $(-10 \pm 2 \text{ mV})$ and can be considered as “neutral”.

5.7 Cellular Internalization of Liposomes

Experiments carried out using confocal microscope demonstrated that fluorescently labeled by PE-Lissamine-Rhodamine B dye (red fluorescence) liposomes were efficiently internalized into the bronchial epithelial cells CFBE41o- cells **Figure 5.7**. The quantitative evaluation of liposome internalization efficiency was based on the emission intensity. Cell nuclei were stained with nuclear-specific dye (DAPI, blue fluorescence). Superimposition of images allows for detecting of cytoplasmic localization of liposomes. Overall, the result is consistent with the ability of the liposomes to be internalized inside the cell.

6 DISCUSSION

Results from *in vitro* cytotoxicity assay showed a considerable CFBE41o- cytotoxicity (vx-809 alone, vx-770 alone, or in combination) with concentrations of more than 3 μ M **Fig 5.1**. The higher concentrations of vx-809 or vx-770 (24, 12, 6 μ M) decreased cell viability down to 60-70% from the control. The cytotoxicity appeared to decrease with concentrations of ≤ 3 μ M where the cell viability appears to be 80-90% when compared with control (untreated cells). This cytotoxicity appears to be related to the drugs themselves and increases with increasing drug concentrations. Drugs-PEG-NLC, showed slightly lower cellular viability to combined drugs without NLC formulation. This indicates that NLC has no significant cytotoxic effect on CFBE41o- cell viability.

CF patients with class II mutation (F508del) have been shown to have abnormally low to no CFTR protein levels on their cell membrane [127]. This gave rise to the idea to test the effect of lumacaftor (VX809) on CFTR and chloride transport in airway epithelial cells. Recently, VX809 was found to enhance the maturation of Δ F508-CFTR [128] and to increase chloride efflux from Δ F508-CF airway epithelial cells [129]. The mechanism by which VX-809 Δ F508-CFTR maturation, and consequently, CFTR-mediated chloride efflux appears due to a partial correction of F508del-CFTR folding by altering the conformation of MSD1 [130]. Several evidences have been shown to support this mechanism [130]: A) VX-809 biochemically stabilizes CFTR fragments that contain only MSD1. B) VX-809 alters the conformation of MSD1 to protect it from proteolytic digestion with trypsin. C) CFTR fragments fold to a conformation that is stabilized by VX-809 after biosynthesis of amino acid F374. F374 is found in the linker between MSD1 and NBD1 and enhances stabilization of MSD1 in conformation that is sensitive to this small molecule.

Data from Cl^- efflux assay **Fig 5.2A** showed that VX-809 was able to correct the F508del-CFTR and partially restored CFTR function in CFBE41o-cells which are homozygous for F508del-CFTR. Although treatment with VX-809 could help in a partial correction of Δ F508-CFTR and enhance moving CFTR channel to cell membrane, these channels may be unable to open properly and that in part due to the Δ F508-CFTR mutation itself which may cause a defect in chloride channel activity [131]. Therefore, the ability of VX-809 to correct F508del-CFTR is enhanced when the drug is combined with agents that improve the channel open probability. These agents act as CFTR potentiators that increase the flow of ions throughout CFTR channels. In the present

study, Ivacaftor (VX-770) was selected as a potentiator for further increasing in F508del-CFTR-mediated chloride transport. For VX-770 to act, CFTR must be at the cell surface [132, 133]. Data showed that combination of VX-770 with VX-809 caused further increase in Cl^- transport across cell membrane which is consistent with the hypothesis that a potentiator may enhance the channel gating activity of VX-809-corrected F508del-CFTR [134]. However, CFBE41o- cells treated with VX-770 alone showed only a slight enhancement in Cl^- transport across cell membrane. This might be caused in part by the fact that cells with this mutation (F508del) are characterized by little to no CFTR channel on cell membrane. Therefore, treatment with ivacaftor alone could enhance the channels that are on cell membrane without need for correctors. Furthermore, measurement with higher concentrations ($\geq 10 \mu\text{M}$) did not show further improvement in Cl^- transport possible because the all available channels were already involved in the reaction and there were no unaffected CFTR channels left. Overall, this *in vitro* experiment presented here suggests that combination of VX-770 with VX-809 further enhance F508del-CFTR-mediated chloride transport.

Regarding cells treated with drugs-PEG-NLC, a further improvement in ion transport was found with $3 \mu\text{M}$ concentration compared to drugs combination with same concentration **Fig 5.2B**. This in part may be due to the ability of PEG-NLC to penetrate the mucus, and enhance drug delivery through the cell membrane of CFBE41O-bronchial epithelial cells. In addition, NLC obtained in this study could potentially be exploited as a carrier with improved drug loading capacity and controlled drug release properties.

It is important to stress that the results of MTT assay showed a lesser cytotoxic effect with 3 μ M concentration, and the results from chloride measurement showed an increase in chloride efflux comparing to control at that concentration, accordingly a concentration of 3 μ M of both drugs had been chosen and loaded in PEG-NLC.

Results of Real-Time Quantitative Polymerase Chain Reaction (RT-QPCR) showed that the 16HBE41o-cells characterized by higher CFTR-mRNA expression than the CFBE41o-cells. This indicates that cystic fibrosis cells have a suppressed CFTR channels and, as a result, affected Cl^- transport which might be effectively improved by the studied drugs.

When F508del-CFTR exits from the ER, it will pass through the Golgi apparatus for glycosylation, where the molecular weight of CFTR protein will be increased (from a 135–140 KDa band to a 170–180 KDa band). Immunoblot techniques were used to examine the change in the molecular weight of CFTR after treatment with lumacaftor VX-809 alone, ivacaftor VX-770 alone and a combination of both VX-809 and VX-770. When the CFTR protein leaves the Golgi apparatus, the mature and complex-glycosylated CFTR will move to cell surface. Therefore, it is important to allow sufficient time for *de novo* synthesis, ER processing, and cellular trafficking of F508del-CFTR to reach steady state. Therefore, CFBE41o- cells were incubated with drugs for 48 h before measurements. Immunoblot results for 16HBE41o- cells showed a high intense band with a molecular weight of 180 KD (band c) **Fig 5.4A**. This position of band reflects that these cells have mature and complex-glycosylated CFTR protein (wild type CFTR). In contrast, the immunoblot for CFBE41o- cells shows less intense band with molecular weight of 150 KD (band b) and there was no visible band can be detected at

position of maturation (band c). This indicates that these cells have an immature form of CFTR. These results support the hypothesis that CFBE41o- cells produce immature CFTR proteins as a result of protein folding defect due to F508del mutation [129]. CFBE41o- cells treated with VX-809 alone for 48hours showed band b together with band c (1:1 ratio). This means that VX-809 could enhance the maturation of some CFTR protein which appears as a mature CFTR. On the other hand, CFBE41o- cells treated with vx-770 showed no mature form (band c). This is consistent with fact that vx-770 enhances channel activity when it is on the cell membrane and has no effect on maturation of F508del-CFTR [61]. The immunoblot of cells treated with both vx-809 and vx-770 showed similar bands for that cells treated with vx-809 alone.

Overall, treatment with lumacaftor alone resulted in formation of a modest amount of mature protein in CF CFBE41o- cultures, which was not present in vehicle- or ivacaftor-treated CF cells. This further indicates that ivacaftor has limited role in maturation of CFTR protein.

As a part of this study, we prepared the PEG-NLC which is considered as a novel drug delivery nanocarrier. After loading the PEG-NLC with both drugs, we examined these carriers for the particle size, charge, and entrapment efficiency. We found that they characterized by particles with desirable size, higher zeta potential value which may reflect greater repulsion between the particles i.e. particle aggression is less likely to happen. Moreover, HPLC analysis of drugs-PEG-NLC indicated that this nanocarrier characterized by a high entrapment efficiency and drug loading capacity.

We further prepared another form of nanotechnology-based carriers - liposomes that also are capable to efficiently deliver the drugs into the cytoplasm of cells, where the CFTR

protein is located. To determine whether the liposomes were able to efficiently deliver drugs into lung cells, we performed a microscopic imaging of fluorescently labeled liposomes. Analysis of these images demonstrated that the fluorescence from labeled liposomes was localized primarily in the cytoplasm of the cells. The liposomes were uniformly distributed in the cytoplasm from the top to the bottom of the cells **Figure 5.5** which is consistent with the fact that the electrostatic interactions between the negatively charged cell membrane and positively charged particles can enhance their cellular uptake [135]. Consequently, the proposed liposomes can be used in the future experiments for the delivery of drugs into pulmonary cystic fibrosis cells.

7 CONCLUSIONS

In summary, a combination of both drug corrector (lumacaftor) and drug potentiator (ivacaftor) had beneficial effects in protecting the CFTR from lysosomal degradation, stabilizing the protein on cell membrane, and restoring protein function. In addition, PEG-NLC and PEGylated liposome may play an important role in enhancing the delivery of drugs to the bronchial epithelial cell. This is due to the ability of mucoinert particle to penetrate well without an attractive force with the viscus mucus. Therefore, these formulations present as a possible option for an effective inhalation delivery of drugs for patients with cystic fibrosis. Understanding the mechanisms of such effects as well as their *in vivo* testing is the goal for the further more extensive studies.

8 FUTURE DIRECTION

- 1) *In vivo* experiment using animal model with deltaF508 cystic fibrosis mutation to examine the inhalation delivery of PEG-NLC and PEG liposome formulations.
- 2) Comparative study of these drugs formulations (PEG-NLC and PEG liposome) with gene therapy.
- 3) Stability studies for these drugs formulations (PEG-NLC and PEG liposome) in the storage and after nebulization.

9 ILLUSTRATIONS

TABLES

Mutation	Frequency
Δ F508	66%–70%
G542X	2.4%
G551D	1.6%
N1303K	1.3%
W1282X	1.2%
All others	27.5%

Table 2.1 Different types of mutation with different frequencies among people wide world [27].

