Challenges in Gene Delivery

Module Objective

The use of biomimetic strategies to ensure delivery of oligonucleotides in to a cell has been discussed in detail. At the end of the module, the learner will be able to understand the nuances involved in gene delivery using nanoparticles that exhibit virus-like strategies to enter a cell, escape the endosome and deliver the cargo.

Preface

With growing emphasis on developing treatment strategies for diseases in the pre-symptomatic stage, gene therapy has assumed significance in recent years. However, its implementation has been restricted due to various issues of stability and off-targeting. "*An inefficient virus kills its host. A clever virus stays with it*", said the famous environmentalist Dr. James Lovelock. The solution for many gene delivery problems can be obtained by understanding the *modus operandi* of a virus to gain entry and integrate its genetic material into the host cell. Lecture 1 provides an overview of the challenges encountered in gene therapy and importance of gene delivery. The requisites of a gene carrier system are also highlighted using bio-inspired strategies. Lecture 2 provides an insight into the mode of entry of the virus into a cell while Lectures 3 and 4 discusses about different cationic surfactant carriers that are under development as virus-mimics.

This lecture provides an insight in to the need for gene delivery and the challenges that arise in delivering oligonucleotides.

1 Why do we need to deliver genes?

Gene therapy deals with inserting an essential gene or substituting or suppressing a defective gene using exogenously introduced oligonucleotide sequences. The suppression of a rogue gene is known as gene silencing or anti-sense therapy and has been explored extensively as part of cancer treatment strategies. Gene therapy is broadly classified into **germline gene therapy** and **somatic gene therapy**. In germ line therapy, the cells targeted are the sperms and ovaries that are involved in transmission of hereditary characters. In somatic gene therapy, the genetic intervention is directed to the body cells. Any alterations in the genetic make-up in these cells will not be transmitted to the individual's progeny but will only be reflected by the individual.

The concept of gene therapy ushered in a new paradigm in treatment of many diseases, especially inherited disorders. Gene therapy enabled one to address the disease even before the onset of symptoms opening up the possibility of treating diseases even at a

molecular level. Moreover, it offered new promises to treat disorders that were earlier considered untreatable such as thalassemia, cystic fibrosis, many forms of cancers, AIDS, Huntington's disease, colour blindness etc. The process of introducing a genetic material into a cell is referred to as **'transfection'**. Though the concept of gene therapy had originated in the early 70s, the first reported treatment using gene therapy was in 1992. However, during multi-centric trials conducted during 2000-2002 for treatment of SCID (Severe combined immune disease or 'bubble boy' disease), which arises due to the deficiency of the enzyme adenosine deaminase, it was discovered that among the ten patients being treated, two of them developed leukemia. This side-effect was a result of the gene being delivered to a non-specific target. This indicated the importance and necessity of site-specific delivery of genes. As a result, concerted efforts were directed towards achieving target-specific gene delivery. However, in spite of many years of research in this direction, success continues to elude scientists in developing an effective gene delivery system. Why? In the following section, we will discuss some major pitfalls that plague the gene therapy strategies.

2 Challenges in gene delivery

There are several challenges that limit the application of gene therapy. These can be broadly categorized in to **oligonucleotide-dependent barriers** and **system-dependent barriers**.

