Chapter 2:

Literature Review

2.1 Cystic Fibrosis:

Cystic fibrosis (CF) is the most common, life-shortening genetic disease in Caucasians. It affects the transport of salt and water across cells and affects different organs such as lungs, intestines, pancreas and liver. However, lung disease dominates the burden of care and clinical picture. The clinical symptoms involve difficulty in breathing, coughing mucus and frequent lung infections and loss of life. The disease is common cause of fear in northern European ancestry. There has not been much progress in treatment alternatives of the disease, however, the disease was found to be inherited and gene that causes the disease was identified and sequenced way back in 1989.

2.1.1 Epidemiology

CF comes under the category of gene disorder with autosomal recessive dominance of hereditary pattern. It is known to be the most common lethal genetic disease of Caucasian population. One in every 29 individuals is reported to be the carrier of the disease. Prior to 1960s the median length of survival of CF patient was less than 5 years; however, with dramatic gains in effective treatment, the current expected median survival has increased to 41.4 years. More than 10 million Americans are untraced, symptomless carriers of the defective CF gene [1, 2]. As per the estimates more than 30000 children and adults in US (70000 worldwide) have CF, while each year more than 1000 new cases are diagnosed. The worst part of the scenario is that more than 75% of CF cases are diagnosed at age 2 and consequently nearly half of the population is aged near 18 or older [2-4].

The major population is localized in Europe, and North America while in Asia and Africa the condition is less prevalent. Though the exact prevalence in Europe is not reported it has been estimated around 8000 to 10000 individuals [5, 6]. Moreover, CF is increasingly being reported in non-white populations, in regions familiar with CF as well in South and East Asia, Africa, and Latin America. It occurs equally in male and female and there is no gender based predisposition. Though it is reported to most be common in Caucasian of Northern European ancestry it occurs in persons of all racial and ethnic backgrounds. Due to the high frequency of occurrence, from 2010 onwards it became necessary to subject the newborn to screening for CF, as it may also help in early diagnosis and to reinforce with immediate treatment measures to reduce cost associated

with disease complication if diagnosed lately. The average cost for CF treatment in US in 2006 was more than $48000. Along with hospitalization the medications also form big part of expenditure. Every year 1 in 3 patients get hospitalized due to pulmonary exacerbation resulting from infections and has to be treated with intravenous antibiotics. Besides cost the treatment burden is also huge as an average CF patient spends more than two hours a day for undergoing requisite therapies to maintain their health.

2.1.2 Pathogenesis

CF is caused by mutation in a gene. The genetic defect was unidentified until 1989 [7]. However, with the advent of positional cloning, mutations were localized to 250000-bp gene on chromosome 7 [8]. The gene product of 1480-amino acids was called as cystic fibrosis transmembrane conductance regulator (CFTR). There are more than 1000 mutations reported to be responsible for CF which are grouped according to the structural and functional effects on CFTR. The most common mutation accountable for 70% of CF alleles is a 3-bp deletion in axon 10 resulting in deletion of phenylalanine at position 508 in CFTR protein (Δ508) [9].

The CFTR is a chloride channel regulated by cAMP dependent protein kinase and adenosine triphosphate. The channel is expressed in apical membrane of epithelial cells [10]. All the mutations hamper chloride secretion through defect in protein production, protein processing, regulation and conduction. In addition, they also interact with sodium channel to control the water- and salt-content of the liquid protecting the airways called as airway surface liquid (ASL). CFTR exchanges chloride ions between the cytoplasm and the airway lumen [11]. The water- and salt-content of airway secretion is also based on epithelial sodium channel (ENaC). The defective CFTR causes an increased ENaC-mediated sodium uptake from the luminal secretions of the airways, depleting the ASL and making it thick and viscous which leads to a defective mucociliary clearance characterizing the pathophysiologic complications of CF [12].

CF is a multisystem disease affecting one or more organs such as the pancreas, lungs, liver and reproductive organs. However, lungs are clinically most inflicted organs, accounting for 90% of deaths due to lung disease. In normal lungs, mucocilliary clearance is responsible for removing particulates, inorganic debris, airborne bacteria, and viruses [13]. The inefficient clearing of particulates and inflammatory reactions result

in mucus plugging and pulmonary obstruction. The hyperinflation and airway obstruction lead to bronchiectasis. As a consequence, the airways are progressively colonized by specific pathogenic bacteria such as *P aeruginosa*, followed by *Staphylococcus aureus, Haemophilus influenzae,* and *Stenotrophomonas maltophilia.* Ultimately, chronic bronchopulmonary infection occurs which damages lungs and leads to death [14].

2.1.3 Co-morbidities with CF

As stated earlier, CF is a multisystem disease which also leads to pathological changes in organs that express CFTR, including secretory cells, sinuses, lungs, pancreas, liver, and reproductive tract. This results in co-morbidities such as:

Cystic fibrosis-related diabetes (CFRD)

The primary effect of CF leading to exocrine insufficiency makes CFRD increasingly common in adults with CF. It affects 40–50% of adult CF patients and 20% of adolescents, and has impact on declining pulmonary function and overall mortality.

Fertility

The fertility of both males and females is affected in CF with different pathophysiological processes. Approximately, 95% of males with CF suffer from obstructive azoospermia due to congenital bilateral absence of the vas deferens.

CF liver disease (CFLD)

CFLD affects approximately 30% of CF adults due to CFTR malfunction in the epithelial lining of the bile ducts and gallbladder. However, only a smaller number succumb to cirrhosis and decompensated disease.

Osteoporosis

The decreased physical activity, decreased exposure to sun, and decreased absorption of both calcium and vitamin D in CF patients may lead to osteoporosis.

2.1.4 Diagnosis

CF was referred to in medieval folklore, which mentions infants with salty skin who were considered "bewitched" because they routinely died an early death. Salty skin is now recognized as a sign of CF. The clinical signs for the purpose of diagnosis include:

- Chronic productive cough,
- Airway colonization with pathogens (*S aureus, mucoid P aeruginosa*)
- Persistent abnormalities on chest radiograph,
- Airway obstruction, Clubbing,
- Pan sinusitis,
- Nasal polyps

The diagnostic tests involve neonatal screening and combination of common clinical tests while confirmation is done through genetic and CFTR functional tests. The neonatal screening involves measuring immunoreactive trypsinogen at the time of the neonatal heel prick test. If the concentration is raised, genetic testing and a sweat test will be performed to confirm the diagnosis. The genetic testing involves genotyping the most common CFTR mutations. In sweat test, a chloride concentration > 60 mmol/L in sweat is considered indicator of CF. Normal values for chloride concentration are < 30 mmol/litre. The functional test for CFTR involves testing of nasal potential difference (NPD). The NPD measures the transepithelial potential difference / voltage generated by the transport of charged ions across the nasal membranes. The subjects are identified from the pattern of NPD tracing.

2.1.5 Treatment

The advent of technology has led to many new ideas for causative treatment, but at present treatment of CF is largely symptomatic.

2.1.5.1 Airway clearance techniques

The CF patient require to be managed by multidisciplinary group of experienced healthcare professionals in specialist center. Airway clearance techniques are important aspect of CF treatment [15]. Activated cycle of breathing technique and autogenic drainage are also among these techniques. The techniques are supported by a number of

devices to create positive expiratory pressure with or without airway oscillation. High-frequency chest wall oscillation vests is similar kind of technique [16].

2.1.5.2 Chronic pulmonary drugs

In order to reduce the viscoelasticity of sputum mucolytic agents can be used. Recombinant human DNase (rhDNase) is the only licensed mucolytic. The reports suggest that rhDNase could prolong life in CF [17, 18]. Osmotic agents such as hypertonic saline, 7% twice daily, have been reported to reduce exacerbation rate by drawing water to the cell surface [19] Inhaled mannitol also benefits mucociliary clearance and has been licensed in Europe in 2011[20]. Long-term azithromycin, is a well-established treatment for CF proven for reducing lung function decline and exacerbation rate [21].

2.1.5.3 Gene therapy

Despite important therapeutic advances, the definitive treatment for CF remains elusive. CF is a good candidate for gene therapy because it is relatively common, lethal and monogenic and it does not have adequate treatment options. Further, the heterozygotes appear to be phenotypically normal, expression of CFTR is low and lungs are accessible through non-invasive techniques [22].

2.2 CF Gene Therapy

This basic strategy for gene therapy involves complementation or augmentation of mutant alleles with wild-type CFTR. One of the most straight forward approach involves delivery of wild-type CFTR gene to lung epithelium. Such attempts in transgenic animals have shown correction of chloride transport. As lung is the center of CF pathophysiology, gene can be administered ex vivo – where cells are harvested first, genetically modified, and then returned to the body (used for adenosine deaminase deficiency). However, ease of accessibility of lung allows direct lung gene therapy through use of gene therapy vectors applied directly to the epithelial cells leading to expression of wild-type CFTR.

Preclinical studies of gene therapy using both viral and non-viral gene delivery vectors have been shown to correct chloride ion transport in transgenic mice. The vector can be classed into: adenovirus, adenovirus associated virus and cationic lipids or

polymers. These trial have used lung as target for gene delivery due to its direct relevance to afflicted organs, as well as upper respiratory system including nasal and maxillary sinus epithelium [23, 24]. The virus mediated gene transfer involves administration of the virus to the lung epithelium. However, the results reported are not always consistent and only few show some changes in chloride transport. They also report dose dependent mild local inflammation and progressive lack of expression following repeated administration [25]. Thus, none of these defective gene correction have been found very promising from clinical point of view.

2.2.1 Problems with CFTR gene transfer

Cells to Target

The epithelial cells at all the levels of distal airways contain the CFTR protein, however, the highest levels are found in submucosal glands of the proximal cartilaginous airways. This finding raises the problem about which cells in the entire airway should be targeted for gene correction. Nebulization or aerosol treatments most often reach the surface epithelium, but is less likely to submucosal glands. Moreover, the terminally differentiated nature of surface epithelium results in loss of gene expression even after successful transfection after the death of transfected cell, which is generally about days. There are certain putative stem cells present in respiratory tract, however, they are not exposed to airway lumen and are difficult to access. Researchers are searching for other such cells. Recently the Clara cells, present distally on the surface of smaller airways have been identified as a progenitor cell [26]. Trials need to be conducted on effects of gene transfer to such cells to evaluate their potential.

Degree of Gene Transfer

The CFTR levels found in overall lung are low compared to other organs where CFTR is expressed such as kidney. Studies on normal phenotype of CF heterozygotes indicates that CFTR levels of ~50% are sufficient to induce CFTR function recovery. Some studies have in fact suggested that even lower levels, i.e. less than 10% of wild type cell correction with CFTR gene may correct for chloride ion transport abnormality of CF monolayer. However, none of the studies till date have observed correction of sodium ion

hyperabsorption even after correction of chloride ion transport. These findings and observations *in vitro* have led to conclusion that almost 100% of cells should be corrected for wild-type CFTR so as to get normalized sodium ion hyperabsorption [27]. Thus, gene transfer may result in correction of the cellular defect, but the levels required to correct the manifestations at phenotype and functions levels are difficult to achieve.

2.3 ENaC as therapeutic target CF

The ENaC channel expressed in the apical membranes mediate the absorption of Na^+ in epithelial lining of distal nephrons, ducts of secretory glands, distal colon and respiratory airways [28]. It mediate uptake of Na^+ from the luminal side into the cells using a gradient maintained by basolateral Na^+/K^+-ATPase to. High affinity to the K^+ sparing diuretic amiloride (K 1/2 ~ 0.1 μM) is also a characteristic of these channels. They have a very slow gating in range of seconds as depicted by their very low values of single-channel conductance of around 3–5 pS [29]. A total of four subunits, called (α, β, γ and δ), sharing a homology of 35% at the amino acid level assemble to form the complete channel [30]. However, it was observed that, α-subunit alone can form a low conductance functional channel, the importance of β-and γ–subunits is for regulatory mechanism required for proper channel gating.

The channel activity can be upregulated by hormones involved in sodium homeostasis such as vasopressin and aldosterone [31, 32]. the δ-ENaC subunit can also form amiloride-sensitive Na^+ channel, when co-expressed with β and γ accessory subunits, but with different currents than that of the classical (α, β and γ) subunit composition [32]. It also differ in tissue distribution as δ-subunit is expressed mainly in brain, heart and pancreas and is also detected in respiratory epithelium [33]. A tetrameric structure of two α-subunits, one β-and one γ –subunit is argued to be the functional unit of ENaC, however, nonameric (α3, β3 and γ3) and trimeric are also among speculated channel stoichiometries [34, 35]. Apart from CF, the ENaC defects is also responsible to cause disease such as Liddle's syndrome and pseudohypoaldosteronism [36].

CF epithelia are pathologically characterized for an extreme increase of Na^+ absorption mediated through ENaC. The hyperabsorption of Na^+ mediated through ENaC by β-ENaC overexpression in mouse model was sufficient to develop symptoms of cystic fibrosis lung disease [37]. Attempts have been made to correct Na^+ hyperabsorption using

ENaC inhibitors such as amiloride and benzamil [38]. Moreover, positive benefits were reported on airway inflammation in β-ENaC-overexpressing mice when amiloride therapy was used as a preventive therapy in structurally normal lung [39]. On the other hand, effects of amiloride and benzamil provided transient relief to patients already suffering from CF symptoms. It has also been reported that amiloride inhalation therapy leads to an improvement in the rheological properties of mucus favouring airway mucus clearance and can retard the decline in lung function [40].

