Polymeric Delivery Vehicle for Gene Therapy

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Kaushik Mishra : I have always looked forward to taking up responsibilities and giving my best to everything that I do irrespective of the field of work. My ambition is to have a double PhD (technical + management). Early Future plans would be to get an industrial summit done in my college, which would ensure to get UICT known amongst the masses. My vision contemporarily revolves around the industry-academic symbiosis for the betterment of the society that would bring about more practical knowledge transfer while also bringing about better productivity cum efficiency in both the fields.

Abstract

Synthetic gene therapy vectors must be designed to safely and efficiently escort DNA from outside the cell to the nucleus and to overcome several barriers that are obstacles to internalization, escape from endocytic vesicles, movement through the cytoplasm and transport into the nucleus. Non-viral transfection systems based on the complexes of DNA and polycations ('polyplexes') are evaluated with respect to their effectiveness, toxicity and cell type dependence in a variety of models. Overall, the article demonstrates good potential of structurally diverse polyplex systems as transfection reagents with relatively low cytotoxicity.

Keywords: Polyplex, Drug Delivery System, Bio-Medical Applications, Polymer, Pharmacology

1. Introduction

Genes are storage facilities, which serve as repositories for the amino acid sequences of the cell's workhorses- the proteins. Proteins, in turn, control cell physiology and cell biochemistry. Mutated genes can give rise to nonfunctional proteins or pathogenic proteins and ultimately disease. Such disease causing genes may be used as therapeutic targets in gene therapy. Gene therapy is important not just because it is an alternative means of treating disease but also because it offers the hope of treatments for currently incurable diseases such as cystic fibrosis, sickle cell anemia, and cancer. Such diseases are either hereditary or acquired and can be either monogenetic in origin (due to the mutation of a single gene) or can originate from the malfunctioning of more than one gene. In gene therapy, the therapeutic exogenous gene encodes for the replacement copy of a missing or faulty gene. Alternatively, as in the treatment of certain cancers, the therapeutic gene may encode for an enzyme capable of specific activation of a prodrug. Another aspect of gene therapy is the use of genes as vaccines. Genes used in the prevention of infectious diseases are genes encoding for specific antigens that will ultimately produce prophylactic antibodies.

Although it is possible to combat diseases with gene therapy, bringing this concept into practice is rather problematic. One key difficulty lies in the delivery of the therapeutic gene. The gene therapeutic must evade degradation by extracellular nucleases, resist deactivation by other extracellular components, and traverse both the plasma and nuclear membranes intact in order to access the transcription machinery and produce the therapeutic protein. Each of these stages is fraught with a huge potential for failure, and to achieve effective gene therapy, these transport barriers must be overcome with the aid of delivery systems. It is in the delivery of genes that polymers

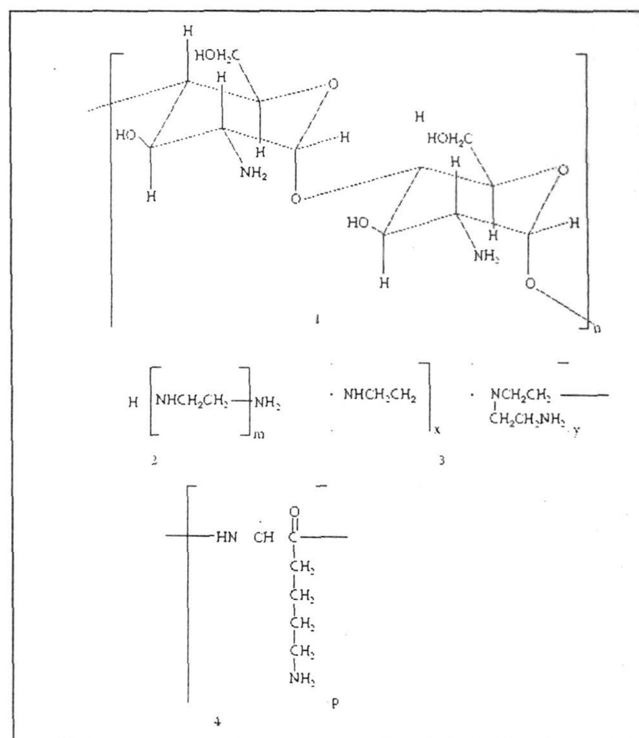


Figure 1 The more commonly used cationic gene delivery polymers. 1. Chitosan, 2.linear poly (ethylenimine), 3.branchedpoly (ethylenimine), 4.poly (L-lysine). Various derivatives of polymers 1–4 have also been used to deliver genes for gene therapy. [Ref. 1]

are applied. Delivery systems fall into two main classes: viruses and synthetic compounds (nonviral systems). Nonviral gene delivery systems may be prepared from polymers, lipids, or dendrimers. Generally viral vectors are thought to have superior transfection

efficiencies over that seen with nonviral vectors, and as such the former have been most frequently investigated in clinical trials. However, serious safety issues have been associated with the use of viral vectors over the last five years, such as insertional mutagenesis with retroviral vectors and a fatal response to the use of adenoviral gene delivery. These events make the search for safe, effective and preferably nonviral gene-transfer systems even more important than was previously thought, especially as polymers, lipids, and dendrimers are envisaged to offer a superior safety profile to the use of viruses. Currently gene therapy research is focused on achieving biodegradable gene delivery options that offer specificity of targeting to the desired cells on systemic administration, transfection efficiencies on par with viruses, and long-term gene expression by sustained-release mechanisms. Polymeric gene delivery systems (cationic) are usually positively charged at physiological pH (pH=7.4), and the most commonly used polymers are shown in Figure 1. Cationic polymers, by virtue of their possession of protonable groups at physiological pH (amine groups), are able to undergo electrostatic interactions with DNA, the latter of which is anionic at physiological pH. DNA is compacted within the electrostatic complex, a process termed DNA condensation, and the colloidal particles that result from this process are known as *polyplexes* (Figure 2). It is these polyplexes that enable the transport of DNA across the various biological barriers to its nuclear destination.