FIGURES

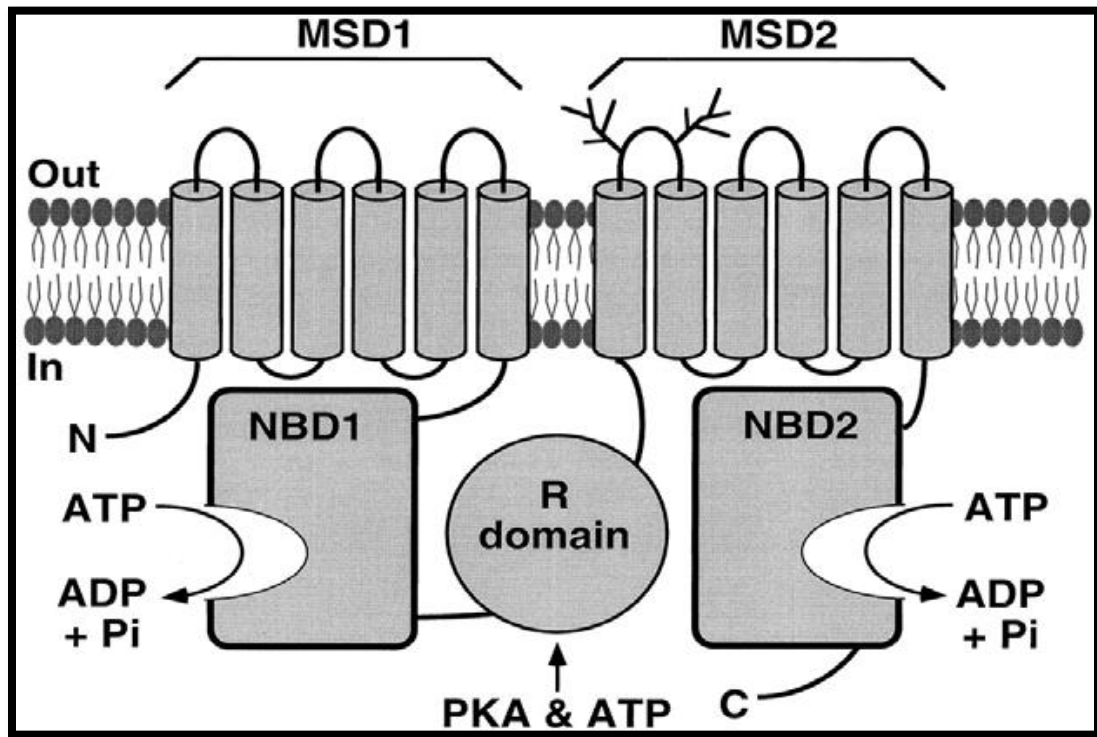


Figure 2.1 Structure of CFTR [29].

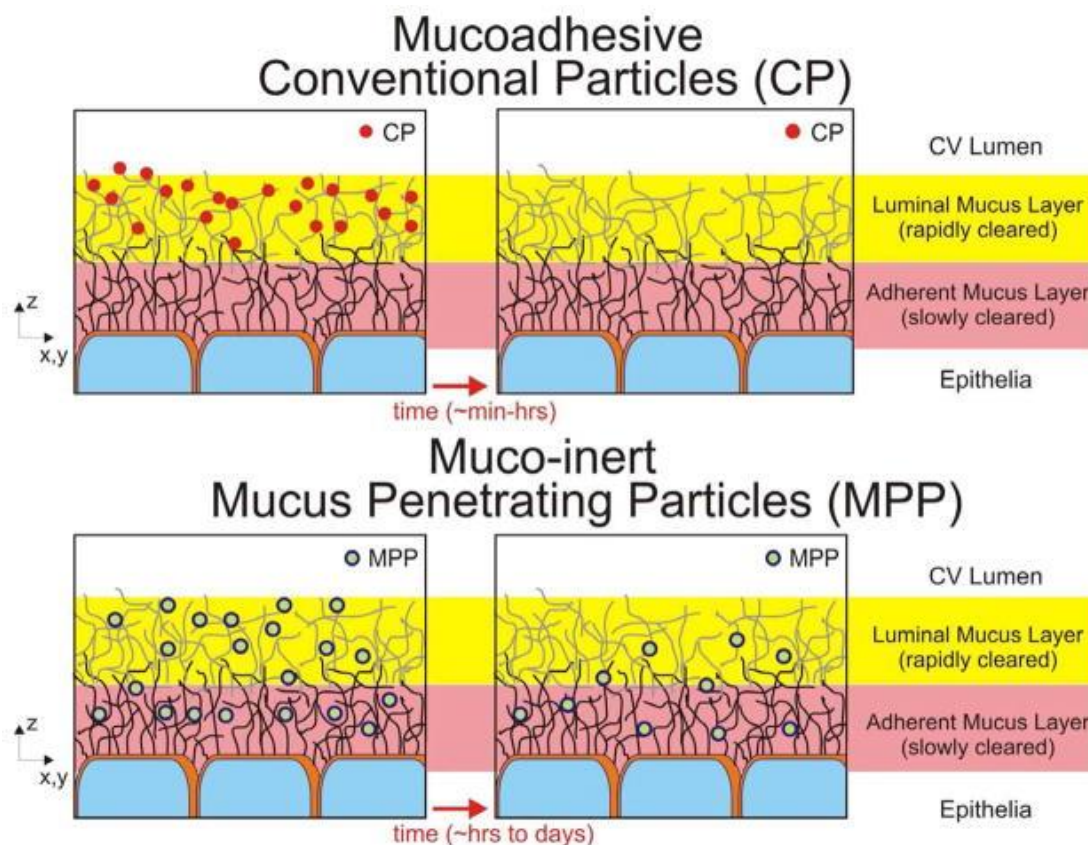


Figure 2.2 Behavior of mucoadhesive and muco-inert particles in mucus layers. Muco-inert particles do not possess any attractive forces with CF mucus like mucoadhesive conventional particles and therefore have greater penetration. Muco-inert particles present as an optimal option for inhalation delivery for patients with cystic fibrosis [95].

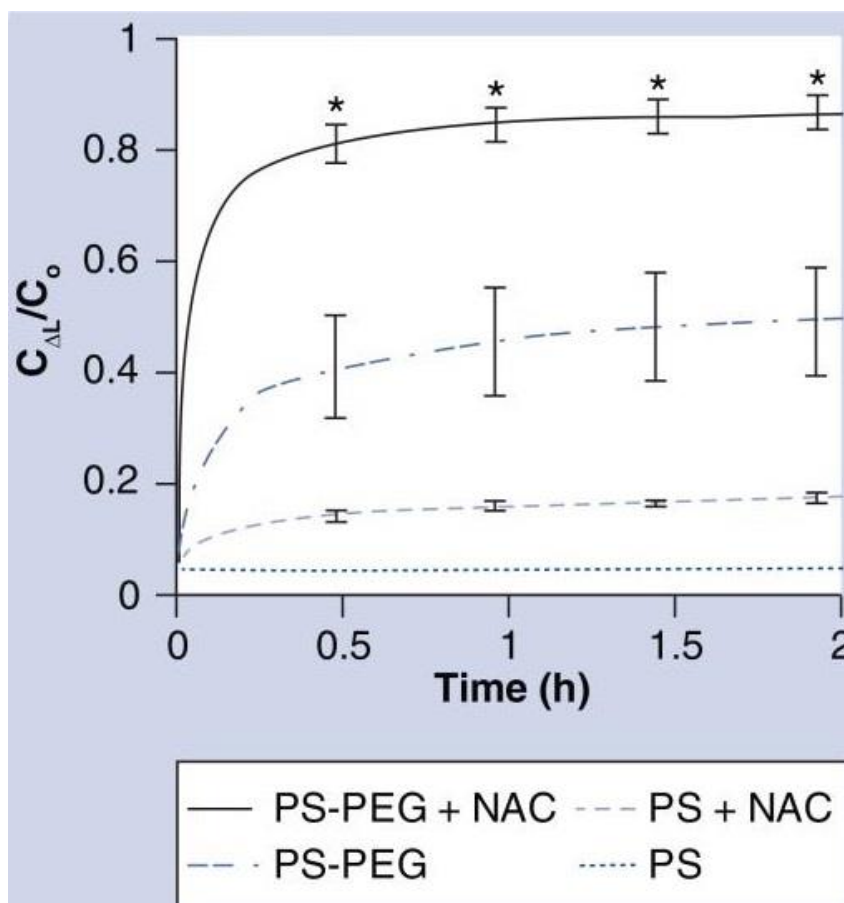


Figure 2.3 Penetration of polystyrene 200 nm nanoparticles through CF sputum. Polystyrene nanoparticles densely coated with low MW PEG (PS-PEG) have greater penetration through CF sputum compared to non-PEGylated particles (PS). Treatment with N-acetyl cysteine (NAC) increases penetration of both coated and non-coated particles. Mean \pm SD are shown [101].

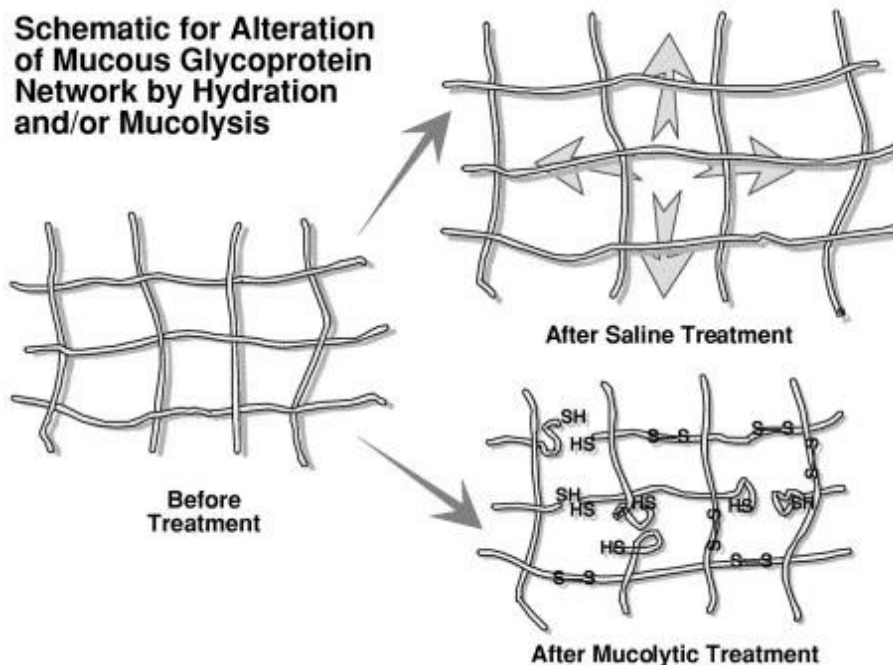


Figure 2.4 An influence of mucolytic treatment on the mucus mesh. Treatment with hypertonic saline increases the spacing within the mucus mesh. Treatment with a mucolytic such as N-acetyl cysteine breaks disulfide bonds within the mucosal mesh network. The increase in opening can increase particle penetration [101].