Sobczak etc al. have reported specific antisense oligonucleotides (AON) directed against the α-subunit of ENaC can block nonselective cation channels in rat alveolar type II cells and human B lymphocytes [41]. As there are no ideal animal model for ENaC hyperabsorption the studies were performed on primary cultured cells. The study concluded that ENaC-specific AON could overcome limitations of the conventional amiloride inhalation therapy in CF [42]. The importance of ENaC mediated increased Na^+ absorption in airway and subsequent ASL volume depletion in progress and pathogenesis of CF has been demonstrated by pulmonary phenotype studies on βENaC-overexpressing mouse. This also indicated involvement of ENaC in epithelial necrosis, transient airway eosinophilia and emphysema observed in CF and suggested a possible benefit of amiloride, amiloride, benzamil and phenamil in CF [43].

However, the trial with aerosol inhalation therapy with classical ENaC blockers failed to show significant therapeutic benefits [44]. This has been attributed to short elimination time and lack of potency of amiloride. Further, the extent to which the treatment can reverse the symptoms of CF is not reported anywhere including the effects on metaplasia of goblet cell, hypersecretion of mucus, inflammation and remodelling of tissue in chronically diseased lung. Therefore, it was hypothesized that the therapeutic response would have been better if the treatment was used as preventive in structurally normal lung rather than using on chronic diseased state. Zhou et al. subsequently tested the hypothesis and found better effects of amiloride therapy with intranasal administration in β-ENaC overexpressing mice model treated from the first day after birth [39]. The intranasal administration of amiloride resulted in reduced pulmonary mortality, airway mucus obstruction, epithelial necrosis, goblet cell metaplasia, and airway inflammation in mice.

Jain et al. tried to manipulate the expression of ENaC protein using ASOs targeting each of the three subunits and evaluated by patch-clamp technique to study the effect on single-channel functioning [41]. The results showed that α-subunit is the primary component of non-selective lung epithelial cation channels. The different channels might be composed of different forms of ENaC family, however, in each at least one protein, α-subunit, is common. Hummler et al. reported that α-subunit can independently lead to assembly and expression of functional active sodium channel while beta and gamma subunits by themselves are unable to induce an amiloride-sensitive Na^+ current. Due to which, inactivating the mouse α-ENaC gene leads to defective neonatal lung liquid clearance leading to respiratory distress and death within 40 h of birth [45]. Canessa et al. and Ismailov et al. reported the primary structure of the α-subunit of the rat ENaC by expression cloning in *Xenopus laevis* oocytes and found that α-subunit itself can form fully functional amiloride-sensitive Na^+ channels, whereas the same is not possible with β- and γ-subunits individually or in combination [32, 46]. Kizer et al. studied α-subunit expressed in the ENaCs from osteoblasts null cell line (LM TK2) and reported presence of a nonselective cation channel with conductance of 24.2 pS. They concluded that the α-subunit inhibition reduces cation-channel density but same was not with inhibition of β- and γ-subunits [47].

2.4 RNA interference

RNA intereference (RNAi) is part of endogenous enzymatic machinery of most eukaryotic cells based on small double stranded RNA (dsRNA) to control the gene activity. Small interfering RNA (siRNA) siRNA are 21-22 bp long dsRNA molecules which are capable of recognizing endogenous machinery with help of characteristic 2 nucleotide 3' overhang and complementary binding leading to degradation of target mRNA molecules. In recent times researchers showed inclination towards use of siRNA for knockdown of gene by RNAi, since it is much more potent than other antisense approaches such as antisense DNA, ribozymes and oligonucletides [48].

siRNAs are also endogenously produced by RNaseIII endonuclease dicer mediated cleavage of longer dsRNA precursors. Dicer cleaves dsRNA and pre-miRNA into siRNA and microRNA respectively. It then activates the RNA-induced silencing complex (RISC) containing the catalytic component named Argonaute 2, which is an

endonuclease that cleaves the target mRNA molecules backbone between bases 10 and 11 from the 5' end of the antisense siRNA strand [49, 50]. Argonaute 2 unwinds the double stranded siRNA loaded into it and cleaves the passenger strand/sense strand. The differential thermodynamic stability at ends of the siRNAs decide the selectivity of strand loading into RISC. The unwinding is directed from the less thermodynamically stable end of the 5' end of the guide strand which binds to the PIWI domain of Ago-2. The activated form of RISC now contains the single-stranded guide/antisense strand that directs the complementary binding by intermolecular base pairing and brings out degradation of the target mRNA [51, 52]. The activated RISC complex can destroy additional mRNAs, which further potentiates gene silencing [53]. This effect can extend the therapeutic benefit for 3–7 days in rapidly multiplying cells, and even for several weeks in non-multiplying cells [54]. Concomitantly, siRNA gets diluted below therapeutic concentration or degraded within the cells, which demands repeated administration for sustained effect.

A perfect to near perfect complementarity to the guide RNA strand is enough to be recognized and cleaved by Argonaute 2. Partial complementarity has been reported to suppress translation or destabilize transcripts as in case of micro-RNAs. These are 60-70 bp hairpins and are derived from pri-miRNAs by endogenous processing within the nucleus by the microprocessor [55]. In cytoplasm RNase III Dicer further processes it and one of the two strands is loaded into RISC. The miRNAs differ from siRNA as they bear only a partial complementarity with target sequences in the 3'UTR of mRNAs. While the primary mechanism of action was reported to be translational repression along with mRNA degradation. RNAs have potential advantages over the other therapeutic methods such as specificity, safety and the generation of the interferon response of antisense therapy [56].

Antisense oligonucleotides technology (AONs) is also an anti-mRNA strategy, used to prevent its translation into protein. Similar to RNA interference, AON have same effect that leads to the degradation of target mRNA, but the mechanism of action of AON in mammalian cells differs from RNAi technology. AON are single stranded-DNA molecules, consisting of 15-20 nucleotides, which are complementary to a specific target mRNA. AONs act by several mechanisms and a single mechanism cannot be defined

which mostly depends on the chemical composition of AON [57, 58]. Mostly after reaching cell, the single stranded AON induce RNase H to bind mRNA-AON complex in cytoplasm and nuclease to cleave mRNA progressively. On the other hand, siRNA act in an elegant fashion through four sequential steps involving incorporation into RISC protein complex, ATP dependent unwinding, ATP independent binding to mRNA and irreversible cleavage by RNase-III-type activity initiating mRNA degradation [59, 60]. The site of action of AON is in both cytoplasm and nucleus while that of siRNA is in cytoplasm only. The efficacy of AON is directly proportional to AON-target duplex stability [61]. The efficiency of siRNA is function of sequence feature and is inversely proportional to siRNA-target duplex stability [62].

Bertrand et al. have compared the efficiencies of a nuclease resistant AON and of siRNA in cell culture and they reported that siRNAs were quantitatively more efficient as its effect lasts for a longer time in cell culture than AON [63]. Small single stranded antisense RNAs may also be loaded into RISC to target mRNA cleavage directly but the low effectiveness of this approach limits its use [64]. The crucial factor in therapeutic outcome is the higher potency of RNAi that means the effector molecules may function at much lower concentrations of siRNA as compared to AON or ribozymes [64]. Some studies have described that it is promising to administer synthetic siRNAs *in vivo* and knockdown an endogenous target without inducing an interferon response [65]. Thus, siRNA appear to be a better logical choice over AON for suppression of gene expression from therapeutic point of view.

Any disease which involves one or a few genes in its pathogenesis is amenable for RNAi-based therapy. The RNAi machinery can be exploited to treat any of such diseases with appropriate design of siRNA, making it broad therapeutic agent than the typical small-molecules. There are many reports and studies conducted on use of synthetic siRNA to knock down defective genes in various diseases such as hypercholesterolaemia, liver cirrhosis, hepatitis B virus (HBV), human papilloma-virus, ovarian cancer and bone cancer [66-70].

2.5 Delivery of siRNA

Though the structure of siRNA and DNA contains basic units of sugar-phosphate backbone connected with nitrogen bases arranged in a double-stranded helical structure,

the difference at chemical level makes it difficult for researchers to extrapolate the hypothesis on DNA to siRNA delivery. siRNA being a RNA, contains ribose sugar rather than deoxyribose which contains 2'-hydroxy group making it more susceptible to hydrolysis by serum nucleases than plasmid DNA [71]. In contrast to plasmid DNAs which are usually large and of the order of several kilo base pairs, siRNAs are often 19-21 base pairs long. This alters charge expression at molecular level and thus requiring a different condensation chemistry for the development of stable non-viral vector for delivery. Compared to DNA, siRNA forms loose complexes with cationic vector as because of its stiff structure and low charge density [72, 73]. From the delivery aspects, the site of action of plasmid DNA is intra-nuclear, while that of siRNA is in cytosol which demands vectors differing in their intracellular trafficking. These differences in physicochemical and biological characteristics of siRNA and DNA make it mandatory to separately choose and optimize a delivery vector for both [74].

2.5.1 Barriers in siRNA delivery

The delivery of macrolmolecules to the target site, tissue or organ is major hurdle in development of siRNA therapies. Further, the unfavourable physicochemical properties makes it a challenging task for researchers. siRNA are negatively charged and have large molecular weight and size due to which it cannot readily cross the cellular membrane. Naked siRNA is also highly unstable in plasma with plasma half-lives of only 10 min [70]. Further, after endocytosis, siRNA is transported to early endosomes which convert into late endosomes and finally fuse with lysosomes, where it gets degraded.

The complexity and accessibility to the desired site of action is major determinant of siRNA efficacy. Mostly, the local or regional tissue targets tender fewer barriers compared to systemic delivery of siRNA. Bitko et al. attempted intranasal inhalation of siRNA against respiratory syncytial virus, they reported that siRNA was having similar effectiveness in reducing the viral infection, in either naked or encapsulated in polycationic liposomes [75]. There are similar reports of delivery to eye, skin, lung, and brain [76]. When DNA encoding for short hairpin RNA (shRNA) was delivered to the brain following intravenous administration with PEGylated immunoliposomes in weekly intravenous doses enables a 90% knockdown of the human epidermal growth factor receptor [77].

The chemical instability of siRNA is another potential concern in development of siRNA delivery systems. Due to presence of 2'-hydroxy group siRNA backbone is chemically labile to hydrolysis. Further, naked siRNA is readily degraded by serum endonucleases and can be eliminated by glomerular filtration, leading to a short plasma half-life of <10 min [78, 79]. The problem of chemical instability has been partially overcome by introducing chemical modifications in RNA backbone which do not adversely affect the bioactivity of siRNA [80]. The introduction of 2'F, 2'O-Me and 2'H substitutions in the RNA backbone can increase serum stability. However, more convenient approach would be to encapsulate or complex the nucleic acid using nano-sized carriers which can protect them.

Tissue transport to the cellular target has always been a hurdle for macromolecular therapeutics. Small molecules undergo ttransport through the interstitial space by diffusion (governed by concentration gradient), whereas large molecules have to travel by convection (governed by hydraulic conductivity and pressure difference). In case of tumours, the lack of lymphatic system increase interstitial pressure and thereby blocks convective flow. The higher interstitial pressure results in outward convective pressure from the core of the tumour to the surrounding normal tissues [81].

Crossing the anionic cell membrane as such is limited by negative charge of siRNA. In addition, the high molecular weight, large size worsens the task. Passive diffusion, as in case of small molecules, is difficult for siRNA. Therefore, the only way for internalization is through endocytosis, a natural phenomenon of "cell eating", wherein, macromolecules can be internalized by forming part of cell membrane. The presence of cell surface receptors can be taken as advantage to increase the efficiency of endocytosis or specifically target a particular cell in a tissue [82, 83]. In case of siRNA, the cationic vectors are capable of interacting with oppositely charged anionic proteoglycans on the cell membrane surface, which subsequently forms an endocytic vesicle, and finally enters the cells through endocytosis [84].

Even after successful internalization siRNA face the challenge of escaping the endocytic vesicles before getting degraded in the late endosomes. The nanoparticle is transported along micro-tubules to lysosomes which are co-localized with micro-tubule organizing centre. The endo cytotic vesicle fuses with early endosomes which convert

into late endosomes and finally fuse with lysosomes. The type of surface receptors decides the destiny of the internalized molecules taken up inside the vesicle which may become: recycled to the cell membrane surface, degraded inside harsh environment of lysosomes, or released to other intracellular compartments such as cytosol [85]. Various approaches have been listed to induce endosomal escape, such mechanisms include: endosomal membrane pore formation, pH-buffering (proton sponge hypothesis) effect of protonable groups and lipid bilayer fusion with endosomal membrane [86]. Several bacterial and viral proteins are reported to manipulate this process. There are chemical agents and photochemical methods reported to induce bursting of endosomal membrane. A lot of research has been focused on use of biomimetic peptides and polymers with improved endosomal escape capacity. However, each strategy has specific requirements and challenges which need to be individually dealt with when applying them for escape from endosomes.