2.1 Oligonucleotide-dependent barriers

One of the major issues is the **stability** of the oligonucleotides to be delivered. Once the oligonucleotides are introduced into the circulation, they are susceptible to degradation by the exo- and endonucleases. Hence there is a need to shield them from the nuclease activity *in vivo*. The next challenge is the **diffusivity** of the oligonucleotide. Generally the double stranded DNA is linear and has poor mobility when compared to globular entities.Why? The double stranded DNA with its numerous hydrogen bonds is **rigid**which reduces its mobility. Another factor that retards its diffusion is its **size.** This factor is especially dominant in the case of plasmids where apart from the gene of interest, additional sequences for promoting the gene expression are also integrated. This results in an increase in the number of base pairs and consequently an increase in the **molecular weight**. Enhanced molecular weight will slow the diffusion of plasmid. Any oligonucleotide sequence with more than 1000 base pairs has been shown to be practically immobile especially in the more viscous cytosol. Also, all oligonucleotides possess **negative charges** due to the phosphate backbone. This will cause formation of an additional layer consisting of counter ions as well as water molecules that will further impede diffusion of the oligonucleotide. The negative charges also prevent folding of single stranded or double stranded oligonucleotides due to electrostatic repulsion and as a result the oligonucleotide remains linear leading to lower diffusivity.

Why is slow diffusion of oligonucleotides a disadvantage? Well, anything that moves slowly in circulation, can be more easily degraded by enzymes (in this case, nucleases). Also, slow moving entities activate the immune system more quickly and strongly. Activation of the immune system results in quicker elimination of the oligonucleotide! Another key limitation in gene therapy is the **immunogenicity** of the oligonucleotide. The activation of immune system can be due presence of a foreign gene or gene fragment or it may be dependent on the sequence of the oligonucleotide being delivered. Unmethylated CpG (cytidine paired with guanosine) segments can elicit an acute immune response, as the frequency of such pairs is high in viral and bacterial genomes. Thus, if the oligonucleotide being delivered contains numerous CpG pairs, then the magnitude of immune activation will be correspondingly high. The CpG pairs activate the toll-like receptors found in natural killer cells, dendritic cells, macrophages, monocytes etc., which in turn activates the T-cells and finally trigger antibody production. The charged surface presented by the oligonucleotides further initiate selective adsorption of cationic proteins leading to a phenomenon called as '***opsonization***'*(Discussion on opsonization process is given in detail in Module 6).* The opsonization process leads to activation of the complement system and other components of the immune cascade.

2.2 System-dependent barriers

In order to overcome the stability and mobility issues of the naked oligonucleotides, a suitable delivery system could be used. However, several challenges still limit the applicability of the gene delivery systems. The carrier system should enable maximum encapsulation of the oligonucleotide. As the oligonucleotides are negatively charged, the carrier should possess positive charges to be able to effectively complex the oligonucleotides. Greater the positive charges in the carrier, better will be complexation. However, cationic carriers are extremely **toxic** to the biological system due to their ability to fuse with cell membranes. The cell membranes possess glycoproteins that have a negative charge and hence these tend to attract cationic carriers resulting in non-specific interactions and consequently toxic manifestations. Another detrimental effect triggered by the cationic nature of the carriers is their **immunogenic potential**. Due to the high concentration of charges, they induce inflammatory reactions. Currently, approaches to mask the surface charge of the carriers have been attempted to reduce charge-driven toxicity and inflammation. However, the inherent immunogenicity of the carrier will remain a concern.

An important requirement in gene delivery is target-specific delivery of the oligonucleotide. In order to achieve targeting, a target-specific ligand can be linked to the carrier system. However, this presents a new challenge. The presence of the targeting ligand on the carrier ensures cell uptake via receptor-mediated endocytosis. This necessitates that the carrier system should **escape from the endosomal pathwa**y, else the carrier along with the oligonucleotide will be degraded as the endosome progressively

acidifies and transforms in to a lysosome. This represents a big bottleneck even today in designing superior gene delivery systems.

Yet another unique challenge exists in the case of plasmid delivery systems. The site of action for a plasmid is in the nucleus and not in the cytosol. Hence, it is not sufficient to deliver the plasmid to the target cell, but ensure that the plasmid escapes the endosome and reaches the nucleus. This necessitates an additional **nucleus-targeting** strategy to be incorporated in the carrier. The **size restrictions** for the carrier are also very critical in the case of gene delivery systems. Generally, sizes below 100 nm are preferred and if plasmid delivery is to be achieved, the size limit further drops to below 30 nm to ensure passage in to the nucleus. Figure 1 presents a snapshot of the various challenges involved in gene delivery.