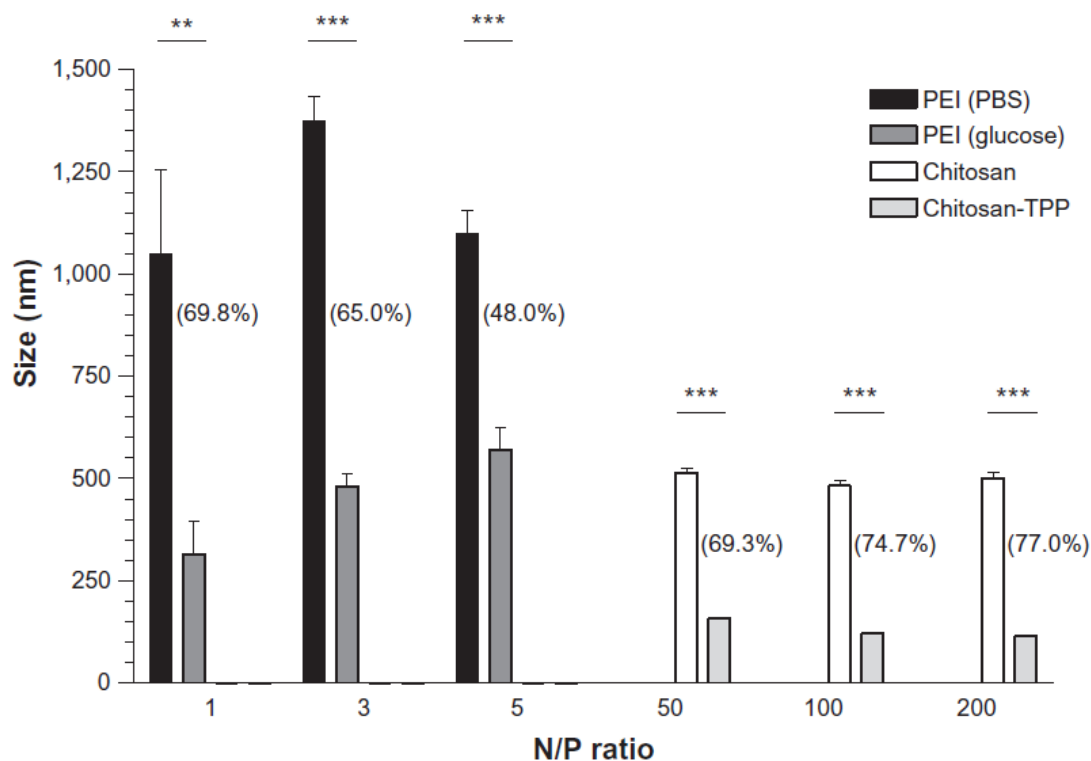


Figure 2.5 Mean size (nm) of premiR-126-poly (ethyleneimine) (PEI) complexes prepared using phosphate-buffered saline (PBS) and 5% glucose, or premiR-126 chitosan complexes and premiR-126 chitosan-TPP nanoparticles. The percentage reduction in nanoparticle size using using glucose or tripolyphosphate (TPP) for PEI and chitosan respectively at each N/P ratio, are shown in brackets.

Notes: Data are represented as mean \pm standard error of the mean and were compared by *t*-test (non-parametric, one-tailed). Differences were considered significant at $P \leq 0.05$. ** $P \leq 0.01$. *** $P \leq 0.001$.

Abbreviations: miR, microribonucleic acid; N/P, nitrogen/phosphate ratio [110].

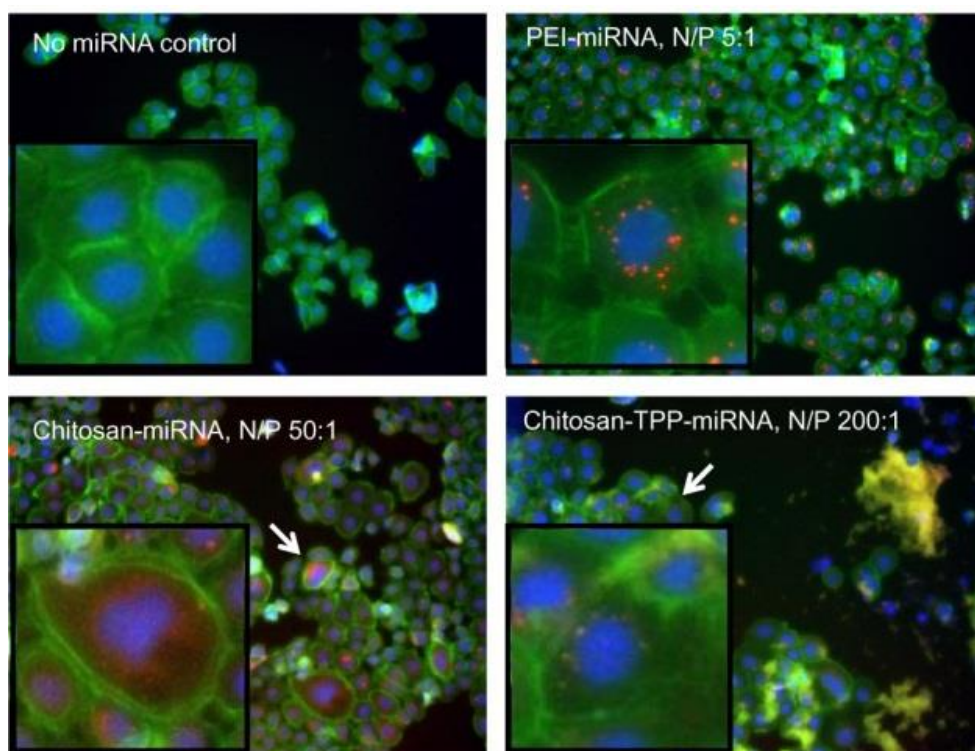


Figure 2.6 Images for miRNA nanoparticles (red) internalized by CFBE cells (blue: nucleus, green: f-actin, arrow: area shown in higher zoom box) visualized using IN Cell Analyzer 1,000[110].

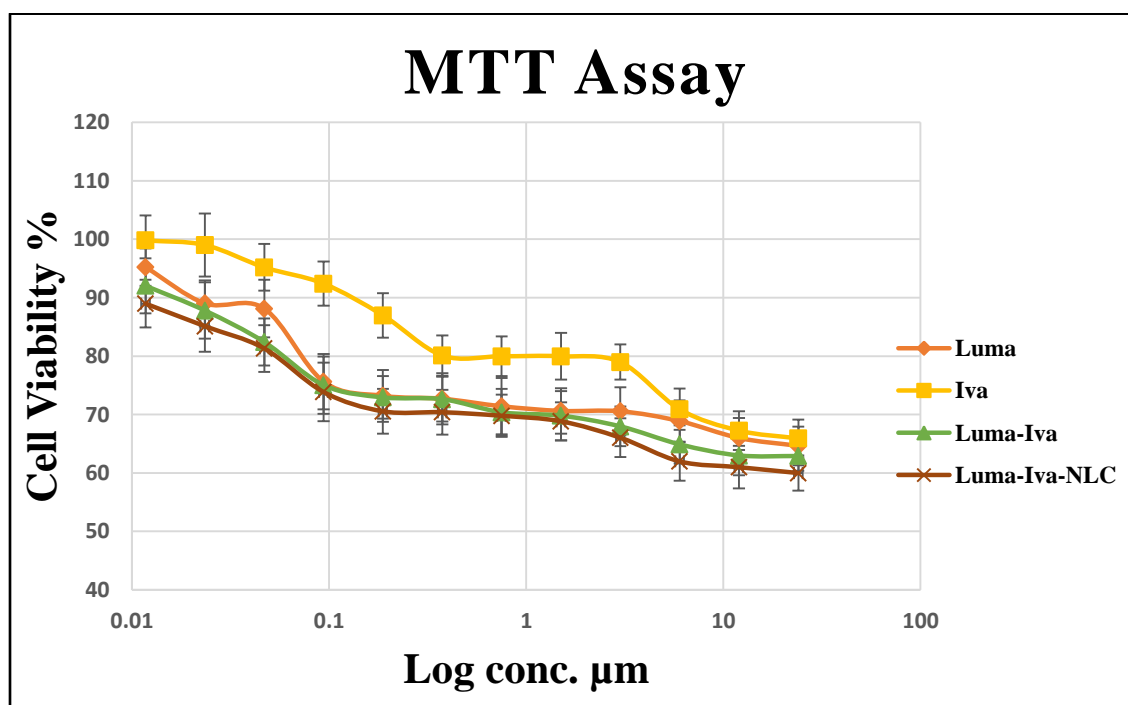


Figure 5.1 *In vitro* cytotoxicity assay for CFBE41o- cells after 48 h treatment with lumacaftor (Luma, VX-809), ivacaftor (Iva, VX-770), a plain combination of both drugs, or NLC-PEG-combination of lumacaftor (Luma) and ivacaftor (Iva). Untreated cells were used as the controls. The plot shows the percent of viable cells versus different drug concentrations. The mean \pm SD are shown.