The mechanisms of endosomal escape:

1. Pore formation in the endosomal membrane: This mechanism is found in the endosomal escape mediated by bacterial endotoxins [87]. The cationic amphiphilic peptides when bound to lipid bilayer reduces line tension responsible for closing the pore which leads to creation of pores in the lipid membrane. The peptide cluster gets oriented perpendicular to the plane of the lipid bilayer to stabilize the formation of the pore [88, 89].
2. Proton sponge effect: This effect is induced by agents with high buffer capacity in the endosomal pH range and can swell when protonated. Protonation in endosomes neutralizes the pH and causes extensive influs of ions and water to counterbalance the effect which ultimately leads to build up of osmotic pressure, swelling and rupture of the endosomal membrane leading to release of entrapped components [90]. The chemical agents known for this effect are: tertiary amine groups with hydrophobic structural segment, histidine rich molecules, poly(amido amine) polymers etc. [91].
3. Fusion in the endosomal membrane: Fosogenic peptides is a class of peptides which cause destabilization of the endosomal membrane with change in their conformation. Conventionally, viruses have single integral membrane peptides

which respond to change in pH through conformational changes [92]. This conformational change helps the protein to stimulate the fusion in lipid bilayer. Haemagglutinin, influenza virus coat peptide is present as an anionic hydrophilic coil at pH 7.4, which gets altered to a hydrophobic helical conformation in response to acidic endosomal pH. The α-helical structural conformation brings about fusion of viral membrane with cell membrane [93].

4. Photochemical disruption of the endosomal membrane: Several photosensitizers, such as TPPS4, TPPS2a, AlPcS2a and dendrimer-based photo-sensitizer (dendrimer phthalocyanine are confined predominantly in the endosomal or lysosomal membrane [94]. When triggered by light these induce reactive singlet oxygen formation having short lifetime which destroys the membranes of endosomes or lysosomes. The contents of the organelles usually remain intact behind and are released into the cytosol. This technique has been found useful in delivery based on lipid carriers [95] and polymers [96].

The typical dose requirements and dosing regimen for siRNA should depend on transfection efficiency and type of transfected cells. A transiently transfected cell in culture can show gene silencing for 3-5 days. However, a non-dividing cell can display sustained silencing for several weeks [97]. Most of the trials include duplexed 21-mer RNA species; however, there are reports that use of longer (25-27mer) RNA species which may modify the potency of siRNA. As they undergo intracellular processing by channeling the duplexes through Dicer, which hands overs siRNAs to RISC and may reducing the required concentrations of siRNAs for achieving a therapeutic effect and thereby such processing can increase the potency [98, 99].

2.5.2 Role of vectors in delivery of siRNA

Vectors can be broadly classified into: viral and non-viral. The non-viral vectors are preferred over viral vectors due to potential toxicity issues with viral vectors. Most of the non-viral vectors are prepared by complexing anionic siRNA with positively charged carriers such as, cationic cell penetrating peptides, cationic polymers, dendrimers, and cationic lipids or conjugating siRNA with cholesterol, bile acids and lipids, antibodies or encapsulating siRNA in nanoparticulate preparations. However, the final choice of

delivery systems depends on characteristics of siRNA, target cell or tissue/organ and delivery route chosen for *in vivo* application.

2.5.2.1 Liposomes and lipid-like materials.

Phospholipids in aqueous environments have a tendency to form lipid bilayer spheres with aqueous cores. They are composed of two sets of polar head groups which form the outer surface of bilayer sphere and inner surface of core. The phospholipids with cationic head groups can be used for siRNA delivery. The examples of such lipids include: DOTAP-- Dioleoyl-trimethylammoniumpropane, DOTMA-- N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl-ammonium chloride, DORIE-- 1,2-dioleoyloxypropyl-3-N,N'-dimethyl-N'-hydroxyethyl ammonium bromide, DOSPA-- N,N-dimethyl-N-[2-sperminecarboxamido)ethyl]- 2,3-bis(dioleyloxy)-1-propanimium pentachloride, DOGS- dioctadylamidoglycylspermine, GAP-DLRIE --N-(3-aminopropyl)-N,N-dimethyl- 2,3-bis(dodecyloxy)-1-propaminium bromide [74].

The transfection efficiency and toxicity of liposomal delivery systems is influenced by the nature of cationic head group, the carbon chain length of the tail group, and linker. In general, lipids with small hydrophilic head group and bulky alkyl chains favor the endosomal escape and enhance the transfection efficiency. The positive head groups can efficiently condense siRNA on the surface. The condensation can be optimized based on lipid and siRNA ratio so as to retain fraction of residual positive charge to facilitate its interaction with the cell membrane and the subsequent endocytosis. In addition, use of neutral helper lipids such as DOPE, due to its molecular shape, can promote formation of inverted hexagonal phase (promoting inverted micelle formation) [70, 100]. The neutral helper lipids induce membrane fusion with the endosomes and assist in endosomal escape. Few have attempted to overcome the poor circulation life using PEGylation. However, it has been reported that PEGylation leads a decrease in cell internalization capacity or surface receptor recognition.

More recently, stable nucleic acid–lipid particle (SNALP) have been suggested for siRNA delivery. siRNA against hepatitis B virus RNA was incorporated in specialized liposome to form SNALP and administered through intravenous route in mice at a dose of 3 mg/kg/day for three days. This resulted in reduction of serum HBV levels by one log unit and it lasted for 7 days after dosing [101]. Liposomes have been among the preferred

nucleic acid delivery carriers; however, they suffer from some issues regarding safety from therapeutic point of view. Cationic lipid have been reported to be toxic both *in vitro* and *in vivo*. The success of commercial liposomal formulations such as doxorubicin liposome, amphotericin liposome, vincristine liposome etc. shows the potential of liposomal siRNA delivery for future clinical use.

2.5.2.2 Polymers

As the success of siRNA based therapy is largely dependent on the development of carrier which should deliver siRNA to the target site efficiently, safely, and repeatedly if required [102]. A model carrier for siRNA therapy should be capable to bind, condense and protect siRNA to facilitate its intracellular uptake into the target cells following the escape from the endosome/lysosome, release of siRNA into cytosol, and lastly should induce effective gene knockdown. Engineering of polymeric carriers so as to inculcate all or some of these attributes is being tried, and had been widely used for siRNA delivery. Cationic polymers, specifically with linear or branched backbone chemistry are reported to be very efficient in transfection due to their ability to bind and condense macromolecular gene/nucleic acids into nanoparticles.

Though the cationic charge forms the basis of application of polymers for gene delivery, the cationic charge is also responsible for toxicity associated with polymers. Further, excessive positive charge may lead to difficulties in release of nucleic acid at the site of action. Therefore, as an alternative to electrostatic condensation, nucleic acid can be entrapped within biodegradable matrices. Such biodegradable material contains labile ester linkage along their backbone e.g. PLGA, which can hydrolytically degrade to oligomeric or monomeric components. Further, they are also amenable for release rate manipulation through alteration of polymer characteristics. However, potential limitation such as low loading efficiencies, entrapment efficiency, nucleic acid degradation from low microenvironment pH and processing difficulties limit the application of biodegradable polymers in nucleic acid delivery.

Therefore, several attempts have been made to prepare biodegradable cationic polymers or subject the cationic polymers to chemical modifications to decrease their toxicity and enhance transfection efficiency [103-105]. Though they have been shown to yield better performance, most of the cases involve tedious synthetic route for

preparation. Therefore, simple modification yielding superior vector properties is always desired [106]. However, it should be noted that polymer modification approaches based on charge reduction to reduce toxicity may destroy the cell interaction and buffer capacity of the polymer. Therefore, the modifications should be aimed to favorably induce the physiochemical properties without affecting the essential ones. For chemical modification PEI is of very much interest due to its proven efficacy but high toxicity is a limitation from clinical application point. Similarly, biodegradable polymers such as chitosan have been subjected to modifications to improve their limited transfection capacity.

2.5.3 Polyethylenemine

Various cationic polymers such as polyethylenimine (PEI) [107, 108] poly-(L-lysine) [109], polyamidoamine [110], poly(2-(di-methylamino)ethyl methacrylate) [111] have been reported for gene delivery. Compared with cationic liposomes, they have the obvious advantage of compressing DNA/siRNA molecules to a robust and relatively small size which can sustain in harsh environmental conditions specifically applied during applications such as inhalation and aerosolization techniques. PEI is one of the most effective non-viral vectors among all the polymer based gene delivery vectors till date [112]. It is a synthetic polymer composed of ethyleneimine monomers. PEI was initially used for waste water treatment during pulp manufacture [113]. However, it was since 1990s, that PEI was recognized as a gene carrier because of its polycationic characteristics.

There are two different forms of PEI i.e. branched and linear. The linear form solely consists of secondary amines in the backbone and primary amine at terminal end. In contrast branched form contains all three 1°, 2°, and 3° amines in particular ratio of 1:2:1 respectively [114, 115]. This ratio is important from transfection point. The efficiency of PEI originates from two primary aspects i.e. the cationic charge for interaction with nucleic acid and negatively charged cell membranes, and excellent buffer capacity in endosomal pH range to induce endosomal escape [91, 107]. The amines of PEI backbone form a buffer system containing primary secondary and tertiary amines of polymer, which resists change in pH in response to endosomal acidification. The proton

uptake is counter-balanced with influx of chloride which leads to build up of osmotic pressure, swelling of endosome and subsequent rupture.

The difference in physico-chemical properties leads to change in transfection, and toxicity profile of the polymer. The linear form has been reported to be more efficient *in vitro* and *in vivo* than the branched when complexes were prepared in salt containing buffer due to kinetic instability under salt conditions [116]. However, branched PEI shows higher transfection *in vitro* because of stronger condensation [108]. The transgene expression after treatment with 22 kDa/ DNA/glucose was 22 kDa/ DNA/glucose due to their small size and stability [117, 118]. The linear PEI showed more transgene expression after intravenous injection; however, the transgene expression of the branched PEI/gene complex may be more efficient in other tissues such as kidney [119, 120]. Wiseman et al. stated that linear PEI have higher transfection efficiency, both *in vitro* and *in vivo*, compared to branched PEI of a similar molecular weight [121]. They compared effect of airway delivery using linear 22 or 25 kDa PEI with DCChol/DOPE liposome after intranasal instillation. The luciferase expression with 22 kDa PEI was 350 fold in lung, 180-fold in the nose and 85-fold in the trachea compared to liposome. However, the 25 kDa form of PEI was insignificant compared to liposome.

However, exposure to cationic charge results in disruption of biological membranes. This has been attributed as primary reason for observed toxicity of PEI and cationic materials. In PEI the positive charge at physiological pH is result of protonation of most basic primary amines [122]. Thus the branched PEI, with more primary amines per polymer would be conceivably more toxic than linear PEI. Coll et al. reported that LPEI-mediated gene transfection to the lung demands application of near-lethal dose of LPEI that transfection is unavoidably accompanied by activation of the endothelium of lung. The high toxicity was apparent within 15 min after injection of 50 mg of LPEI/DNA in form of shock, which was tolerated by 95% of the Swiss nude mice. When dose was increased to 100 mg further enhancement of transfection was observed, however, animals faced significant liver injury. The linear form is devoid of any branching on the flexible backbone of the chain. It has been reported that flexible polymers are better at DNA condensation than rigid polymers [123]. This property is

particularly important in maintaining integrity of siRNA in application requiring harsh conditions such as dry powder inhalation, aerosol and sprays etc. [124].

Modifications of polymeric backbone that reduce the positive charge of PEI have been attempted to reduce the toxicity and improve transfection efficiency of the polymer. Recent studies have been focused on the acetylation of PEI in order to reduce the membrane disruptiveness and associated toxicity. However, this approach, though reduced toxicity, was found to reduce the buffering capacity required during endosomal capacity [125]. Further, PEGylation of PEI was also reported to reduce surface charge, increased dispersion stability at high concentrations, decrease protein binding and erythrocyte aggregation, and prolong blood circulation. However, such modification lead to decrease in binding affinity between DNA/siRNA and the polymer which may need higher quantities of polymer to deliver same required dose and subsequently higher body exposure to non-biodegradable polymer [126-128]. Kichler et al. prepared covalent conjugate of the LPEI with polyethyleneglycol, they reported a decrease in binding affinity, however; the observed improvements were not significant compared to naked siRNA [129]. Further some have reported backbone modification to reduce the transfection efficiency [130].

The ionization behavior of polymers is major determinant of vector performance as it governs the properties such as particle size and surface charge, proton sponge, transfection and toxicity. In spite of this, there has been very scarce work done on determination of protonation behavior of PEI, both free in solution and bound with DNA. The reported values in literature are inconsistent. Von Harpe et al. and Choosakoonkriang et al. have reported the chain length dependence of pKa's for PEI samples in range of 8.2 to 9.5 [131, 132]. Ogris et al. have reported more than 90% protonation at physiologic pH [133]. A comparison of protonation behavior for linear, star-like, and comb-like PEI by a group of researchers using titrimetric methods found that any form of PEI, at pH = 7.4, contains ~50% of the amine groups in protonated form. Further, doublet and triplet interactions, as a result of protonation of nearest neighbor amine groups may complicate the protonation behavior with pH. Studies on polyamines structurally similar to PEI showed that they have no less than 50% of the amine group protonated at physiological pH.