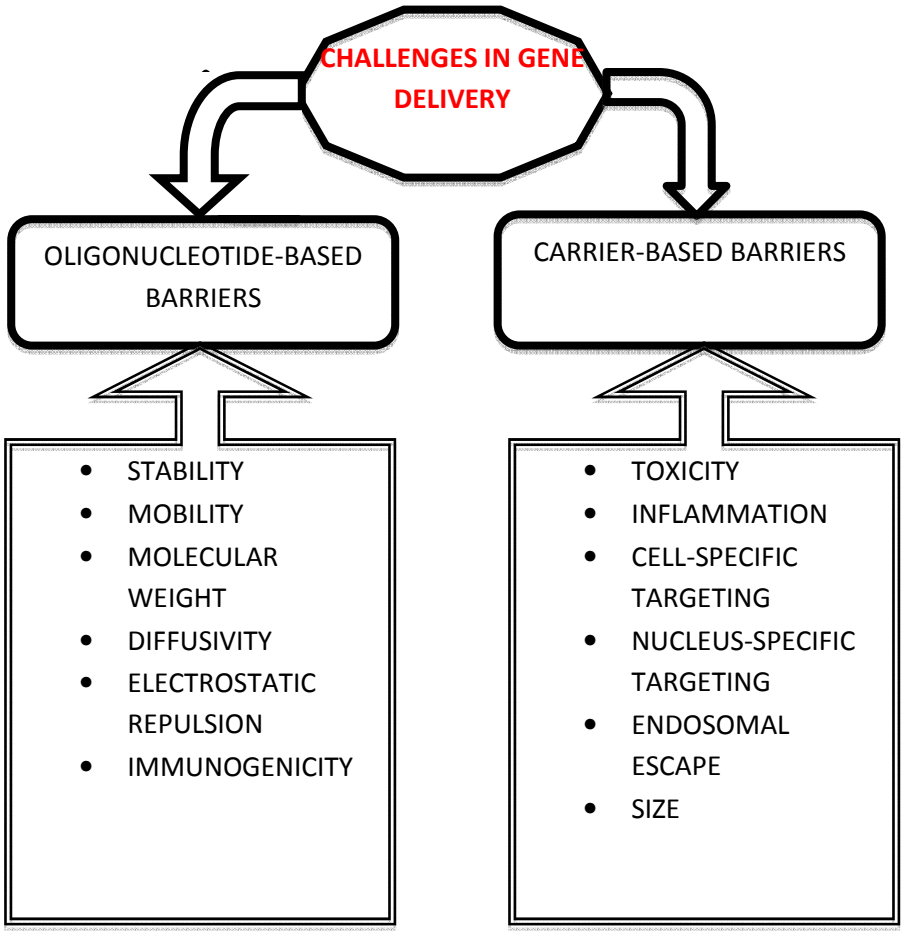

Fig. 1: Challenges in gene delivery

Thus, a carrier for the oligonucleotides should possess the following characteristics:

- ✓ *Should be sufficiently cationic to ensure high complexation*
- ✓ *Should not elicit high immune response*
- ✓ *Should be able to selectively target the cells of interest*
- ✓ *Should escape the endosome*
- ✓ *Should ensure release of the oligonucleotide at the site of action (cytosol or nucleus).*

These widely diverse requirements seem nearly impossible to be met using a single carrier. However, nature provides an interesting solution to fulfill all these requirements! The best and most effective gene delivery system till date in the world is a **VIRUS**!