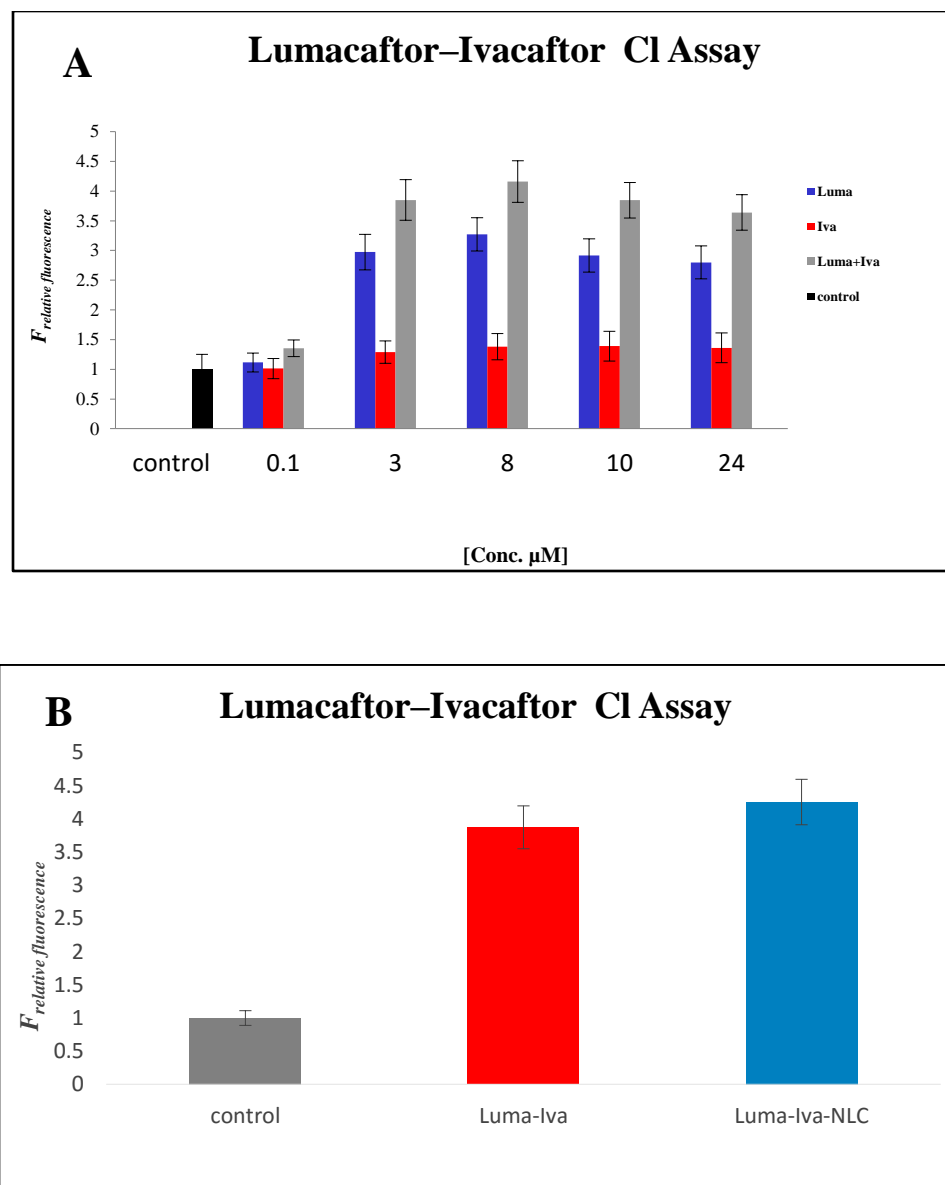


Figure 5.2. Chloride efflux measurements for CFBE41o- cells after treatment of with **A.** lumacaftor (Luma) in combination with ivacaftor (Iva) and separately. **B.** Plain combination of lumacaftor (Luma) and ivacaftor (Iva), or NLC–PEG–combination of lumacaftor (Luma) and ivacaftor (Iva). The blot shows the relative fluorescence of MQAE dye versus different concentrations of drugs. The mean \pm SD are shown.

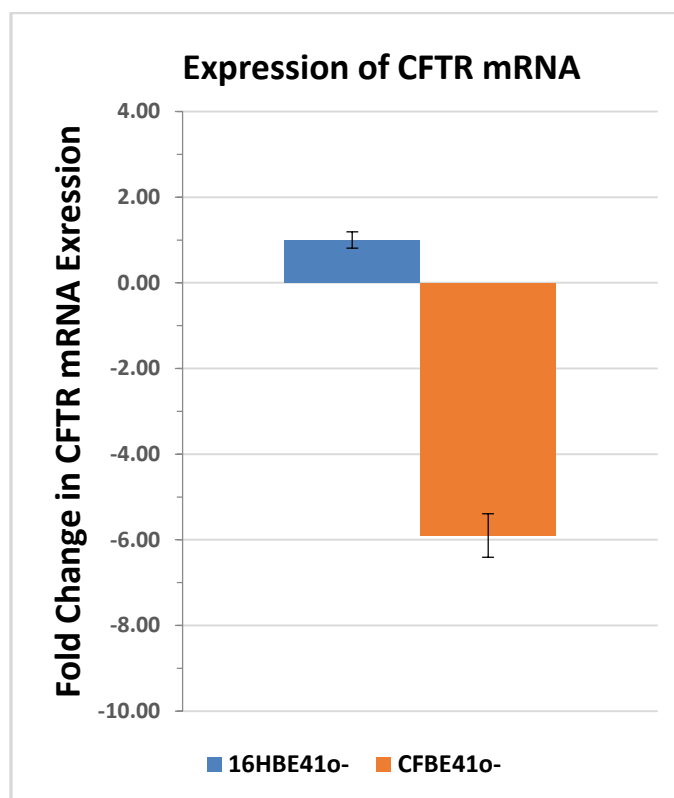


Figure 5.3 The relative quantity of CFTR gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method. CFTR mRNA expression value of 16HBE14o- cells was defined as 1 and expression of CFBE41o-cells were normalized to this value. CFTR mRNA levels of the CFBE41o-cell lines were represented as a fold change relative to 16HBE14o-. GAPDH used as an internal reference. Means \pm SD are shown

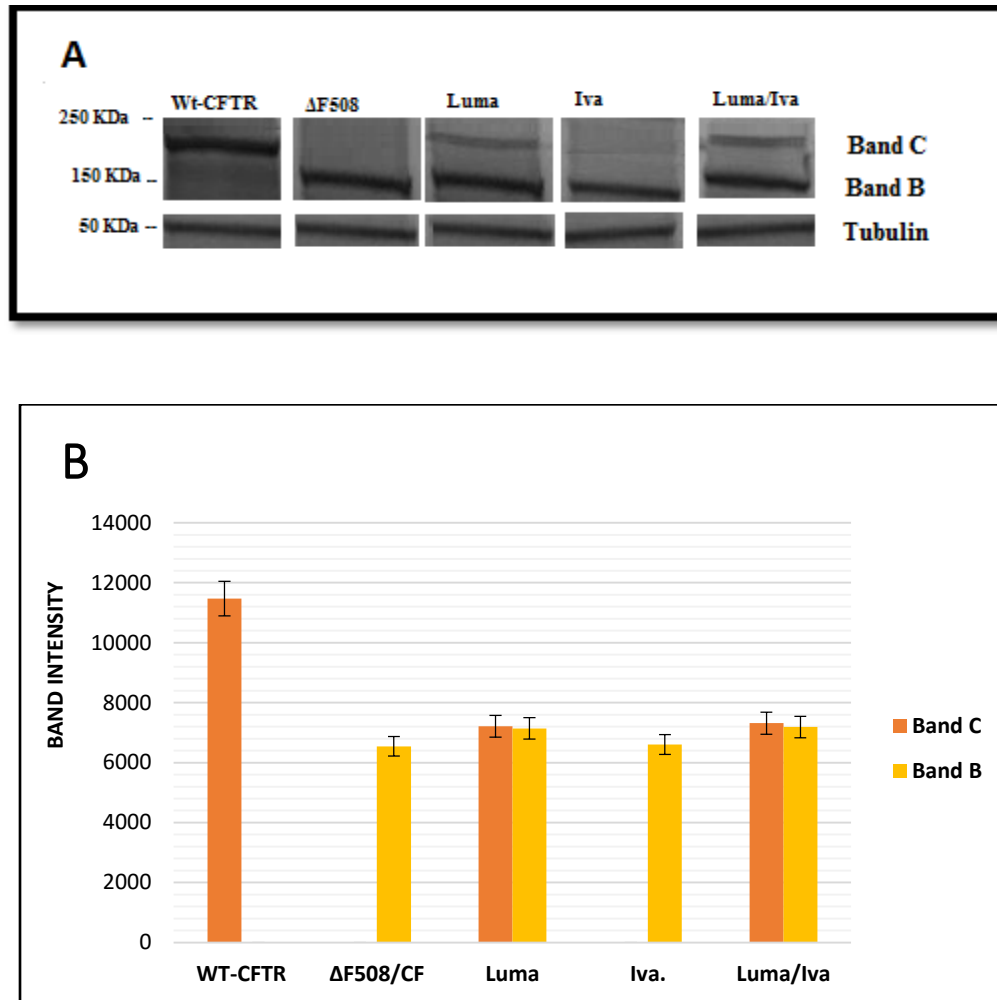


Figure 5.4 A. Western blot of wild type CFTR and $\Delta F508$ -CFTR after treatment of CFBE41o- cells with 3 μ M of lumacaftor (Luma, VX-809) with or without 3 μ M of ivacaftor (Iva, VX-770) for 48 hrs. Band C indicates the mature, complex glycosylated form of CFTR, Band B indicates the immature form of CFTR. Anti- α -tubulin antibodies were used for normalization of the Western blot data. **B.** Quantification of the band from immunoblot A, it shows the band intensity of WT-CFTR and $\Delta F508$ -CFTR (before and after treatment).