In contrast to small molecules, which are separate in solution so that ionization behavior is independent of neighboring molecules, the ionization of polyelectrolytes is influenced by ionization of neighbor group. This is because due to close proximity of atoms protonation state of a site is influenced by the electrostatic affects from the protonation of nearby sites. When in aqueous solution, with decrease in pH, LPEI becomes charged as secondary amines get protonated as shown in fig. 1.

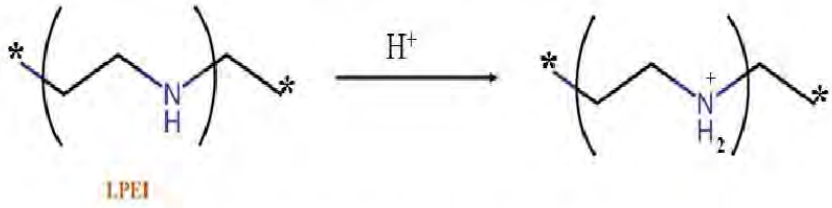

Fig. 2.1: Protonation state of LPEI backbone

However, due to structural peculiarity of LPEI, all the charges are positioned directly on the backbone of the chain. Computational analysis indicated that LPEI contains nearly ~55% amines protonated at physiological conditions and the charges generated on LPEI chain are present in nearly alternating fashion. Implying that any two charged amine are separated by 7 Å, a distance equivalent to Bjerrum length in water. The maximum distance between the neighbor charges is considerably small than any other polyelectrolytes with bulkier charge carriers groups. Due to small distance and axial distribution of charges they are not fully screened by dielectric effects of solvents. When two charged nearest-neighbor monomeric groups become charged they are called doublet which further dominate electrostatic effects [134, 135]. Further, the presence of oppositely charged object is reported to induce ionization and influence the degree of ionization of polyelectrolyte at a given pH. Thus, presence of negatively charged nucleic acid molecule can induce ionization in cationic polymer. This fact should be considered in design of polyelectrolyte based vector development.

Now as chain becomes progressively charged, there would be a cumulative build-up of multiple charge-charge interactions. These interaction over the small distance, is source of significant deviations from protonation behavior of polyelectrolyte from that of an independent single site. This deviation leads to an apparent shift in pKa values (if we

still monitor protonation of individual site) [134]. The relative impact of shift in pKa can be understood from small molecules with known pKa for individual site. Table 2.1 shows effect of distance of separation between amine nitrogen on second protonation constant for multi-amines.

Table 2.1: Comparison of shift of second protonation constant in multi-amines

Diamine	Distance	Experimental Data**	
		pKa_1, pKa_2**	ΔpKa
NH_2-$(CH2)_2$-NH_2	3.5	9.89, 7.05	2.81
NH_2-$(CH2)_3$-NH_2	4.6	10.56, 8.76	1.80
NH_2-$(CH2)_4$-NH_2	5.6	10.72, 9.44	1.28
NH_2-$(CH2)_5$-NH_2	6.7	10.78, 9.85	0.93
NH_2-$(CH2)_6$-NH_2	7.5	10.97, 10.09	0.88

*Average distance between amine nitrogens from atomistic molecular dynamics simulation

**Data from ref [136]

For example, ethylenediamine, the ΔpKa is large, meaning that a significant neighbor group interaction leading to suppression of ionization of second amine when first is protonated. In contrast, ΔpKa value for propylenediamine is small due to increased distance of separation between neighbor groups which reduced the electrostatic interaction between the two neighbor sites. As shown in table 1, the ΔpKa becomes smaller as distance between amines is increased. Finally, from pentamethylenediamine to hexamethylenediamine there is insignificant change in ΔpKa. Thus, impact of protonation states of neighboring site amines in LPEI will be significant due to small distance of separation as only two methylene units separate the nitrogen and a significant deviation from individual pKa can be expected.

Therefore, a reasonable way to maintain the balance between efficiency and cytotoxicity is to alter the ionization behavior of LPEI. The modification should be attempted to reduce the charge density however, it should not adversely affect properties such, binding affinity of polymer to siRNA/DNA, hydrophilicity, colloidal stability, buffer capacity and endosomal escape, and consequently the transfection capacity.

2.5.4 Chitosan

Chitosan is a linear copolymer of β (1, 4) bound 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose units which is produced by chitin alkaline N-deacetylation process (1, 4). Chitosan can be characterized by a number of physicochemical properties such as molecular weight, viscosity, degree of deacetylation and crystallinity [137].The solution properties of chitosan are function of molecular weight, content of acetylated amino groups and its distribution, ionic strength and pH. The pKa value for the amine in chitosan has been reported to be around 6.5 [138, 139]. It has been reported that the distribution of acetylated and deacetylated units are random along the chain [140, 141]. The reported LD_{50} of chitosan in rats is 16 g/kg [142] which depicts its non-toxic nature in animal [143] and also in human [144].

Recently, chitosan based non-viral vectors were used as a potential carrier for gene therapy i.e. plasmid DNA, siRNA and oligonucleotide, mainly because of its safe, low immunogenic, biocompatible properties and transfection efficiency owing to its high positive charge density [145, 146]. This high positive charge is capable of forming polyelectrolyte complexes through electrostatic interaction with negatively charged pDNA, siRNA and oligonucleotide. However, formulation related parameters significantly affect the efficiency of chitosan for gene delivery. Chitosan is insoluble at neutral and alkaline pH and soluble in acidic media such as acetic acid, glutamic acid, citric acid, hydrochloric acid, aspartic acid and lactic acid. In physiological conditions enzymes such as lysozymes or chitinase can easily digest chitosan, which are produced by the normal flora in the intestine [147, 148] or are present in the blood [149].

Several studies have shown the prospectus of chitosan as a carrier for DNA and influencing expression of reporter genes *in vitro* and *in vivo* [150]. Katas and Alpar [151] was the first group to explore the applications of chitosan as a delivery vector for siRNA delivery *in vitro*. The high susceptibility of RNA to enzymatic degradation as compared to DNA may pose difficulties to chitosan based RNA transfer. Additionally, the structure and size of siRNA are less than the pDNA that also can influence the performance of vector and thus various formulation parameters necessities to be optimized in relation to the physicochemical and biological properties of the prepared siRNA complex.

Many studies have been done to correlate MW and cytotoxicity of the chitosan but the results were still debatable. A sequence of toxicity studies reported the concern over toxicity of chitosan, which was proposed to be dependant on its molecular weight, degree of deacetylation and salt form [152, 153]. On the other hand, some investigators claimed that the toxicity of chitosan was minor [154, 155]. Additionally, chitosan of 10–50 kDa molecular weight was reported to be promising as gene transfer vector because chitosan with molecular weight >100 kDa showed a reduction in transfection efficiency compared to 15 and 52 kDa [156].

pKa of the amine functionality of chitosan is related by inverse function to the degree of deacetylation (DD). The effect is further accentuated with increase in the extent of amine protonation [157]. The pKa values of the amine functional group on chitosan (when extrapolated to 100% amine protonation) are shown in fig. 2.2. It can be seen that, from pH 7.2 to 4.4 as the DD value increases from 54 to 100% the pKa value of the amine units on chitosan drops progressively.

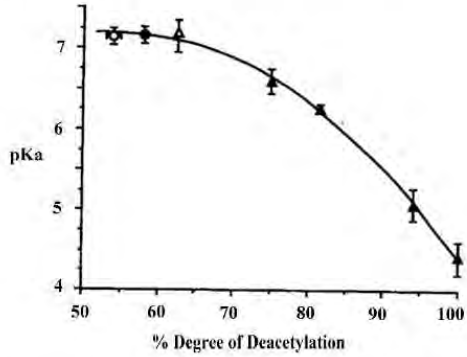

Fig. 2.2: pKa values of chitosan with degree of deacetylation

However, most of the commercially obtainable chitosan have quite large MWs. Generally, low molecular weight chitosan can be prepared from high molecular weight chitosan by depolymerization using enzymatic degradation method, oxidative degradation, acidic cleavage and ultrasonic degradation. The rate of molecular weight degradation has been reported to be inconsistent during the time course of ultrasound treatment. On the other hand, nitrous acid (HONO) based deplymerization offers several advantages for preparing low molecular chitosan compared to chemical and non-

chemical methods reported [158]. The reaction is reproducible due to its selective nature, further it is rapid and can be readily controlled. Moreover, the chemistry of treatment of HONO with carbohydrate amines is traditionally been used to elucidate the structure of sugars and therefore, the reaction products, conditions and stoichiometry and are well established [158]. The mechanism of depolymerization of chitosan proceeds through a rate-limiting nitrosation step involving attack of nitrous acidium ion on unprotonated amine of chitosan. The following Fig. 2.3 depicts the overall stoichiometry of the reaction between HONO and chitosan.

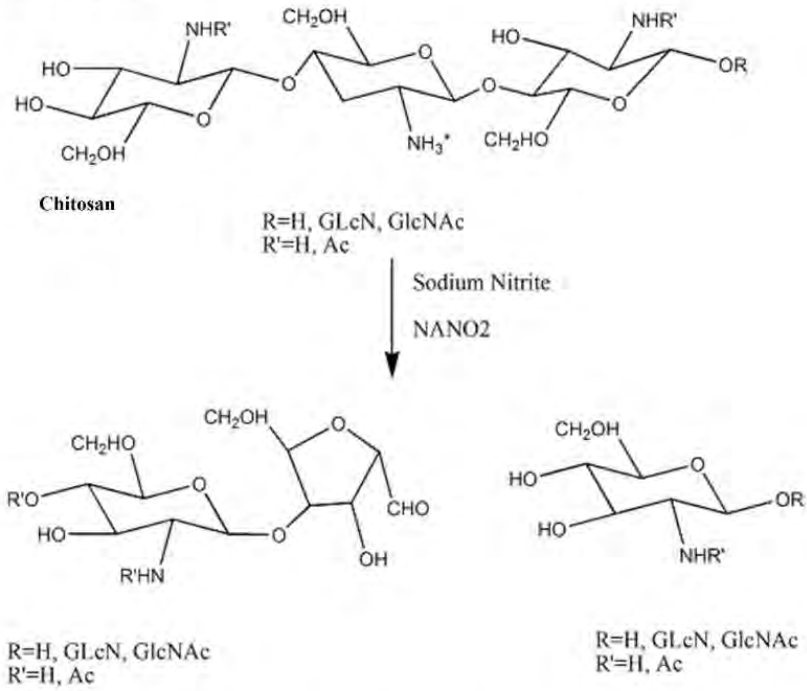

Fig. 2.3: Mechanism of nitrous acid mediated depolymerization of chitosan

Nitrous acidium ion (nitrosating species) attacks the amine, and not N-acetyl group, leading to cleavage of the 3-glycosidic bond. HONO and amine group react at 1:1 mole ratio to form a 2,5-anhydro-D-mannose unit at the reducing end of the cleaved polymer. A number of deamination products have been reported to be formed with carbohydrate amines, no such side reactions have been reported with chitosan [159, 160]. Even in mildly acidic solutions and at room temperature conditions the

deamination reaction proceeds very rapidly [161, 162]. Moreover, due to solubility of chitosan in dilute solutions, the reaction with HONO is homogeneous.

Liu et al compared $NaNO_2$, H_2O_2, and HCl induced depolymerization of chitosan and reported that $NaNO_2$ was best choice amongst the three [163]. Chitosan with larger molecular weight were found to be more susceptible to depolymerization. Moreover, during depolymerization process there was no structural change which was proved by IR spectrum and 1H NMR spectroscopy. According to the literature, depolymerization using nitrous acid does not change the DD, because HONO attacks the amine groups, rather than the N-acetyl group, and consequently cleaves the β-glycosidic linkages, without producing any side reactions. The solubility of chitosan was increased with decrease in molecular weight due to reduction in inter chain interactions. However, it was reported that molecular weight has no effect on the cytotoxicity of chitosan as observed in MTT assay. Chitosan was non-toxic in cell lines when the concentration was as high as 1 mg/ml.

The stability of chitosan/pDNA polyplexes should be enough to protect them against nuclease degradation and colloidal instability. Using ethidium bromide exclusion experiments and laser light scattering technique, it has been shown that under acidic and low ionic strength conditions the chitosan/pDNA polyplexes were very stable. But, after changing the physiological conditions, these polyplexes tend to form aggregate and decrease in binding affinity for pDNA. When polyplexes were prepared in 15 mM acetate buffer with pH 5.5, unmodified chitosan was most efficient in DNA condensation when compared to other modified forms [164].

Depolymerization of HMWC can be advantageous to reduce the dose dependent cytotoxicity of chitosan and it also improves its solubility [165]. However, this may diminished the DNA-chitosan complexes stability [166], which presumes that the complexes must resist the attack from cellular environment during the transport towards the target. In many studies, DNA release from weak complexes formed with depolymerized chitosan improved transfection efficiency, compared to that achieved with HMWCs [166].