3 What are viruses?

Virus represents a unique class of organisms that probably form the interface between live and dead. While they share certain common characteristics of living organisms, they fail to exhibit certain traits that define a living thing!Viruses do not respire nor exhibit growth! In fact, they do not possess elaborate metabolic pathways that are characteristic of a living organism. They have a DNA or RNA based genome and a protein coat but do not possess the ability to replicate by themselves. Hence, they utilize a host cell's replication machinery to proliferate. All viruses therefore are parasitic. What is intriguing about viruses is their ability to infect a specific host using sophisticated and highly efficient mechanisms of entry and integration. Therefore, understanding the mechanism of virus entry into the host cell can throw light on the possible strategy that could be developed for efficient entry in to cells, endosomal escape and gene delivery.

4 How does a virus enter a cell?

There are different classes of virus namely adenovirus, rotavirus, flavivirus, coronavirus, retrovirus, rhinovirus etc. Though their host specificity varies, they all have a common strategy to enter a host cell. They utilize a specific cell surface receptor to internalize in to the cell. The cell membrane contains a mosaic of phospholipids, glycoproteins, transmembrane proteins that can be harnessed to gain entry in to the cell. If the strategy employed by a virus into a membrane is deciphered, it will be possible to design a virus mimic to achieve a carrier system with best cellular uptake and capable of endosomal escape.

Let us take the example of an adenovirus. Adenovirus is a human pathogenic virus commonly implicated in diseases such as conjunctivitis, tonsilitis, viral meningitis, gastroenteritis, bronchiolitis, hemorrhagic cystitis etc. This class of virus has no outer lipid bilayer coating and hence is known as a non-enveloped virus. It has a icosahedron shape and its size ranges between 90 and 100 nm. The surface of the adenovirus contains

many spike-like projections on the surface, which it uses intelligently to anchor itself on the host cell membrane. The adenovirus possesses a double stranded DNA as its genetic material.It uses a **clathrin-dependent mechanism** to enter the cell.How does this mechanism operate? Let us see in the following section.

4.1 Mechanism of cell entry of an adenovirus

The entry of an adenovirus in to a host cell involves numerous proteins each of which has a key role to play. To avoid any confusion to the learner, the important players are highlighted in bold. Initially, the virus binds to a specific receptor known as **coxsackie-adenovirus receptor** (CAR) in the host cell. The name of the receptor is slightly misleading as its primary function is not to transport the coxsackievirus or adenovirus in to the cell! Rather, the CAR is expressed in cells, especially those of the developing neural tissue as a cell adhesion molecule and anchoring the cellsby interacting with the extracellular matrix proteins like fibronectin, agrin, laminin and tenascin.This receptor unfortunately also allows preferential binding of the adenovirus as well as coxsackie virus and hence the name. The binding of the virus to the CAR receptor activates the adaptor proteins such as **AP180** and **epsin**. These in turn activate the migration of the proteins **adaptin** and **clathrin**.

Clathrin is a protein that consists of three heavy chains and three light chains. Once it arrives at the site of the receptor-ligand complex, it starts polymerization. Simultaneously the membrane domain containing the CAR-virus complex invaginates and forms a vesicle over which the clathrin chains polymerize resulting in formation of a clathrin coat over the vesicle. The protein **dynamin**, a GTPase enzyme, assists in the separation of the clathrin-coated vesicle from the membrane. Now the vesicle is inside the cytosol. Here, the clathrin coat starts depolymerizing and the vesicle fuses with the **endosome**. The endosome is an intracellular organelle, which has an acidic pH that progressively decreases as the endosome matures from the early endosome to the late endosome and finally fuses with the lysosome where degradation of the endosomal contents occurs. For this reason, lysosomes are referred to as the '*suicide compartments*' of the cell because it is involved in the destruction or death of the molecules that enter it!It is obvious that entering the highly lytic environment of the lysosome is detrimental to any molecule or organism and hence it is important to escape from the endosome itself to avoid degradation. How does the adenovirus escape from the endosome? This mystery will be unraveled in the following lecture.

5 Reference

Smart Nanoparticles in Nanomedicine (The MML series, Vol. 8), Editors: Reza Arshady& Kenji Kono, Kentus Books, 2006

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