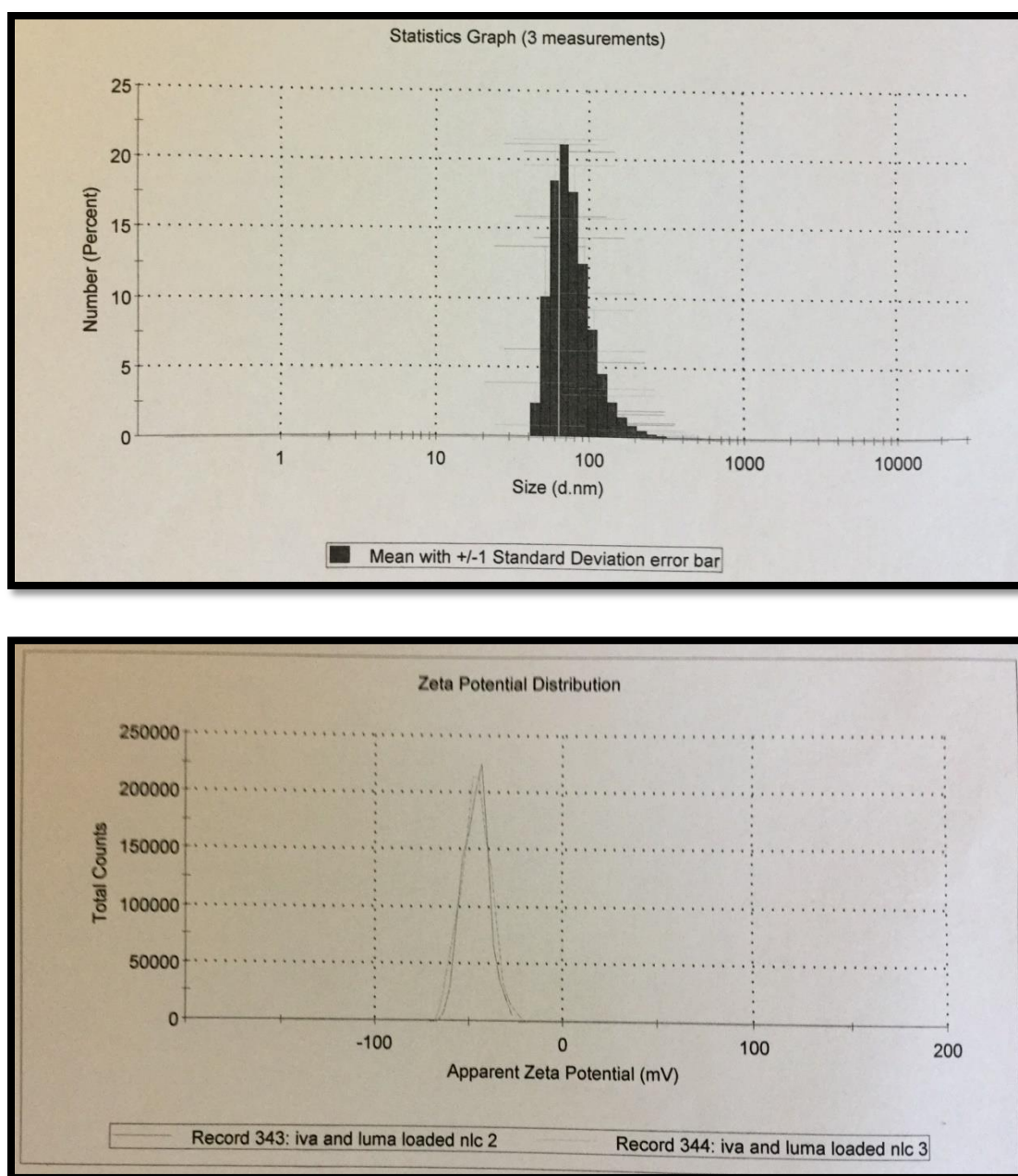


Figure 5.5 **A.** Particle size distribution curve of PEG-NLC particles. **B.** Zeta potential distribution graph for PEG-NLC particles.

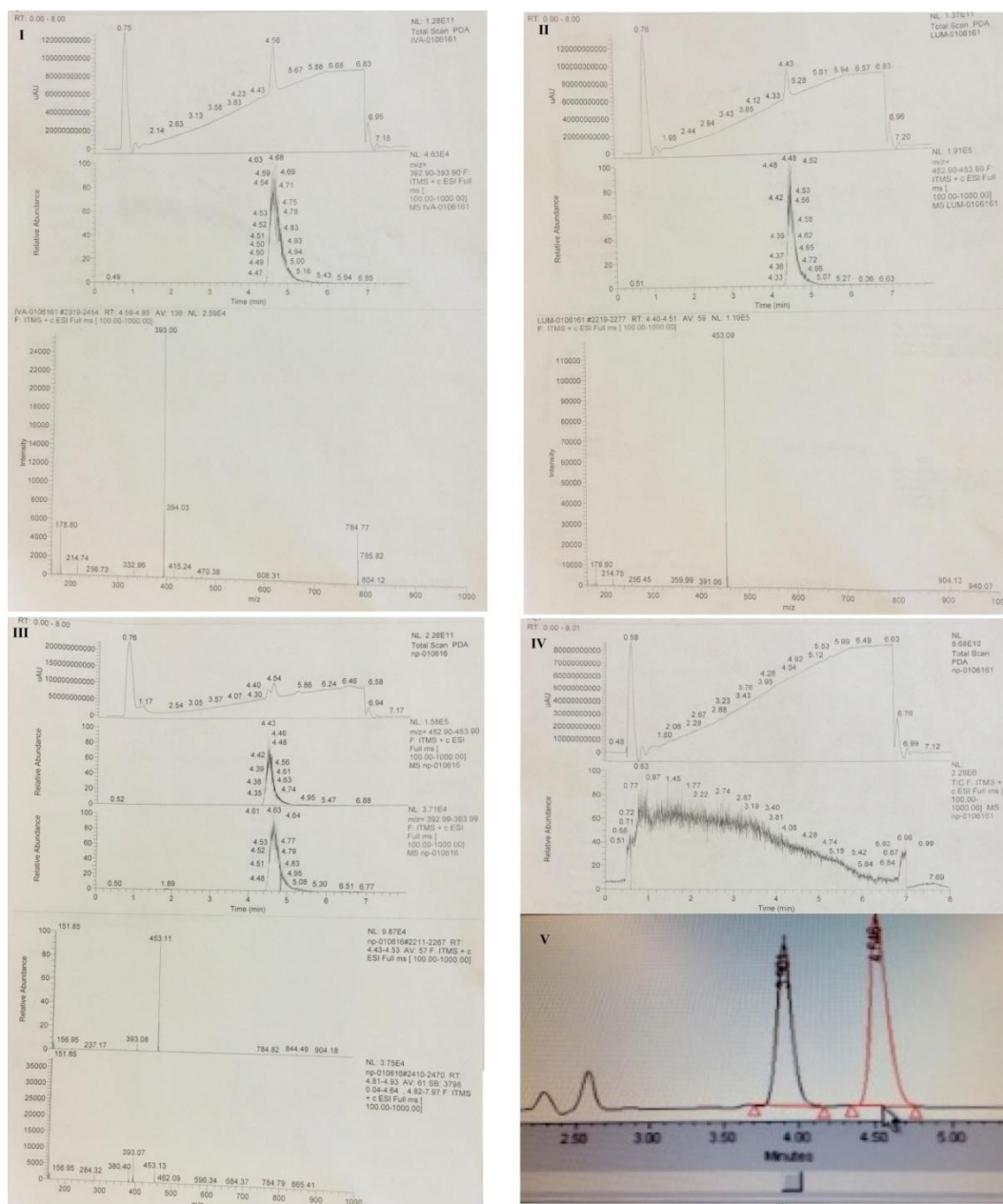


Figure 5.5C. I. LCMS graphs for Ivacaftor **II.** LCMS graphs for Lumacaftor **III.** LCMS graphs for Lum/Iva-PLG-NLC **IV.** LCMS graphs for sample flow through. **V.** HPLC graph for Lum/Iva-PLG-NLC.

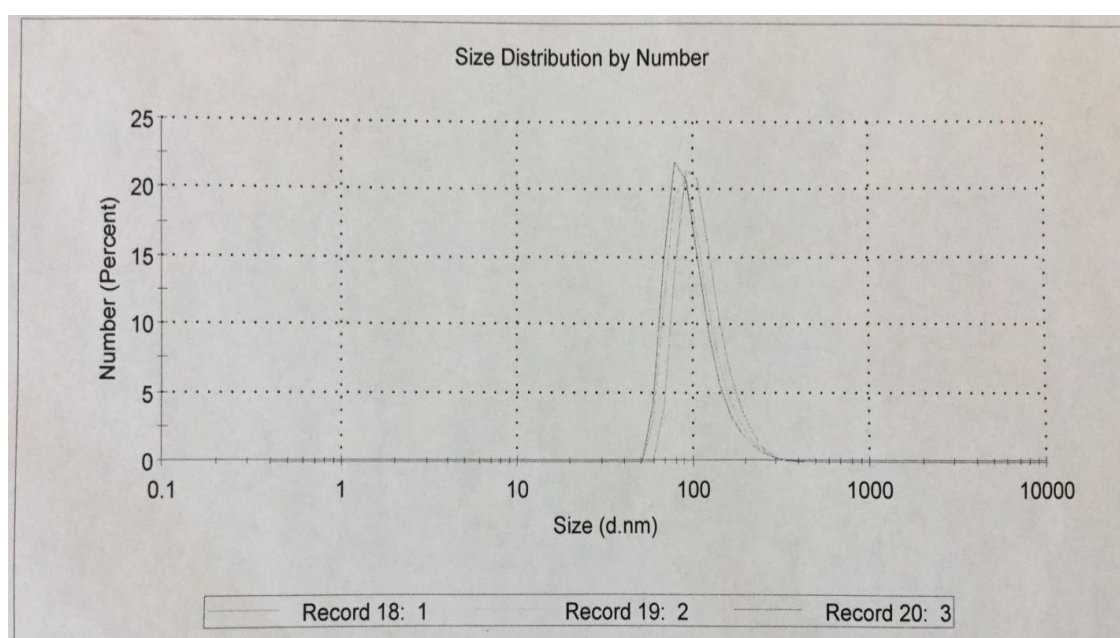


Figure 5.6 Particle size distribution of liposome.