Contrary to these results, failure of depolymerized chitosan to give protection to condensed DNA from degradation by enzymes in serum milieu and poor uptake of DNA

complexes have also been shown [167], which is responsible for their low transfection efficiency [167]. The depolymerization chitosan is accompanied with a reduction in binding affinity with the nucleic acids. HMWC effectively condenses siRNA at lower weight ratios due to its large molecular weight. Literature values of dissociation constant (Kd) obtained through isothermal titration calorimetry are less than 1 for chitosan with MW 44 kDa, which sharply increases to 1.5 and 1.9 for MW of 63 and 93 kDa respectively, indicating high affinity [168]. This results in premature release of nucleic acids due to displacement from completion from endogenously present polyanions which subsequently get degraded by nucleases. Therefore, attempts in direction to overcome limitations of depolymrized chitosan is cotemporary requisite.

2.6 Pulmonary delivery

The pulmonary route has gained much attention for delivery of therapeutics and genomics due to its capability for local delivery of sufficiently high concentrations of the drug, with minimal side effects [169, 170]. Further, due to rich blood supply, thin absorptive mucosal membrane and large absorptive surface area, the route is of interest for systemic treatments as well [171]. The peculiar anatomical organization demands aerosol as primary dosage form.

For delivery of siRNA there are several ways for local delivery. Amongst them inhalation is most preferred and easiest method for delivery. The liquid aerosol or dry powder aerosols are most compatible for siRNA application. In addition to inhalation, intranasal and intra-tracheal routes are often used mostly in animal studies.

2.6.1 Physiological considerations

The respiratory system consists of two regions:

1) Nasal cavity, pharynx, trachea, bronchi and bronchioles form the conducting region which are responsible for air conductance; while,

2) Respiratory bronchioles and alveoli form the respiratory region which is responsible for gaseous exchange.

The major anatomical barrier to delivery point is the high degree of branching. According to lung model for aerosol delivery, as devised by Wiebel, there are 23 bifurcations in total forming a network of airways with varying length and diameter [172-174]. In addition,

lower region of the lungs are mostly affected in the lung disorders, making it the actual target for delivery. Further the site of deposition is significantly influenced by particle aerodynamic size. Depending on their aerodynamic size the particles are deposited by either of inertial impaction, sedimentation, diffusion (Table 2). In addition the table shows that, airstream characteristics and residence time also governs collection efficiency at every stage of lung. Thus, at larger size, inertial impaction and sedimentation are important while at nano-scale the diffusional mobility plays an important role. For deposition in lower respiratory tract the optimal particle size is between 1-5 μ [175].

Table 2.2: Factors governing deposition of particles in lung

	Diffusion	**Sedimentation**	**Inertial impaction**
Particles	Small particles < 0.5 μ	Intermediate particle	Large and dense > 6 μ
Collection efficiency	Size and Residence time	Aerodynamic size, residence time	Aerodynamic size, velocity,
Airstream characteristic	High residence times	Low velocity and high residence time	High velocity and turbulent
Location	Alveolar region	Bronchiole and alveoli	Oropharynx, larynx, airway bifurcations

Another potential physiological barrier is mucociliary clearance action of the ciliated epithelial cells, and the presence of mucus, alveolar fluid and macrophages. The ciliated cells will rapidly remove any particle deposited on its surface which are eventually coughed up or swallowed. Further, the respiratory epithelium is lined with mucus from nasal cavity to the terminal bronchioles. It presents the physiologic barrier as a result of high viscosity of lung epithelial surface limiting the drug penetration and diffusion. The alveoli are bathed in thin layer of surface liquid consisting of phospholipids and other surfactant proteins. These pulmonary surfactant have been reported to severely interfere with the lipid based nucleic acid delivery systems compared to polymer based delivery systems. This implies that polymer based systems would perform better than lipid based systems in lung delivery [176-178]. The alveolar macrophages are capable of engulfing foreign particles, including nucleic acids by phagocytosis, and it is degraded inside the cells. Moreover, the physiologic state of lung

is also influenced by disease state and underlying pathophysiology. Infection and inflammatory diseases lead to increase in mucus secretion and impaired ciliary clearance. The thickness of airway surface fluid, viscosity and composition may also get affected in some disease.

All these factors should be considered while designing delivery systems. Various delivery platforms attempt to overcome these barrier. The perfect choice of aerodynamic diameter helps to deposit in desired region of lung. The macrophage uptake can be avoided by creating large porous particles over 10 μm in geometric diameter but aerodynamic diameter meeting desired deposition site. Mucolytic agents have been used to breakdown mucus or with use of mucus inhibitors [179]. Inhaled mannitol has been clinically reported to increase the mucus clearance in patients with cystic fibrosis or bronchiectasis [180]. Due to its osmotic properties it also improves hydration and surface properties of sputum [181]. Thus, mannitol inhalation prior to the delivery of siRNA or using mannitol as adjuvant in siRNA formulation may thus be beneficial.

After overcoming the extracellular barriers siRNA reaches to the surface of target cells of respiratory tract. Here, it depends on endocytosis for entry inside the cells. Particles smaller than 150 nm are more efficient in endocytosis. Further, this size can also escape macrophage uptake and delay lung clearance. If particles undergo clathrin-mediated endocytosis they enter the cell enclosed in clathrin-coated vesicles. These are subsequently converted into early endosomes, which fuse to become late endosomes and subsequently converted into lysosomes. In order to be effective, the siRNA must be able to escape from the endosomes/lysosomes and get released into cytoplasm before getting degraded.

2.6.2 Pulmonary delivery platforms

2.6.2.1 Inhalation route

This is most popular due to non-invasive nature of administration. There are three major aerosolization systems; nebulizers, meter dose inhaler (MDI), and dry powder inhaler (DPI). With suitable modifications these devices can be made compatible for siRNA delivery.

Nebulizers

These are the oldest aerosol devices and still have the importance for generating continuous stream of liquid droplets for easy penetrability in size range of 1-5 um. They offer ease of use due to no need of synchronization of actuation and inhalation which also eliminates any training for users [182]. It is most preferred method for administering high dose antibiotics [183]. However, during nebulization high shear stress is generated repeatedly as 99% of the generated aerosol droplets are recirculated back into the reservoir, which can induce degradation of nucleic acid. This combined with lesser stability of biomolecules in liquid form than dry form makes it unsuitable for siRNA delivery otherwise the vector chosen should be capable of protecting it from high shears.

Metered dose inhalers

These are designed to delivery discrete doses to respiratory tract in the form of aerosol. It uses an actuator to dispense a metered dose of 25 - 100 µL of liquid containing suitable amount of active ingredient [184]. The propellant undergoes flash evaporation from discharged liquid droplets to produce drug having desired aerodynamic size [185]. These are considered to be "the most intricate dosage form used in medicine today" as their performance is result of combination of formulation, container, metering valve and actuator performance [186]. However, the compatibility of propellant with formulation is a potential concern. The formulation is generally presented in the form of suspension or solutions. The suspensions are the preferred one, as propellants are non-polar liquids in which most drugs have poor solubility. Similar to nebulizers, MDIs also present high shear to the formulation, and therefore, may not be the best direction for developing inhalable siRNA.

Dry Powder Inhaler

DPIs presents drugs for inhalation in the form of clouds of dry particles in air stream which is drawn through the device by inspiratory action of patient. In contrast to MDIs they are devoid of dependence of coordination between drug aerosolization and inspiration. This method has been successfully used to deliver therapeutic macromolecules such as insulin [187], parathyroid hormone [188].

However, formulating as DPI for siRNA presents considerable challenges as it demands not only flowability and dispersibility of the powders but also the retention of biochemical efficacy of the conformationally sensitive macromolecules. The problem can be addressed by formulating macromolecules using lyophilization or spray drying and subsequently processing them into flowable and dispersible powder as reported in literature [189-192]. Spray drying is economically more feasible process than lyophilisation. However, for formulating siRNA in DPI a need for suitable vector for protecting it from shear of spray drying is required. The size of lyophilized or spray dried product can be carefully adjusted to improve deposition in respiratory tract. Improved stability and sterility of macromolecules is the key benefit offered by DPIs liquid aerosols. The delivery performance of PDIs also varies with different device designs. However, for final efficacy of the formulation the patient inspiratory flow rate also needs to be taken into consideration. In addition, the problems of de-aggregation and agglomeration of dry powders should be addressed.

Although inhalation becomes the most preferred way to deliver siRNA to the lungs; however, none of the clinical study on siRNA therapy is administered by inhalation. Intratracheal or intranasal route has been used in most of the *in vivo* studies, which could be due to difficulty in developing effective inhalable siRNA, retaining bioactivity during processing and storage [193].

2.6.2.2 Intratracheal route

This is most commonly used for administering to respiratory tract of animals. However, the method is described as non-physiological and surgery based uncomfortable makes it unsuitable for human administration. In case of animals, they have to be anaesthetized and trachea is exposed through which an endotracheal tube is or needle is inserted projecting its tip at a defined position just before tracheal bifurcation. Using a microsyring the drug solution can be instilled into the airways [194].

A non-invasive method has been described using a microsprayer inserted endotracheally to deliver the aerosol into lungs under anaesthesia. Otherwise, animal intubation through mouth and trachea using a catheter or needle can be used to instil solution or suspension form of the medicine. As these procedures are done through mouth these are called as oro-tracheal administration [195]. Many studies have reported intra-

tracheal route for administration of siRNA [196-198]. The intratracheal route results in minimal loss of drug and provides high delivery efficiency. This is a good advantage for any proof-of-concept study. However, since this route is an artificial way to deliver drugs and it results in no-uniform deposition of drug compared to inhalation [172]. It also eliminates oropharynx deposition and concomitant drug loss. All these factor obscure the effect of aerosol size, the critical factor in DPI development, on lung deposition making it difficult to compare and evaluate the delivery efficiency of particulate formulation.

2.6.2.3 Intranasal route

This is another non-invasive route of administration to lungs and it has been reported in number of studies [199-202]. To the deeply anaesthetized animal the formulations are administered drop-wise to the naris to be breathed. Zang et al. used this route to administer naked siRNA to inhibit the expression of HO-1 gene in injured lung of mouse [201]. However, as there is significant difference in the anatomy of physiology of mice and human lungs the efficacies observed in mice cannot be extrapolated to human use. Since mice are obligate nasal breathers, a high proportion of nasal dose is deposited in lung. Further, the anaesthetics have been reported to impair the mucociliary clearance in these animals which might overestimate the *in vivo* efficacy of formulations [203]. Heyder et al. tried the feasibility of this route in humans and found that majority of particles deposited in nose while only 3% of 1 –5 μm particles deposited in bronchial airways through nose breathing [204]. However, this route has been used in clinical trial to deliver siRNA in treatment of diseases of upper respiratory tract such RSV infection. In addition the large surface area offered by this route has long remained an incentive to explore for systemic delivery of siRNA.

2.7 References

1. De Boeck K, Zolin A, Cuppens H, Olesen HV, Viviani L. The relative frequency of CFTR mutation classes in European patients with cystic fibrosis. J Cyst Fibros 2014; 13(4): 403-9.
2. FitzSimmons SC. The changing epidemiology of cystic fibrosis. The Journal of pediatrics 1993; 122(1): 1-9.
3. Cystic fibrosis Epidemiology, http://www.datamonitorhealthcare.com/files/2014/08/Sample-package-Cystic-Fibrosis-Epidemiology.pdf, accessed on
4. De Boeck K, Zolin A, Cuppens H, Olesen HV, Viviani L. The relative frequency of CFTR mutation classes in European patients with cystic fibrosis. Journal of cystic fibrosis 2014; 13(4): 403-09.
5. Farrell PM, Rosenstein BJ, White TB, Accurso FJ, Castellani C, Cutting GR, et al. Guidelines for diagnosis of cystic fibrosis in newborns through older adults: Cystic Fibrosis Foundation consensus report. The Journal of pediatrics 2008; 153(2): S4-S14.
6. Ouyang L, Grosse SD, Amendah DD, Schechter MS. Healthcare expenditures for privately insured people with cystic fibrosis. Pediatr Pulmonol 2009; 44(10): 989-96.
7. Riordan JR. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Trends in Genetics 1989; 5: 363.
8. Rommens JM, Iannuzzi MC, Kerem B-s, Drumm ML, Melmer G, Dean M, et al. Identification of the cystic fibrosis gene: chromosome walking and jumping. Science 1989; 245(4922): 1059-65.
9. Wagner JA, Gardner P. Toward cystic fibrosis gene therapy. Annu Rev Med 1997; 48: 203-16.
10. Stutts MJ, Canessa CM, Olsen JC, Hamrick M. CFTR as a cAMP-dependent regulator of sodium channels. Science 1995; 269(5225): 847.
11. Welsh MJ, Smith AE. Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. Cell 1993; 73(7): 1251-54.

12. Dalemans W, Barbry P, Champigny G, Jallat S, Jallat S, Dott K, et al. Altered chloride ion channel kinetics associated with the ΔF508 cystic fibrosis mutation. Nature 1991; 354(6354): 526-28.
13. Smith JJ, Travis SM, Greenberg EP, Welsh MJ. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. Cell 1996; 85(2): 229-36.
14. Pier GB, Grout M, Zaidi TS, Olsen JC, Johnson LG, Yankaskas JR, et al. Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections. Science (New York, NY) 1996; 271(5245): 64.
15. Ramsey BW. Management of pulmonary disease in patients with cystic fibrosis. New England Journal of Medicine 1996; 335(3): 179-88.
16. Kerem E, Conway S, Elborn S, Heijerman H, Committee C. Standards of care for patients with cystic fibrosis: a European consensus. Journal of cystic fibrosis 2005; 4(1): 7-26.
17. Shah PL, Scott SF, Knight RA, Marriott C, Ranasinha C, Hodson ME. In vivo effects of recombinant human DNase I on sputum in patients with cystic fibrosis. Thorax 1996; 51(2): 119-25.
18. Fuchs HJ, Borowitz DS, Christiansen DH, Morris EM, Nash ML, Ramsey BW, et al. Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. The Pulmozyme Study Group. N Engl J Med 1994; 331(10): 637-42.
19. Elkins MR, Robinson M, Rose BR, Harbour C, Moriarty CP, Marks GB, et al. A controlled trial of long-term inhaled hypertonic saline in patients with cystic fibrosis. New England Journal of Medicine 2006; 354(3): 229-40.
20. Bilton D, Daviskas E, Anderson SD, Kolbe J, King G, Stirling RG, et al. Phase 3 randomized study of the efficacy and safety of inhaled dry powder mannitol for the symptomatic treatment of non-cystic fibrosis bronchiectasis. CHEST Journal 2013; 144(1): 215-25.
21. Saiman L, Marshall BC, Mayer-Hamblett N, Burns JL, Quittner AL, Cibene DA, et al. Azithromycin in patients with cystic fibrosis chronically infected with

Pseudomonas aeruginosa: a randomized controlled trial. Jama 2003; 290(13): 1749-56.

22. 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(11): 1373-93.
23. Crystal RG, McElvaney NG, Rosenfeld MA, Chu CS, Mastrangeli A, Hay JG, et al. Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. Nat Genet 1994; 8(1): 42-51.
24. Perricone MA, Morris JE, Pavelka K, Plog MS, O'Sullivan BP, Joseph PM, et al. Aerosol and lobar administration of a recombinant adenovirus to individuals with cystic fibrosis. II. Transfection efficiency in airway epithelium. Hum Gene Ther 2001; 12(11): 1383-94.
25. Harvey BG, Leopold PL, Hackett NR, Grasso TM, Williams PM, Tucker AL, et al. Airway epithelial CFTR mRNA expression in cystic fibrosis patients after repetitive administration of a recombinant adenovirus. J Clin Invest 1999; 104(9): 1245-55.
26. Warburton D, Wuenschell C, Flores-Delgado G, Anderson K. Commitment and differentiation of lung cell lineages. Biochemistry and cell biology 1998; 76(6): 971-95.
27. Johnson LG, Boyles SE, Wilson J, Boucher RC. Normalization of raised sodium absorption and raised calcium-mediated chloride secretion by adenovirus-mediated expression of cystic fibrosis transmembrane conductance regulator in primary human cystic fibrosis airway epithelial cells. Journal of Clinical Investigation 1995; 95(3): 1377.
28. Alvarez de la Rosa D, Canessa CM, Fyfe GK, Zhang P. Structure and regulation of amiloride-sensitive sodium channels. Annu Rev Physiol 2000; 62: 573-94.
29. Kellenberger S, Schild L. Epithelial sodium channel/degenerin family of ion channels: a variety of functions for a shared structure. Physiol Rev 2002; 82(3): 735-67.

30. Rossier BC, Pradervand S, Schild L, Hummler E. Epithelial sodium channel and the control of sodium balance: interaction between genetic and environmental factors. Annu Rev Physiol 2002; 64: 877-97.
31. Waldmann R, Champigny G, Bassilana F, Voilley N, Lazdunski M. Molecular cloning and functional expression of a novel amiloride-sensitive Na+ channel. J Biol Chem 1995; 270(46): 27411-4.
32. Canessa CM, Schild L, Buell G, Thorens B, Gautschi I, Horisberger JD, et al. Amiloride-sensitive epithelial Na+ channel is made of three homologous subunits. Nature 1994; 367(6462): 463-7.
33. Bangel-Ruland N, Sobczak K, Christmann T, Kentrup D, Langhorst H, Kusche-Vihrog K, et al. Characterization of the epithelial sodium channel delta-subunit in human nasal epithelium. Am J Respir Cell Mol Biol 2010; 42(4): 498-505.
34. Stockand JD, Staruschenko A, Pochynyuk O, Booth RE, Silverthorn DU. Insight toward epithelial Na+ channel mechanism revealed by the acid-sensing ion channel 1 structure. IUBMB Life 2008; 60(9): 620-8.
35. Snyder PM, Cheng C, Prince LS, Rogers JC, Welsh MJ. Electrophysiological and biochemical evidence that DEG/ENaC cation channels are composed of nine subunits. J Biol Chem 1998; 273(2): 681-4.
36. Schild L. The epithelial sodium channel: from molecule to disease. Rev Physiol Biochem Pharmacol 2004; 151: 93-107.
37. Mall M, Grubb BR, Harkema JR, O'Neal WK, Boucher RC. Increased airway epithelial Na+ absorption produces cystic fibrosis-like lung disease in mice. Nat Med 2004; 10(5): 487-93.
38. Hofmann T, Stutts MJ, Ziersch A, Ruckes C, Weber W, Knowles MR, et al. Effects of topically delivered benzamil and amiloride on nasal potential difference in cystic fibrosis. American journal of respiratory and critical care medicine 1998; 157(6): 1844-49.
39. Zhou Z, Treis D, Schubert SC, Harm M, Schatterny J, Hirtz S, et al. Preventive but not late amiloride therapy reduces morbidity and mortality of lung disease in βENaC-overexpressing mice. American journal of respiratory and critical care medicine 2008; 178(12): 1245-56.

40. Tomkiewicz RP, App EM, Zayas JG, Ramirez O, Church N, Boucher RC, et al. Amiloride inhalation therapy in cystic fibrosis. Influence on ion content, hydration, and rheology of sputum. Am Rev Respir Dis 1993; 148(4 Pt 1): 1002-7.
41. Jain L, Chen XJ, Malik B, Al-Khalili O, Eaton DC. Antisense oligonucleotides against the alpha-subunit of ENaC decrease lung epithelial cation-channel activity. Am J Physiol 1999; 276(6 Pt 1): L1046-51.
42. Sobczak K, Segal A, Bangel-Ruland N, Semmler J, Van Driessche W, Lindemann H, et al. Specific inhibition of epithelial Na+ channels by antisense oligonucleotides for the treatment of Na+ hyperabsorption in cystic fibrosis. J Gene Med 2009; 11(9): 813-23.
43. Mall MA. Role of the amiloride-sensitive epithelial Na+ channel in the pathogenesis and as a therapeutic target for cystic fibrosis lung disease. Exp Physiol 2009; 94(2): 171-4.
44. Pons G, Marchand MC, d'Athis P, Sauvage E, Foucard C, Chaumet-Riffaud P, et al. French multicenter randomized double-blind placebo-controlled trial on nebulized amiloride in cystic fibrosis patients. The Amiloride-AFLM Collaborative Study Group. Pediatr Pulmonol 2000; 30(1): 25-31.
45. Hummler E, Barker P, Gatzy J, Beermann F, Verdumo C, Schmidt A, et al. Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice. Nat Genet 1996; 12(3): 325-8.
46. Ismailov II, Awayda MS, Berdiev BK, Bubien JK, Lucas JE, Fuller CM, et al. Triple-barrel organization of ENaC, a cloned epithelial Na+ channel. Journal of Biological Chemistry 1996; 271(2): 807-16.
47. Kizer N, Guo XL, Hruska K. Reconstitution of stretch-activated cation channels by expression of the alpha-subunit of the epithelial sodium channel cloned from osteoblasts. Proc Natl Acad Sci U S A 1997; 94(3): 1013-8.
48. Morris KV, Chan SW, Jacobsen SE, Looney DJ. Small interfering RNA-induced transcriptional gene silencing in human cells. Science 2004; 305(5688): 1289-92.
49. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 2001; 409(6818): 363-6.

50. Rand TA, Ginalski K, Grishin NV, Wang X. Biochemical identification of Argonaute 2 as the sole protein required for RNA-induced silencing complex activity. Proc Natl Acad Sci U S A 2004; 101(40): 14385-9.
51. Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell 2005; 123(4): 607-20.
52. Ameres SL, Martinez J, Schroeder R. Molecular basis for target RNA recognition and cleavage by human RISC. Cell 2007; 130(1): 101-12.
53. Hutvagner G, Zamore PD. A microRNA in a multiple-turnover RNAi enzyme complex. Science 2002; 297(5589): 2056-60.
54. Bartlett DW, Davis ME. Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. Nucleic Acids Res 2006; 34(1): 322-33.
55. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116(2): 281-97.
56. Dykxhoorn DM, Novina CD, Sharp PA. Killing the messenger: short RNAs that silence gene expression. Nat Rev Mol Cell Biol 2003; 4(6): 457-67.
57. Altmann K-H, Fabbro D, Dean N, Geiger T, Monia B, Müllert M, et al. Second-generation antisense oligonucleotides: structure—activity relationships and the design of improved signal-transduction inhibitors. Biochemical Society Transactions 1996; 24(3): 630-37.
58. Giles RV, Ruddell CJ, Spiller DG, Green JA, Tidd DM. Single base discrimination for ribonuclease H-dependent antisense effects within intact human leukaemia cells. Nucleic Acids Res 1995; 23(6): 954-61.
59. Nykänen A, Haley B, Zamore PD. ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 2001; 107(3): 309-21.
60. Hall J. Unravelling the general properties of siRNAs: strength in numbers and lessons from the past. Nature Reviews Genetics 2004; 5(7): 552-57.
61. Couvreur P, Malvy C. Pharmaceutical aspects of oligonucleotides: CRC Press; 2003.

62. Forte A, Cipollaro M, Cascino A, Galderisi U. Small interfering RNAs and antisense oligonucleotides for treatment of neurological diseases. Current drug targets 2005; 6(1): 21-29.
63. Bertrand J-R, Pottier M, Vekris A, Opolon P, Maksimenko A, Malvy C. Comparison of antisense oligonucleotides and siRNAs in cell culture and in vivo. Biochemical and biophysical research communications 2002; 296(4): 1000-04.
64. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001; 411(6836): 494-98.
65. Heidel JD, Hu S, Liu XF, Triche TJ, Davis ME. Lack of interferon response in animals to naked siRNAs. Nat Biotechnol 2004; 22(12): 1579-82.
66. Frank-Kamenetsky M, Grefhorst A, Anderson NN, Racie TS, Bramlage B, Akinc A, et al. Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates. Proceedings of the National Academy of Sciences 2008; 105(33): 11915-20.
67. Sato Y, Murase K, Kato J, Kobune M, Sato T, Kawano Y, et al. Resolution of liver cirrhosis using vitamin A–coupled liposomes to deliver siRNA against a collagen-specific chaperone. Nature biotechnology 2008; 26(4): 431-42.
68. Song E, Lee S-K, Wang J, Ince N, Ouyang N, Min J, et al. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat Med 2003; 9(3): 347-51.
69. Halder J, Kamat AA, Landen CN, Han LY, Lutgendorf SK, Lin YG, et al. Focal adhesion kinase targeting using in vivo short interfering RNA delivery in neutral liposomes for ovarian carcinoma therapy. Clinical Cancer Research 2006; 12(16): 4916-24.
70. Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. Nat Rev Drug Discov 2009; 8(2): 129-38.
71. Gogotsi Y. Nanomaterials handbook: CRC press; 2006.
72. Kwok A, Hart SL. Comparative structural and functional studies of nanoparticle formulations for DNA and siRNA delivery. Nanomedicine: Nanotechnology, Biology and Medicine 2011; 7(2): 210-19.

73. Hong CA, Nam YS. Functional nanostructures for effective delivery of small interfering RNA therapeutics. Theranostics 2014; 4(12): 1211-32.
74. Vhora I, Patil S, Amrutiya J, Misra A. Liposomes and Lipid envelope-type systems for systemic siRNA delivery. Curr Pharm Des 2015; 21(31): 4541-55.
75. Bitko V, Musiyenko A, Shulyayeva O, Barik S. Inhibition of respiratory viruses by nasally administered siRNA. Nat Med 2005; 11(1): 50-55.
76. Fattal E, Bochot A. Ocular delivery of nucleic acids: antisense oligonucleotides, aptamers and siRNA. Advanced Drug Delivery Reviews 2006; 58(11): 1203-23.
77. Pardridge WM. shRNA and siRNA delivery to the brain. Advanced Drug Delivery Reviews 2007; 59(2): 141-52.
78. Van de Water FM, Boerman OC, Wouterse AC, Peters JG, Russel FG, Masereeuw R. Intravenously administered short interfering RNA accumulates in the kidney and selectively suppresses gene function in renal proximal tubules. Drug metabolism and disposition 2006; 34(8): 1393-97.
79. Hong J, Huang Y, Li J, Yi F, Zheng J, Huang H, et al. Comprehensive analysis of sequence-specific stability of siRNA. The FASEB Journal 2010; 24(12): 4844-55.
80. Chiu Y-L, Rana TM. siRNA function in RNAi: a chemical modification analysis. Rna 2003; 9(9): 1034-48.
81. Jain RK. Transport of molecules in the tumor interstitium: a review. Cancer research 1987; 47(12): 3039-51.
82. Oh N, Park J-H. Endocytosis and exocytosis of nanoparticles in mammalian cells. Int J Nanomedicine 2014; 9(Suppl 1): 51-63.
83. Gao H, Yang Z, Zhang S, Cao S, Shen S, Pang Z, et al. Ligand modified nanoparticles increases cell uptake, alters endocytosis and elevates glioma distribution and internalization. Scientific reports 2013; 3.
84. Goula D, Becker N, Lemkine G, Normandie P, Rodrigues J, Mantero S, et al. Rapid crossing of the pulmonary endothelial barrier by polyethylenimine/DNA complexes. Gene Ther 2000; 7(6).
85. Steinman RM, Mellman IS, Muller WA, Cohn ZA. Endocytosis and the recycling of plasma membrane. The Journal of cell biology 1983; 96(1): 1-27.