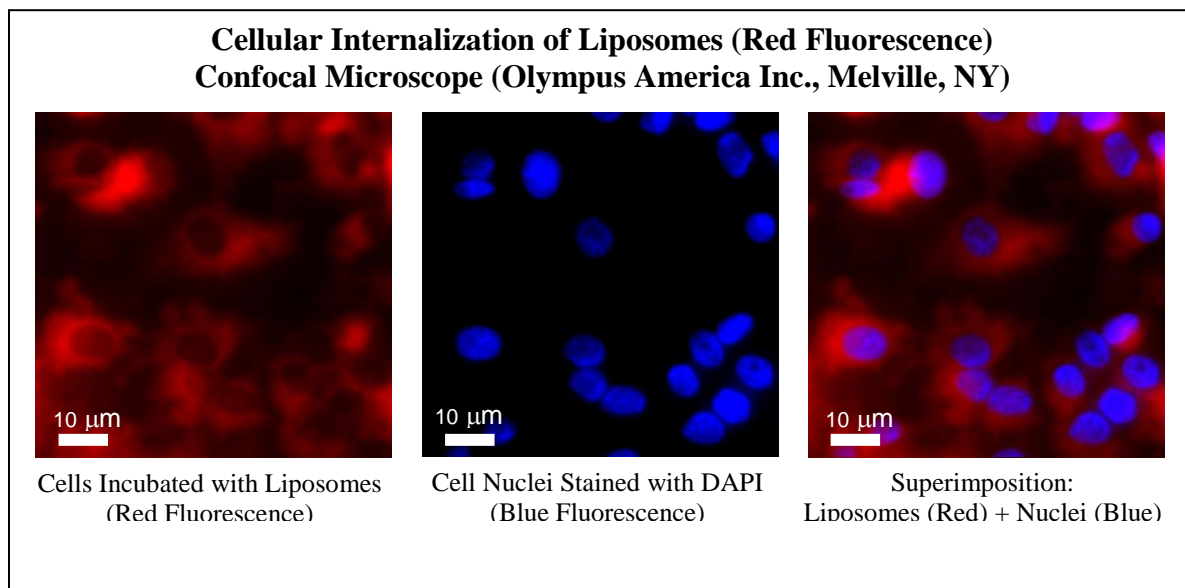


Figure 5.7 Representative images of human bronchial epithelial CFBE41o- cells incubated within 24 h with liposomes (red fluorescence). Cell nuclei were stained with nuclear-specific dye (DAPI, blue fluorescence). Superimposition of images allows for detecting of cytoplasmic localization of liposomes.

10 REFERENCES

- 1- Brennan, Marie-Luise, Iris Schrijver. Cystic Fibrosis: A Review of Associated Phenotypes, Use of Molecular Diagnostic Approaches, Genetic Characteristics, Progress, and Dilemmas. *The Journal of Molecular Diagnostics* 2016;18: 3-14.
- 2- Kollberg H, Carlander D, Olesen H, Wejaker PE, Johannesson M, Larsson A. Oral administration of specific yolk antibodies (IgY) may prevent *Pseudomonas aeruginosa* infections in patients with cystic fibrosis: a phase I feasibility study. *Pediatr Pulmonol* 2003; 35:433-40.
- 3- Waters, V., F. Ratjen. Antimicrobial susceptibility testing for acute exacerbations in chronic infection of *Pseudomonas aeruginosa* in cystic fibrosis. *The Cochrane Library* 2008.
- 4- Farokhzad OC, Langer R. Impact of nanotechnology on drug delivery. *ACS Nano*. 2009; 3:16 -20.
- 5- El-Sherbiny IM, El-Baz NM, Yacoub MH. Inhaled nano- and microparticles for drug delivery, *Global Cardiology Science and Practice* 2015;2
- 6- Caparrotta A, Di Pillo S, Attanasi M, Consilvio NP, Cingolani A, Rapino D, Mohn A, Chiarelli F. et al. Growth failure in children with cystic fibrosis. *J Pediatr Endocrinol Metab*. 2012; 25(5-6):393-405.
- 7- Reaves J, Wallace G. Unexplained bruising: weighing the pros and cons of possible causes. *Consultant for Pediatricians*. . 2010; 9: 201–202.
- 8- Flume PA, Mogayzel Jr PJ, Robinson KA, et al. Cystic Fibrosis Pulmonary Guidelines: Pulmonary Complications: Hemoptysis and Pneumothorax. *Am J Respir Crit Care Med*. 2010;182 (3): 298.
- 9- Mitchell, Richard Sheppard; Kumar, Vinay; Robbins, Stanley L.; Abbas, Abul K.; Fausto, Nelson. 2007. Robbins basic pathology. Saunders/Elsevier.
- 10- Girón RM, Domingo D, Buendía B, Antón E, Ruiz-Velasco LM, Ancochea J. Nontuberculous mycobacteria in patients with cystic fibrosis. *Arch. Bronconeumol*. 2005; 41 (10): 560–565.
- 11- Maldonado M, Martínez A, Alobid I, Mullol J. The antrochoanal polyp. *Rhinology* 2004; 42 (4): 178–182.

- 12- Ramsey B, Richardson MA. Impact of sinusitis in cystic fibrosis. *J. Allergy Clin. Immunol.* .1992; 90 (3 Pt 2): 547–552.
- 13- Eggermont E, De Boeck K. Small-intestinal abnormalities in cystic fibrosis patients. *Eur. J. Pediatr.* 1991; 150 (12): 824–828.
- 14- Kulczycki LL, Shwachman H. Studies in cystic fibrosis of the pancreas; occurrence of rectal prolapse. *N. Engl. J. Med.* 1958; 259 (9): 409–412.
- 15- Cohn JA, Friedman KJ, Noone PG, Knowles MR, Silverman LM, Jowell. Relation between mutations of the cystic fibrosis gene and idiopathic pancreatitis. *N. Engl. J. Med.* 1998; 339 (10): 653–658.
- 16- Malfroot A, Dab I. New insights on gastro-esophageal reflux in cystic fibrosis by longitudinal follow up". *Arch. Dis. Child.* 1991; 66 (11): 1339–1345.
- 17- Khoshoo V, Udall JN. Meconium ileus equivalent in children and adults. *Am. J. Gastroenterol.* 1994; 89 (2): 153–157.
- 18- Williams SG, Westaby D, Tanner MS, Mowat AP. Liver and biliary problems in cystic fibrosis. *Br. Med. Bull.* 1992; 48 (4): 877–892.
- 19- Colombo C, Russo MC, Zazzeron L, Romano G. Liver disease in cystic fibrosis. *J. Pediatr. Gastroenterol.* 2006; *Nutr.* 43 (Suppl 1): S49–S55.
- 20- Moran A, Pyzdrowski KL, Weinreb J, Kahn BB, Smith SA, Adams KS, Seaquist ER. Insulin sensitivity in cystic fibrosis". *Diabetes* 1994; 43 (8): 1020–1026.
- 21- Alves Cde A, Aguiar RA, Alves AC, Santana MA. Diabetes mellitus in patients with cystic fibrosis. *J Bras Pneumol.* 2007; 33 (2): 213–221.
- 22- Haworth CS, Selby PL, Webb AK, Dodd ME, Musson H, McL Niven R, Economou G, Horrocks AW, Freemont AJ, Mawer EB, Adams JE . Low bone mineral density in adults with cystic fibrosis. *Thorax.* 1999; 54 (11): 961–967.
- 23- Vandemergel X, Decaux G. Review on hypertrophic osteoarthropathy and digital clubbing. *Revue Médicale de Bruxelles.* 2003; 24 (2): 88–94.
- 24- Pitts-Tucker TJ, Miller MG, Littlewood JM. Finger clubbing in cystic fibrosis. *Arch. Dis. Child.* 1986; 61 (6): 576–579.
- 25- McCallum TJ, Milunsky JM, Cunningham DL, Harris DH, Maher TA, Oates RD. Fertility in men with cystic fibrosis: an update on current surgical practices and outcomes. *Chest.* 2000; 118 (4): 1059–1062.

- 26- Chen H, Ruan YC, Xu WM, Chen J, Chan HC. Regulation of male fertility by CFTR and implications in male infertility. *Human Reproduction Update*. 2012; 18 (6): 703–713.
- 27- Araújo FG, Novaes FC, Santos NP, Martins VC, Souza SM, Santos SE, Ribeiros-Santos AK. Prevalence of deltaF508, G551D, G542X, and R553X mutations among cystic fibrosis patients in the North of Brazil. *Braz. J. Med. Biol. Res.* 2005; 38 (1): 11–5.
- 28- Lap-Chee Tsui: Profile. Science.ca. 1989-05-09. Retrieved 2013-01-23.
- 29- Rowe SM, Miller S, Sorscher EJ. Cystic fibrosis. *The New England Journal of Medicine*. 2005; 352 (19): 1992–2001.
- 30- Short DB, Trotter KW, Reczek D, Kreda SM, Bretscher A, Boucher RC, Stutts MJ, Milgram SL. An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. *J. Biol. Chem.* 1998; 273 (31): 19797–801.
- 31- Childers M, Eckel G, Himmel A, Caldwell J. A new model of cystic fibrosis pathology: lack of transport of glutathione and its thiocyanate conjugates. *Medical Hypotheses*. 2007; 68 (1): 101–12.
- 32- Moskwa P, Lorentzen D, Excoffon KJ, Zabner J, McCray PB, Nauseef WM, Dupuy C, Bánfi B. A novel host defense system of airways is defective in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 2007; 175 (2): 174–183.
- 33- Conner GE, Salathe M, Forteza R. Lactoperoxidase and hydrogen peroxide metabolism in the airway. *Am. J. Respir. Crit. Care Med.* 2002; 166 (12 Pt 2): S57–61.
- 34- Saiman L. Microbiology of early CF lung disease. *Paediatric Respiratory Reviews* 5 (Suppl A): 2004; S367–69.
- 35- Tümmler B, Koopmann U, Grothues D, Weissbrodt H, Steinkamp G, von der Hardt H. Nosocomial acquisition of *Pseudomonas aeruginosa* by cystic fibrosis patients". *J. Clin. Microbiol.* 1991; 29 (6): 1265–1267.
- 36- Jones AM, Govan JR, Doherty CJ, Dodd ME, Isalska BJ, Stanbridge TN, Webb AK. Identification of airborne dissemination of epidemic multiresistant strains of