86. Varkouhi AK, Scholte M, Storm G, Haisma HJ. Endosomal escape pathways for delivery of biologicals. Journal of Controlled Release 2011; 151(3): 220-28.
87. Mandal M, Lee K-D. Listeriolysin O-liposome-mediated cytosolic delivery of macromolecule antigen in vivo: enhancement of antigen-specific cytotoxic T lymphocyte frequency, activity, and tumor protection. Biochimica et Biophysica Acta (BBA)-Biomembranes 2002; 1563(1): 7-17.
88. Jenssen H, Hamill P, Hancock RE. Peptide antimicrobial agents. Clinical microbiology reviews 2006; 19(3): 491-511.
89. Huang HW, Chen FY, Lee MT. Molecular mechanism of Peptide-induced pores in membranes. Phys Rev Lett 2004; 92(19): 198304.
90. Akinc A, Thomas M, Klibanov AM, Langer R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. J Gene Med 2005; 7(5): 657-63.
91. Creusat G, Rinaldi A-S, Weiss E, Elbaghdadi R, Remy J-S, Mulherkar R, et al. Proton sponge trick for pH-sensitive disassembly of polyethylenimine-based siRNA delivery systems. Bioconjugate chemistry 2010; 21(5): 994-1002.
92. Horth M, Lambrecht B, Khim M, Bex F, Thiriart C, Ruysschaert JM, et al. Theoretical and functional analysis of the SIV fusion peptide. The EMBO journal 1991; 10(10): 2747.
93. Wiley DC, Skehel JJ. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annual review of biochemistry 1987; 56(1): 365-94.
94. Nishiyama N, Arnida, Jang W-D, Date K, Miyata K, Kataoka K. Photochemical enhancement of transgene expression by polymeric micelles incorporating plasmid DNA and dendrimer-based photosensitizer. Journal of drug targeting 2006; 14(6): 413-24.
95. Fretz MM, Høgset A, Koning GA, Jiskoot W, Storm G. Cytosolic delivery of liposomally targeted proteins induced by photochemical internalization. Pharmaceutical research 2007; 24(11): 2040-47.

96. Cabral H, Nakanishi M, Kumagai M, Jang W-D, Nishiyama N, Kataoka K. A photo-activated targeting chemotherapy using glutathione sensitive camptothecin-loaded polymeric micelles. Pharmaceutical research 2009; 26(1): 82-92.
97. Song E, Lee S-K, Dykxhoorn DM, Novina C, Zhang D, Crawford K, et al. Sustained small interfering RNA-mediated human immunodeficiency virus type 1 inhibition in primary macrophages. Journal of virology 2003; 77(13): 7174-81.
98. Rose SD, Kim D-H, Amarzguioui M, Heidel JD, Collingwood MA, Davis ME, et al. Functional polarity is introduced by Dicer processing of short substrate RNAs. Nucleic Acids Res 2005; 33(13): 4140-56.
99. Kim D-H, Behlke MA, Rose SD, Chang M-S, Choi S, Rossi JJ. Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. Nature biotechnology 2005; 23(2): 222-26.
100. Schroeder A, Levins CG, Cortez C, Langer R, Anderson DG. Lipid-based nanotherapeutics for siRNA delivery. Journal of internal medicine 2010; 267(1): 9-21.
101. Morrissey DV, Lockridge JA, Shaw L, Blanchard K, Jensen K, Breen W, et al. Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. Nature biotechnology 2005; 23(8): 1002-07.
102. Kumar LD, Clarke AR. Gene manipulation through the use of small interfering RNA (siRNA): from in vitro to in vivo applications. Adv Drug Deliv Rev 2007; 59(2-3): 87-100.
103. Kim TH, Cook SE, Arote RB, Cho MH, Nah JW, Choi YJ, et al. A degradable hyperbranched poly (ester amine) based on poloxamer diacrylate and polyethylenimine as a gene carrier. Macromolecular bioscience 2007; 7(5): 611-19.
104. Reul R, Nguyen J, Kissel T. Amine-modified hyperbranched polyesters as non-toxic, biodegradable gene delivery systems. Biomaterials 2009; 30(29): 5815-24.
105. Luten J, van Nostrum CF, De Smedt SC, Hennink WE. Biodegradable polymers as non-viral carriers for plasmid DNA delivery. Journal of Controlled Release 2008; 126(2): 97-110.

106. Zintchenko A, Philipp A, Dehshahri A, Wagner E. Simple modifications of branched PEI lead to highly efficient siRNA carriers with low toxicity. Bioconjugate chemistry 2008; 19(7): 1448-55.
107. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proceedings of the National Academy of Sciences 1995; 92(16): 7297-301.
108. Godbey W, Wu KK, Mikos AG. Poly (ethylenimine) and its role in gene delivery. Journal of Controlled Release 1999; 60(2): 149-60.
109. Wolfert MA, Schacht EH, Toncheva V, Ulbrich K, Nazarova O, Seymour LW. Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block co-polymers. Hum Gene Ther 1996; 7(17): 2123-33.
110. Haensler J, Szoka Jr FC. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. Bioconjugate chemistry 1993; 4(5): 372-79.
111. Cherng J-Y, van de Wetering P, Talsma H, Crommelin DJ, Hennink WE. Effect of size and serum proteins on transfection efficiency of poly ((2-dimethylamino) ethyl methacrylate)-plasmid nanoparticles. Pharmaceutical research 1996; 13(7): 1038-42.
112. Abdallah B, Hassan A, Benoist C, Goula D, Behr JP, Demeneix BA. A powerful nonviral vector for in vivo gene transfer into the adult mammalian brain: polyethylenimine. Hum Gene Ther 1996; 7(16): 1947-54.
113. Sarkar JM, Tseng AM, Collins JH. Treatment of pulp and paper wastewater with reducing agent and a polymer for color removal. Google Patents; 1994.
114. Jones GD, Langsjoen A, NEUMANN SMMC, Zomlefer J. The polymerization of ethylenimine. The Journal of Organic Chemistry 1944; 9(2): 125-47.
115. Dick C, Ham G. Characterization of polyethylenimine. Journal of Macromolecular Science—Chemistry 1970; 4(6): 1301-14.
116. Wightman L, Kircheis R, Rossler V, Carotta S, Ruzicka R, Kursa M, et al. Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. J Gene Med 2001; 3(4): 362-72.

117. Goula D, Benoist C, Mantero S, Merlo G, Levi G, Demeneix B. Polyethylenimine-based intravenous delivery of transgenes to mouse lung. Gene Ther 1998; 5(9): 1291-95.

118. Zabner J, Cheng SH, Meeker D, Launspach J, Balfour R, Perricone MA, et al. Comparison of DNA-lipid complexes and DNA alone for gene transfer to cystic fibrosis airway epithelia in vivo. Journal of Clinical Investigation 1997; 100(6): 1529.

119. Bragonzi A, Boletta A, Biffi A, Muggia A, Sersale G, Cheng S, et al. Comparison between cationic polymers and lipids in mediating systemic gene delivery to the lungs. Gene Ther 1999; 6(12): 1995-2004.

120. Cozens A, Yezzi M, Kunzelmann K, Ohrui T, Chin L, Eng K, et al. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. American journal of respiratory cell and molecular biology 1994; 10(1): 38-47.

121. Wiseman JW, Goddard CA, McLelland D, Colledge WH. A comparison of linear and branched polyethylenimine (PEI) with DCChol/DOPE liposomes for gene delivery to epithelial cells in vitro and in vivo. Gene Ther 2003; 10(19): 1654-62.

122. Coll JL, Chollet P, Brambilla E, Desplanques D, Behr JP, Favrot M. In vivo delivery to tumors of DNA complexed with linear polyethylenimine. Hum Gene Ther 1999; 10(10): 1659-66.

123. De Martimprey H, Vauthier C, Malvy C, Couvreur P. Polymer nanocarriers for the delivery of small fragments of nucleic acids: oligonucleotides and siRNA. European journal of pharmaceutics and biopharmaceutics 2009; 71(3): 490-504.

124. Guillaume C, Delépine P, Droal C, Montier T, Tymen G, Claude F. Aerosolization of cationic lipid–DNA complexes: lipoplex characterization and optimization of aerosol delivery conditions. Biochemical and biophysical research communications 2001; 286(3): 464-71.

125. Aravindan L, Bicknell KA, Brooks G, Khutoryanskiy VV, Williams AC. Effect of acyl chain length on transfection efficiency and toxicity of polyethylenimine. International Journal of Pharmaceutics 2009; 378(1): 201-10.

126. Nguyen H, Lemieux P, Vinogradov SV, Gebhart CL, Guerin N, Paradis G, et al. Evaluation of polyether-polyethyleneimine graft copolymers as gene transfer agents. Gene Ther 2000; 7(2).
127. Ogris M, Brunner S, Schüller S, Kircheis R, Wagner E. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther 1999; 6(4): 595-605.
128. Choe JH, Choe JS, Seo HR, Park JS. Effect of poly (ethylene glycol) grafting on polyethylenimine as a gene transfer vector in vitro. Bulletin of the korean chemical society 2001; 22(1): 46-52.
129. Kichler A, Chillon M, Leborgne C, Danos O, Frisch Bt. Intranasal gene delivery with a polyethylenimine–PEG conjugate. Journal of Controlled Release 2002; 81(3): 379-88.
130. Brissault B, Kichler A, Guis C, Leborgne C, Danos O, Cheradame H. Synthesis of linear polyethylenimine derivatives for DNA transfection. Bioconjugate chemistry 2003; 14(3): 581-87.
131. von Harpe A, Petersen H, Li Y, Kissel T. Characterization of commercially available and synthesized polyethylenimines for gene delivery. J Control Release 2000; 69(2): 309-22.
132. Choosakoonkriang S, Lobo BA, Koe GS, Koe JG, Middaugh CR. Biophysical characterization of PEI/DNA complexes. J Pharm Sci 2003; 92(8): 1710-22.
133. Ogris M, Steinlein P, Kursa M, Mechtler K, Kircheis R, Wagner E. The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. Gene Ther 1998; 5(10): 1425-33.
134. Ziebarth JD, Wang Y. Understanding the protonation behavior of linear polyethylenimine in solutions through Monte Carlo simulations. Biomacromolecules 2010; 11(1): 29-38.
135. Suh J, Paik H-j, Hwang BK. Ionization of Poly (ethylenimine) and Poly (allylamine) at Various pH′ s. Bioorganic Chemistry 1994; 22(3): 318-27.

136. Bencini A, Bianchi A, Garcia-España E, Micheloni M, Ramirez JA. Proton coordination by polyamine compounds in aqueous solution. Coordination chemistry reviews 1999; 188(1): 97-156.
137. Kas HS. Chitosan: properties, preparations and application to microparticulate systems. J Microencapsul 1997; 14(6): 689-711.
138. Domard A. pH and cd measurements on a fully deacetylated chitosan: application to Cu II—polymer interactions. International journal of biological macromolecules 1987; 9(2): 98-104.
139. Anthonsen MW, Smidsrød O. Hydrogen ion titration of chitosans with varying degrees of N-acetylation by monitoring induced 1H-NMR chemical shifts. Carbohydrate Polymers 1995; 26(4): 303-05.
140. Varum KM, Anthonsen MW, Grasdalen H, Smidsrod O. Determination of the degree of N-acetylation and the distribution of N-acetyl groups in partially N-deacetylated chitins (chitosans) by high-field n.m.r. spectroscopy. Carbohydr Res 1991; 211(1): 17-23.
141. Varum KM, Anthonsen MW, Grasdalen H, Smidsrod O. 13C-n.m.r. studies of the acetylation sequences in partially N-deacetylated chitins (chitosans). Carbohydr Res 1991; 217: 19-27.
142. Chandy T, Sharma CP. Chitosan--as a biomaterial. Biomater Artif Cells Artif Organs 1990; 18(1): 1-24.
143. Rao SB, Sharma CP. Use of chitosan as a biomaterial: studies on its safety and hemostatic potential. J Biomed Mater Res 1997; 34(1): 21-8.
144. Aspden TJ, Mason JD, Jones NS, Lowe J, Skaugrud O, Illum L. Chitosan as a nasal delivery system: the effect of chitosan solutions on in vitro and in vivo mucociliary transport rates in human turbinates and volunteers. J Pharm Sci 1997; 86(4): 509-13.
145. Shu XZ, Zhu KJ. The influence of multivalent phosphate structure on the properties of ionically cross-linked chitosan films for controlled drug release. European Journal of Pharmaceutics and Biopharmaceutics 2002; 54(2): 235-43.