- Pseudomonas aeruginosa* at a CF centre during a cross infection outbreak. 2003; *Thorax* 58(6): 525–527.
- 37- Høiby N. Isolation and treatment of cystic fibrosis patients with lung infections caused by *Pseudomonas* (Burkholderia) *cepacia* and multiresistant *Pseudomonas aeruginosa*. *Neth J Med*.1995; 46 (6): 280–287.
 - 38- Pihet M, Carrere J, Cimon B, Chabasse D, Delhaes L, Symoens F, Bouchara JP. Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis—a review. *Med Mycol*.2009; 47 (4): 387–397.
 - 39- Rapaka RR, Kolls JK. Pathogenesis of allergic bronchopulmonary aspergillosis in cystic fibrosis: current understanding and future directions. *Med Mycol*.2009; 47 (Suppl 1): S331–7.
 - 40- Mishra A, Greaves R, Massie J. The relevance of sweat testing for the diagnosis of cystic fibrosis in the genomic era. . *The Clinical biochemist. Reviews / Australian Association of Clinical Biochemists*. 2005; 26 (4): 135–153.
 - 41- Davies JC, Alton EW, Bush A. Cystic fibrosis. *BMJ*. 2007; 335(7632): 1255–1259.
 - 42- Ross LF. Newborn screening for cystic fibrosis: a lesson in public health disparities. *The Journal of Pediatrics*. 2008; 153 (3): 308–313.
 - 43- Assael BM, Castellani C, Ocampo MB, Iansa P, Callegaro A, Valsecchi MG. Epidemiology and survival analysis of cystic fibrosis in an area of intense neonatal screening over 30 years. *American Journal of Epidemiology*. 2002; 156 (5): 397–401.
 - 44- Minarowski Ł, Sands D, Minarowska A, Karwowska A, Sulewska A, Gacko M, Chyczewska E. Thiocyanate concentration in saliva of cystic fibrosis patients. *Folia Histochem. Cytobiol*. 2008; 46 (2): 245–6.
 - 45- Stern RC. The diagnosis of cystic fibrosis. *N. Engl. J. Med*.1997; 336 (7): 487–491.
 - 46- Elias S, Annas GJ, Simpson JL. Carrier screening for cystic fibrosis: implications for obstetric and gynecologic practice. *Am. J. Obstet. Gynecol*.1991; 164 (4): 1077–1083.

- 47- Tabor A, Philip J, Madsen M, Bang J, Obel EB, Nørgaard-Pedersen B. Randomised controlled trial of genetic amniocentesis in 4606 low-risk women. *Lancet* 1.1986; (8493): 1287–1293.
- 48- Eddleman KA, Malone FD, Sullivan L, Dukes K, Berkowitz RL, Kharbutli Y, Porter TF, Luthy DA, Comstock CH, Saade GR, Klugman S, Dugoff L, Craigo SD, Timor-Tritsch IE, Carr SR, Wolfe HM, D'Alton ME. Pregnancy loss rates after midtrimester amniocentesis. *Obstet Gynecol.* 2006; 108 (5): 1067–1072.
- 49- Pai VB, Nahata MC. Efficacy and safety of aerosolized tobramycin in cystic fibrosis. *Pediatr. Pulmonol.* 2001; 32 (4): 314–327.
- 50- Westerman EM, Le Brun PP, Touw DJ, Frijlink HW, Heijerman HG. Effect of nebulized colistin sulphate and colistin sulphomethate on lung function in patients with cystic fibrosis: a pilot study. *J. Cyst. Fibros.* 2004; 3 (1): 23–28.
- 51- McCoy KS, Quittner AL, Oermann CM, Gibson RL, Retsch-Bogart GZ, Montgomery AB. Inhaled aztreonam lysine for chronic airway *Pseudomonas aeruginosa* in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 2008; 178 (9): 921–928.
- 52- Ryan G, Singh M, Dwan K. Inhaled antibiotics for long-term therapy in cystic fibrosis. *The Cochrane Database of Systematic Reviews*(3).2011; CD001021.
- 53- Hansen CR, Pressler T, Koch C, Høiby N. Long-term azitromycin treatment of cystic fibrosis patients with chronic *Pseudomonas aeruginosa* infection; an observational cohort study. *J. Cyst. Fibros.* 2005; 4 (1): 35–40.
- 54- Tan KH, Mulheran M, Knox AJ, Smyth AR. Aminoglycoside prescribing and surveillance in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 2003; 167 (6): 819–823.
- 55- van der Schans C, Prasad A, Main E. Chest physiotherapy compared to no chest physiotherapy for cystic fibrosis. In Van Der Schans, Cees P. *Cochrane Database Syst Rev* (2):2000; CD001401.
- 56- Kuver R, Lee SP. Hypertonic saline for cystic fibrosis. *N. Engl. J. Med.* 2006; 354 (17): 1848–51; author reply 1848–1851.
- 57- Lieberman J. Dornase aerosol effect on sputum viscosity in cases of cystic fibrosis. *JAMA.* 1968; 205 (5): 312–313.

- 58- Kellerman D, Rossi Mospan A, Engels J, Schaberg A, Gorden J, Smiley L. Denufosol: a review of studies with inhaled P2Y(2) agonists that led to Phase 3. *Pulmonary Pharmacology & Therapeutics*. 2008; 21 (4): 600–607.
- 59- Moran F, Bradley JM, Piper AJ. Non-invasive ventilation for cystic fibrosis. In Moran, Fidelma. *Cochrane Database Syst Rev* (1). 2009; CD002769.
- 60- Huth MM, Zink KA, Van Horn NR. The effects of massage therapy in improving outcomes for youth with cystic fibrosis: an evidence review. *Pediatr Nurs*. 2005; 31 (4): 328–332.
- 61- Van Goor F, Hadida S, Grootenhuis PD. Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator. VX-770. *Proc Natl Acad Sci U S A*. 2009; 106:18825-18830.
- 62- Clinical trial .gov. Bethesda (MD). National library of Medicine (US). Vertex Pharmaceutical Incorporation. Study of VX-809 alone or in combination with VX-770 in CF patients homozygous or heterozygous for F508del-CFTR mutation. 2010.
- 63- Condren ME, Bradshaw MD. Ivacaftor: a novel gene-based therapeutic approach for cystic fibrosis. *J Pediatr Pharmacol Ther*. 2013; 18:8-13.
- 64- Amaral MD, Kunzelmann K. Molecular targeting of CFTR as a therapeutic approach to cystic fibrosis. *Trend Pharmacol. Sci*. 2007; 28:334-341.
- 65- Barends PJ. Distribution of receptor targets in the lung. *Proc Am Thorac Soc*. 2004; 1:345-351.
- 66- Oakland M, Sinn PL, McCray Jr PB. Advances in cell and gene based therapies for cystic fibrosis lung disease. 2012; 20:1108-1115.
- 67- Zhang L, Button B, Gabriel SE, et al. CFTR delivery to 25% of surface epithelial cells restores normal rate of mucus transport to human cystic fibrosis airway epithelium. 2009; 7: e1000155.
- 68- Dorin JR, Farley R, Webb S, et al. A demonstration using mouse models that successful gene therapy for cystic fibrosis requires only partial gene correction. *Gene Ther*. 1996; 3:797-801.
- 69- Johnson LG, Olsen JC, Sarkadi B, et al. Efficiency of gene transfer for restoration of normal airway epithelial function for cystic fibrosis. 1992; 2:21-25.

- 70- Johnson JS, Gentsch M, Zhang L, et al. AAV exploits subcellular stress associated with inflammation, endoplasmic reticulum expansion, and misfolded proteins in models of cystic fibrosis. 2011; 7: e1002053.
- 71- Ferrari S, Geddes DM, Alton EW. Barriers to and new approaches for gene therapy and gene delivery in cystic fibrosis. *Adv Drug Deliv Rev.* 2002; 54:1373-1393.
- 72- Griesenbach U, Inoue M, Hasegawa M, et al. Viral vector for cystic fibrosis gene therapy: what does the future hold? *Virus Adaptation Treatment.* 2010; 2:159-171.
- 73- Zuckerman JB, Robinson CB, McCoy KS, et al. A phase I study of adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator gene to a lung segment of individuals with cystic fibrosis. *Hum Gene Ther.* 1999; 10:2973-2985.
- 74- Wagner JA, Messner AH, Moran ML, et al. Safety and biological efficacy of an adeno-associated virus vector-cystic fibrosis transmembrane regulator (AAV-CFTR) in the cystic fibrosis maxillary sinus. *Laryngoscope.* 1999; 109(2 Pt 1):266–74.
- 75- Moss RB, Rodman D, Spencer LT, et al. Repeated adenoassociated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: a multicenter, double-blind, placebo-controlled trial. *Chest* 2004; 125:509–21.
- 76- Kondo R, Feitelson MA, Strayer DS. Use of SV40 to immunize against hepatitis B surface antigen: implications for the use of SV40 for gene transduction and its use as an immunizing agent. *Gene Ther.* 1998; 5:575–82.
- 77- Griesenbach U, McLachlan G, Owaki T, et al. Validation of recombinant Sendai virus in a non-natural host model. *Gene Ther.* 2010; 18:182–188.
- 78- Cmielewski P, Anson DS, Parsons DW. Lysophosphatidylcholine as an adjuvant for lentiviral vector mediated gene transfer to airway epithelium: effect of acyl chain length. *Respir Res.* 2010; 1:84.

- 79- Kremer KL, Dunning KR, Parsons DW, Anson DS. Gene delivery to airway epithelial cells in vivo: a direct comparison of apical and basolateral transduction strategies using pseudotyped lentivirus vectors. *J Gene Med.* 2007; 9:362–8.
- 80- Stocker AG, Kremer KL, Koldej R, Miller DS, Anson DS, Parsons DW. Single-dose lentiviral gene transfer for lifetime airway gene expression. *J Gene Med.* 2009; 11:861–7.
- 81- Mitomo K, Griesenbach U, Inoue M, et al. Toward gene therapy for cystic fibrosis using a lentivirus pseudotyped with Sendai virus envelopes. *Mol Ther.* 2010; 18:1173–82.
- 82- Griesenbach U, Inoue M, Meng C, et al. Assessment of F/HNPseudotyped Lentivirus as a Clinically Relevant Vector for Lung Gene Therapy. *Am J Respir Crit Care Med.* 2012; 186(9):846–56.
- 83- Sanders NN, De Smedt SC, Van Rompaey E, et al. Cystic fibrosis sputum: a barrier to the transport of nanospheres. *Am J Respir Crit Care Med.* 2000; 162:1905-1911.
- 84- Khanvilkar K, Donovan MD, Flanagan DR. Drug transfer through mucus. *Adv Drug Deliv Rev.* 2001; 48:172-193.
- 85- Matsui H, Johnson LG, Randell SH, et al. Loss of binding and entry of liposome-DNA complexes decrease transfection efficiency in differentiated airway epithelium cells. *J Bio Chem.* 1997; 272:1117-1126.
- 86- Alton EW, Boyd AC, Cheng SH, et al. A randomized, double-blind, placebo-controlled phase IIB clinical trial of repeated application of gene therapy in patient with cystic fibrosis. *Thorax.* 2013; 00:1-3.
- 87- Oxford University Gene Medicine. GL67A/Pgm169-The consortium's first clinical trial product. 2013.
- 88- Hyde SC, Pringle IA, Abdullah S, et al. CpG-free plasmid confer reduced inflammation and sustained pulmonary gene expression. *Nat Biotechnol.* 2008; 26:549-551.
- 89- Pringle IA, Abdullah S, Connolly MM, et al. 2012. CpG-free plasmid expression cassettes for cystic fibrosis gene therapy. *Biomaterials.* 2012; 33:6833-6842.