146. Lee MK, Chun SK, Choi WJ, Kim JK, Choi SH, Kim A, et al. The use of chitosan as a condensing agent to enhance emulsion-mediated gene transfer. Biomaterials 2005; 26(14): 2147-56.
147. Aiba S. Studies on chitosan: 4. Lysozymic hydrolysis of partially N-acetylated chitosans. Int J Biol Macromol 1992; 14(4): 225-8.
148. Zhang H, Neau SH. In vitro degradation of chitosan by bacterial enzymes from rat cecal and colonic contents. Biomaterials 2002; 23(13): 2761-6.
149. Escott GM, Adams DJ. Chitinase activity in human serum and leukocytes. Infect Immun 1995; 63(12): 4770-3.
150. Liu X, Howard KA, Dong M, Andersen MO, Rahbek UL, Johnsen MG, et al. The influence of polymeric properties on chitosan/siRNA nanoparticle formulation and gene silencing. Biomaterials 2007; 28(6): 1280-8.
151. Katas H, Alpar HO. Development and characterisation of chitosan nanoparticles for siRNA delivery. Journal of Controlled Release 2006; 115(2): 216-25.
152. Sgouras D, Duncan R. Methods for the evaluation of biocompatibility of soluble synthetic polymers which have potential for biomedical use: 1 — Use of the tetrazolium-based colorimetric assay (MTT) as a preliminary screen for evaluation ofin vitro cytotoxicity. Journal of Materials Science: Materials in Medicine 1990; 1(2): 61-68.
153. Carreño-Gómez B, Duncan R. Evaluation of the biological properties of soluble chitosan and chitosan microspheres. International Journal of Pharmaceutics 1997; 148(2): 231-40.
154. Aspden TJ, Adler J, Davis SS, Skaugrud ø, Illum L. Chitosan as a nasal delivery system: Evaluation of the effect of chitosan on mucociliary clearance rate in the frog palate model. International Journal of Pharmaceutics 1995; 122(1–2): 69-78.
155. Aspden T, Illum L, Skaugrud Ø. The effect of chronic nasal application of chitosan solutions on cilia beat frequency in guinea pigs. International Journal of Pharmaceutics 1997; 153(2): 137-46.
156. Sato T, Ishii T, Okahata Y. In vitro gene delivery mediated by chitosan. effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. Biomaterials 2001; 22(15): 2075-80.

157. Lee M, Nah J-W, Kwon Y, Koh J, Ko K, Kim S. Water-Soluble and Low Molecular Weight Chitosan-Based Plasmid DNA Delivery. Pharmaceutical Research 2001; 18(4): 427-31.
158. Allan GG, Peyron M. Molecular weight manipulation of chitosan I: kinetics of depolymerization by nitrous acid. Carbohydr Res 1995; 277(2): 257-72.
159. Hirano S, Kondo Y, Fujii K. Preparation of acetylated derivatives of modified chito-oligosaccharides by the depolymerisation of partially N-acetylated chitosan with nitrous acid. Carbohydr Res 1985; 144(2): 338-41.
160. Sashiwa H, Saimoto H, Shigemasa Y, Tokura S. N-Acetyl group distribution in partially deacetylated chitins prepared under homogeneous conditions. Carbohydr Res 1993; 242: 167-72.
161. Williams JM. Deamination of carbohydrate amines and related compounds. Advances in Carbohydrate Chemistry and Biochemistry 1975; 31: 9-79.
162. Ridd JH. Diffusion Control and Pre—association in Nitrosation, Nitration and. ADV PHYSICAL ORGANIC CHEMISTRY APL 1979; 16: 1.
163. Liu Y. The Preparation of Water-soluble Low-molecular Weight Chitosan and the Complex with Calcium Ions. JOURNAL-FUJIAN TEACHERS UNIVERSITY NATURAL SCIENCE EDITION 1997; 13: 67-70.
164. Germershaus O, Mao S, Sitterberg J, Bakowsky U, Kissel T. Gene delivery using chitosan, trimethyl chitosan or polyethylenglycol-graft-trimethyl chitosan block copolymers: establishment of structure–activity relationships in vitro. Journal of Controlled Release 2008; 125(2): 145-54.
165. Tripathi SK, Goyal R, Kashyap MP, Pant AB, Haq W, Kumar P, et al. Depolymerized chitosans functionalized with bPEI as carriers of nucleic acids and tuftsin-tethered conjugate for macrophage targeting. Biomaterials 2012; 33(16): 4204-19.
166. Mao S, Shuai X, Unger F, Simon M, Bi D, Kissel T. The depolymerization of chitosan: effects on physicochemical and biological properties. International Journal of Pharmaceutics 2004; 281(1): 45-54.
167. Strand SP, Lelu S, Reitan NK, de Lange Davies C, Artursson P, Varum KM. Molecular design of chitosan gene delivery systems with an optimized balance

between polyplex stability and polyplex unpacking. Biomaterials 2010; 31(5): 975-87.

168. Holzerny P, Ajdini B, Heusermann W, Bruno K, Schuleit M, Meinel L, et al. Biophysical properties of chitosan/siRNA polyplexes: profiling the polymer/siRNA interactions and bioactivity. J Control Release 2012; 157(2): 297-304.
169. Agu RU, Ugwoke MI, Armand M, Kinget R, Verbeke N. The lung as a route for systemic delivery of therapeutic proteins and peptides. Respir Res 2001; 2(4): 198-209.
170. Labiris NR, Dolovich MB. Pulmonary drug delivery. Part II: the role of inhalant delivery devices and drug formulations in therapeutic effectiveness of aerosolized medications. British journal of clinical pharmacology 2003; 56(6): 600-12.
171. Kohler D. Aerosols for systemic treatment. Lung 1990; 168 Suppl: 677-84.
172. Sakagami M. In vivo, in vitro and ex vivo models to assess pulmonary absorption and disposition of inhaled therapeutics for systemic delivery. Adv Drug Deliv Rev 2006; 58(9-10): 1030-60.
173. Frijlink HW, De Boer AH. Dry powder inhalers for pulmonary drug delivery. Expert Opin Drug Deliv 2004; 1(1): 67-86.
174. Labiris NR, Dolovich MB. Pulmonary drug delivery. Part I: physiological factors affecting therapeutic effectiveness of aerosolized medications. Br J Clin Pharmacol 2003; 56(6): 588-99.
175. Yang W, Peters JI, Williams RO, 3rd. Inhaled nanoparticles--a current review. Int J Pharm 2008; 356(1-2): 239-47.
176. Rosenecker J, Naundorf S, Gersting SW, Hauck RW, Gessner A, Nicklaus P, et al. Interaction of bronchoalveolar lavage fluid with polyplexes and lipoplexes: analysing the role of proteins and glycoproteins. J Gene Med 2003; 5(1): 49-60.
177. Duncan JE, Whitsett JA, Horowitz AD. Pulmonary surfactant inhibits cationic liposome-mediated gene delivery to respiratory epithelial cells in vitro. Hum Gene Ther 1997; 8(4): 431-8.

178. Merkel OM, Beyerle A, Librizzi D, Pfestroff A, Behr TM, Sproat B, et al. Nonviral siRNA delivery to the lung: investigation of PEG-PEI polyplexes and their in vivo performance. Mol Pharm 2009; 6(4): 1246-60.
179. Ferrari S, Kitson C, Farley R, Steel R, Marriott C, Parkins DA, et al. Mucus altering agents as adjuncts for nonviral gene transfer to airway epithelium. Gene Ther 2001; 8(18): 1380-6.
180. Daviskas E, Anderson SD, Brannan JD, Chan HK, Eberl S, Bautovich G. Inhalation of dry-powder mannitol increases mucociliary clearance. Eur Respir J 1997; 10(11): 2449-54.
181. Daviskas E, Anderson SD, Jaques A, Charlton B. Inhaled mannitol improves the hydration and surface properties of sputum in patients with cystic fibrosis. Chest 2010; 137(4): 861-8.
182. van Beerendonk I, Mesters I, Mudde AN, Tan TD. Assessment of the inhalation technique in outpatients with asthma or chronic obstructive pulmonary disease using a metered-dose inhaler or dry powder device. J Asthma 1998; 35(3): 273-9.
183. Steckel H, Eskandar F, Witthohn K. The effect of formulation variables on the stability of nebulized aviscumine. Int J Pharm 2003; 257(1-2): 181-94.
184. Purewal TS, Grant DJW. Metered dose inhaler technology: CRC Press; 1997.
185. Bisgaard H. What dose fraction represents the respirable dose? Respiratory medicine 1997; 91: 20-21.
186. Newman SP. Principles of metered-dose inhaler design. Respiratory care 2005; 50(9): 1177-90.
187. Mastrandrea LD, Quattrin T. Clinical evaluation of inhaled insulin. Advanced Drug Delivery Reviews 2006; 58(9): 1061-75.
188. Codrons V, Vanderbist F, Verbeeck RK, Arras M, Lison D, Préat V, et al. Systemic delivery of parathyroid hormone (1-34) using inhalation dry powders in rats. Journal of pharmaceutical sciences 2003; 92(5): 938-50.
189. Mahesh Kumar T, Misra A. Formulation and evaluation of insulin dry powder for inhalation. Drug Dev Ind Pharm 2006; 32(6): 677-86.
190. Shah SP, Misra A. Liposomal amikacin dry powder inhaler: effect of fines on in vitro performance. Aaps Pharmscitech 2004; 5(4): 107-13.

191. Shah S, Misra A. Development of liposomal amphotericin B dry powder inhaler formulation. Drug Deliv 2004; 11(4): 247-53.
192. Chougule M, Padhi B, Misra A. Nano-liposomal dry powder inhaler of tacrolimus: preparation, characterization, and pulmonary pharmacokinetics. International Journal of Nanomedicine 2007; 2(4): 675.
193. Lam JK-W, Liang W, Chan H-K. Pulmonary delivery of therapeutic siRNA. Advanced Drug Delivery Reviews 2012; 64(1): 1-15.
194. Driscoll KE, Costa DL, Hatch G, Henderson R, Oberdorster G, Salem H, et al. Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations. Toxicological Sciences 2000; 55(1): 24-35.
195. Bivas-Benita M, Zwier R, Junginger HE, Borchard G. Non-invasive pulmonary aerosol delivery in mice by the endotracheal route. European journal of pharmaceutics and biopharmaceutics 2005; 61(3): 214-18.
196. Moschos SA, Jones SW, Perry MM, Williams AE, Erjefalt JS, Turner JJ, et al. Lung delivery studies using siRNA conjugated to TAT (48-60) and penetratin reveal peptide induced reduction in gene expression and induction of innate immunity. Bioconjugate chemistry 2007; 18(5): 1450-59.
197. Rosas-Taraco AG, Higgins DM, Sánchez-Campillo J, Lee EJ, Orme IM, González-Juarrero M. Intrapulmonary delivery of XCL1-targeting small interfering RNA in mice chronically infected with Mycobacterium tuberculosis. American journal of respiratory cell and molecular biology 2009; 41(2): 136-45.
198. Wang J-C, Lai S, Guo X, Zhang X, De Crombrugghe B, Sonnylal S, et al. Attenuation of fibrosis in vitro and in vivo with SPARC siRNA. Arthritis research & therapy 2010; 12(2): 1.
199. Fulton A, Peters ST, Perkins GA, Jarosinski KW, Damiani A, Brosnahan M, et al. Effective treatment of respiratory alphaherpesvirus infection using RNA interference. PLoS One 2009; 4(1): e4118.
200. Gutbier B, Kube SM, Reppe K, Santel A, Lange C, Kaufmann J, et al. RNAi-mediated suppression of constitutive pulmonary gene expression by small interfering RNA in mice. Pulmonary pharmacology & therapeutics 2010; 23(4): 334-44.

201. Zhang X, Shan P, Jiang D, Noble PW, Abraham NG, Kappas A, et al. Small interfering RNA targeting heme oxygenase-1 enhances ischemia-reperfusion-induced lung apoptosis. Journal of Biological Chemistry 2004; 279(11): 10677-84.
202. Senoo T, Hattori N, Tanimoto T, Furonaka M, Ishikawa N, Fujitaka K, et al. Suppression of plasminogen activator inhibitor-1 by RNA interference attenuates pulmonary fibrosis. Thorax 2010; 65(4): 334-40.
203. Hinchcliffe M, Illum L. Intranasal insulin delivery and therapy. Advanced Drug Delivery Reviews 1999; 35(2): 199-234.
204. Heyder J, Gebhart J, Rudolf G, Schiller CF, Stahlhofen W. Deposition of particles in the human respiratory tract in the size range 0.005–15 μm. Journal of Aerosol Science 1986; 17(5): 811-25.