- 90- McLachlan G, Davidson H, Holder E, et al. Preclinical evaluation of three non-viral gene transfer agent for cystic fibrosis after aerosol delivery to the ovine lung. *Gene Ther.* 2011; 18: 996-1005.
- 91- Davies J, Davies G, Voase N, et al. Evaluation of safety and gene expression with a single dose of PGM169/GL 67A administered to the nose and lung of individuals with cystic fibrosis: the UK Gene Therapy Consortium "Pilot Study". 2009; 64: A 70.
- 92- Manunta MD, McAnulty RJ, Tagalaskis AD, et al. Nebulization of receptor-targeted nanocomplexes for gene delivery to the air epithelium. *PloS one.* 2011; 6: e26768.
- 93- Konstan MW, Davis PB, Wagener JS, et al. Compacted DNA nanoparticles administered to the nasal mucosa of cystic fibrosis subjects are safe and demonstrate partial to complete for cystic fibrosis transmembrane regulator reconstitution. *Hum Gene Ther.* 2004; 15:1255-1269.
- 94- Davis SS. Biomedical application of nanotechnology- implications for drug targeting and gene therapy. *Trends Biotechnol.* 1997; 15:217-224.
- 95- Lai SK, Wang YY, Hanes J. Mucus penetrating nanoparticles for drug and gene delivery to mucosal tissues. *Adv Drug Deliv Rev.* 2009; 61:158-171.
- 96- Jubeh TT, Barenholz Y, Rubinstein A. Differential adhesion of normal and inflamed rat colonic mucosa by charged liposomes. *Pharm Res.* 2004; 21:447-453.
- 97- Parego C, Torres D, Alonso MJ. The potential of Chitosan for the oral administration of peptides. *Expert Opin Drug Deliv.* 2005; 2:843-854.
- 98- Woodley J. Bioadhesion: new possibility for drug administration? *Clin Pharmacokinet.* 2001; 40:77-84.
- 99- Dawson M, Wirtz D, Hanes J. Enhanced viscoelasticity of human cystic fibrosis sputum correlates with increasing microheterogeneity in particle transport. *J Biol Chem.* 2003; 278:50393-50401.
- 100- Suk JS, Lai SK, Wang YY, et al. The penetration of fresh undiluted sputum expectorated by cystic fibrosis patients by non-adhesive polymer nanoparticles. *Biomaterials.* 2009; 30:2591-2597.

- 101-Suk JS, Lai SK, Boylan NJ, et al. Rapid transport of muco-inert nanoparticles in cystic fibrosis sputum treated with N-acetyl cysteine. *Nanomedicine (Lond)*. 2011; 6:365-375.
- 102-Lai SK, O'Hanlon DE, Harrold S, et al. Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus. *Proc Natl Acad Sci U S A*. 2007; 104:1482-1487.
- 103-Sinn PL, Anthony RM, McCray PB. Genetic therapies for cystic fibrosis lung disease. *Hum Mol Genet*. 2011; 20: R79-86.
- 104-Cartiera MS, Ferreira EC, Caputo C, et al. Partial correlation of cystic fibrosis defects with PLGA nanoparticles encapsulating curcumin. *Mol Pharm*. 2010; 7:86-93.
- 105-Vij N, Min T, Marasigan R, et al. Development of PEGlyated of PLGA nanoparticle for controlled and sustained drug delivery in cystic fibrosis. *J Nanobiotechnology*. 2010; 8:22.
- 106-Griesenbach U, Alton EW. Progress in gene and cell therapy for cystic fibrosis lung disease. *Curr Pharmaceut Design*. 2012; 18: 642-662.
- 107-Conese M, Ascenzioni F, Boyed AC, et al. Gene and cell therapy for cystic fibrosis :from bench to bedside. 2011; 10 (suppl 2): S144-128.
- 108-Moss R.B, Mistry SJ, et al. Safety and early treatment effects of the CXCR2 antagonist SB-656933 in patients with cystic fibrosis. *Journal of Cystic Fibrosis*. 2013; 12:241–248
- 109-Hartl D, Latzin P, Hordijk P, Marcos V, Rudolph C, Woischnik M, et al. Cleavage of CXCR1 on neutrophils disables bacterial killing in cystic fibrosis lung disease. *Nat Med* Dec. 2008; 13(12):1423-30.
- 110-McKiernan P, Cunningham O, Greene C, Cryan S. Targeting miRNA-based medicines to cystic fibrosis airway epithelial cells using nanotechnology. *International Journal of Nanomedicine*. 2013; 8:3907–3915.
- 111-Brissault B, Leborgne C, Guis C, Danos O, Cheradame H, Kichler A. Linear topology confers in vivo gene transfer activity to polyethylenimines. *Bioconjug Chem*. 2006; 17:759–765.

- 112- Katas H, Alpar HO. Development and characterisation of chitosan nanoparticles for siRNA delivery. *J Control Release*. 2006; 115:216–225.
- 113- Ehrhardt, Carsten. characterisation of the human bronchial epithelial cell line CFBE41o. *Cell and tissue research* 2006; 405-415.
- 114- Zhang, Yue Ying. Anti-proliferation effect of polypeptide extracted from scorpion venom on human prostate cancer cells in vitro. *J clinical medicine research* 2009; 1: 24.
- 115- Servetnyk, Zhanna. The effect of S-nitrosoglutathione and L-cysteine on chloride efflux from cystic fibrosis airway epithelial cells. *Experimental and molecular pathology* 2011; 90: 79-83.
- 116- Dragomir, Anca, and Godfried M. Roomans. Increased chloride efflux in colchicine-resistant airway epithelial cell lines. *Biochemical pharmacology* 2004;68: 253-261.
- 117- Favia, Maria. Na⁺/H⁺ exchanger regulatory factor 1 overexpression-dependent increase of cytoskeleton organization is fundamental in the rescue of F508del cystic fibrosis transmembrane conductance regulator in human airway CFBE41o-cells. *Molecular biology* 2010; 21:73-86.
- 118- Illek, Beate. Cl transport in complemented CF bronchial epithelial cells correlates with CFTR mRNA expression levels. *Cellular Physiology and Biochemistry* 2008;22: 57-68.
- 119- Bebok, Zsuzsa. Failure of cAMP agonists to activate rescued Δ F508 CFTR in CFBE41o–airway epithelial monolayers. *Journal of physiology* 2005;569: 601-615.
- 120- Bangel-Ruland, Nadine. Cystic fibrosis transmembrane conductance regulator-mRNA delivery: a novel alternative for cystic fibrosis gene therapy. *J gene medicine* 2013;15: 414-426.
- 121- Cholon, Deborah M. Potentiator ivacaftor abrogates pharmacological correction of Δ F508 CFTR in cystic fibrosis. *Science translational medicine* 2014;6: 246ra96-246ra96.

- 122- Carlile, Graeme W. Correction of F508del-CFTR trafficking by the sponge alkaloid latonduine is modulated by interaction with PARP. *Chemistry & biology* 2012;19: 1288-1299.
- 123- Wang, Lei. PEGylated nanostructured lipid carriers (PEG-NLC) as a novel drug delivery system for biochanin A. *Drug development and industrial pharmacy* 2014;0: 1-9.
- 124- Kassebaum, Nicholas J. Global, regional, and national levels and causes of maternal mortality during 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. *The Lancet* 384.9947 2014: 980-1004.
- 125- Pakunlu RI, Wang Y, Saad M, Khandare JJ, Starovoytov V, Minko T. In vitro and in vivo intracellular liposomal delivery of antisense oligonucleotides and anticancer drug *J Control Release*. 2006;114(2):153-62.
- 126- Garbuzenko OB, Saad M, Betigeri S, Zhang M, Vetcher AA, Soldatenkov VA, Reimer DC, Pozharov VP, Minko T. Intratracheal versus intravenous liposomal delivery of siRNA, antisense oligonucleotides and anticancer drug *Pharm Res*. 2009;26(2):382-94.
- 127- Grasemann, Bernhard, Harry Fritz, and Jean-Claude Vannay. Quantitative kinematic flow analysis from the Main Central Thrust Zone (NW-Himalaya, India): implications for a decelerating strain path and the extrusion of orogenic wedges. *Journal of Structural Geology* 1999; 21: 837-853.
- 128- Servetnyk, Zhanna. Activation of chloride transport in CF airway epithelial cell lines and primary CF nasal epithelial cells by S-nitrosoglutathione. *Respiratory research* 2006;7: 124.
- 129- Peier, Andrea M. A TRP channel that senses cold stimuli and menthol. *Cell* 2002; 108: 705-715.
- 130- Brudey, Karine. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC microbiology* 2006; 6: 23.
- 131- Boucher RC. Cystic fibrosis: A disease of vulnerability to airway surface dehydration. *Trends Mol Med*. 2007; 13: 231–240

- 132- Choi JY. Synergistic airway gland mucus secretion in response to vasoactive intestinal peptide and carbachol is lost in cystic fibrosis. *J Clin Invest.* 2007; 117: 3118–3127.
- 133- Van Goor, Fredrick. Correction of the F508del-CFTR protein processing defect in vitro by the investigational drug VX-809. *Proceedings of the National Academy of Sciences* 2011; 108: 18843-18848.
- 134- FDA Draft Briefing Document for the Cardiovascular and Renal Drugs Advisory Committee (CRDAC)
- 135- Helwa, Youssef, Neeshma Dave, and Juewen Liu. "Electrostatically directed liposome adsorption, internalization and fusion on hydrogel microparticles." *Soft Matter* 9.26 (2013): 6151-6158.