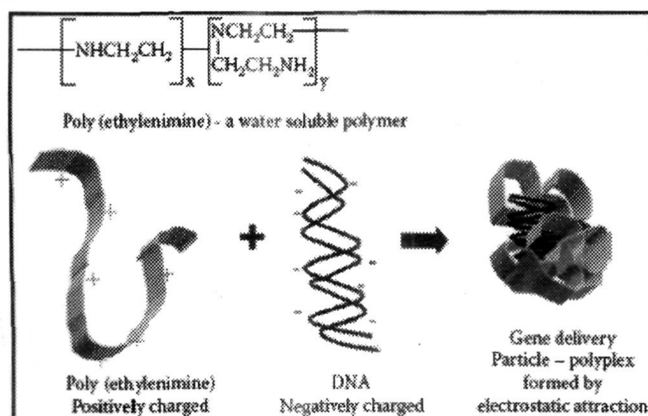


Figure 2 **Polyplex formations on electrostatic binding of genes and polymers. Source: Ref. 1.**

2. Barriers to Polymeric Gene Transfer

Polymeric gene delivery systems carrying an exogenous gene payload face a number of biological barriers that hamper cellular entry of the therapeutic gene. Biological barriers may be broadly classed as being of an extracellular (Figure 3a) and intracellular nature (Figure 3b). Examples of the extracellular barriers to gene delivery include: susceptibility to enzymatic degradation by nucleases in the serum and extracellular fluid, the presence of a mucus coat on certain cell surfaces, vulnerability to inactivation by interactions with blood components, the possibility of recognition and elimination by immunological defense systems, and the possibility of uptake by nontarget organs and tissues. Once inside the cell the intracellular barriers to gene expression come into play, and these include: entrapment and degradation within the endosomes and lysosomes, vulnerability to cytoplasmic nuclease enzymatic attack, and the relative impenetrability of the nuclear membrane. Once safely inside the nucleus the genes are transcribed to produce messenger RNA (mRNA), which in turn, is transported to the cytoplasm and eventually translated into the therapeutic protein. The biological barriers described here must be overcome in order to achieve a clinically relevant level of gene expression.

2.1 Extracellular Barriers

2.1.1 Interactions with Plasma and Blood Components

Upon entering the systemic circulation, the injected exogenous gene will be enzymatically degraded by the nucleases present in the serum. However, upon incorporation of the gene within polyplexes, the gene acquires resistance to degradation by serum nucleases. The polyplexes then need to travel to their target site of action. One factor that is crucial in allowing the polyplexes to travel unhindered to their target site is their particle size. A colloidal (< 1 mm) particle size is ideal. On intravenous injection of colloidal polyplexes, there is an interaction with serum proteins that leads to polyplex aggregation and an increase in particle size. This size increase is believed to hinder transport through the fine capillaries and tissues, and results in polyplexes being entrapped within the first capillary bed encountered - that of the lung. Polyplexes must resist aggregation prior and subsequent to *in vivo* application. However, most polyplexes tend to aggregate at physiological salt concentrations and are also bound by electrostatic attractions to negatively charged blood components such as serum proteins and blood cells. Specifically, when negatively charged serum proteins are bound to the polyplexes, charge neutralization occurs, leading to an increase in polyplex particle size. Such an increase in the size of the polyplex not only results in gene expression occurring predominantly in the lung but also ultimately reduces the level of gene transfer in the target region. Polyplex aggregation may be suppressed by the covalent attachment of poly (ethylene oxide) (poly (ethylene glycol) – PEG) moieties to the polyplex.

2.1.2 Uptake by the Target Cell

DNA faces significant difficulty in entering the cells owing to its hydrophilic nature, large size and polyanionic character. The surface charge of the cell membrane is negative and it is envisaged that this cell surface will repel the approach of the large anionic DNA molecule. Polyplexes can assist in facilitating DNA uptake (endosomal uptake) because they usually carry a positive charge due to the excess of positively charged polymer components they carry. However, the electrostatic binding to the anionic cell surfaces is of a nonspecific nature, and can often lead to inefficiency in transfection because of electrostatic interactions with nontargeted cells when polyplexes are given by an intravenous route. In order to increase the specificity of uptake, homing devices are incorporated in the vector–DNA complex. Examples of such homing devices include ligands such as galactose, antibodies and protein transferrin. These ligands will obviously be more efficient if the nonspecific interactions with blood components and cells are reduced. A commonly used strategy to reduce nonspecific interactions is the shielding of cationic surface charges using poly (ethylene oxide) moieties.

2.2 Intracellular Barriers

Upon arrival in the cell, the exogenous gene is once again faced

with a number of hurdles that prevent it reaching the nucleus, which is the site of transcription. The polyplex is usually taken up by endocytosis, and DNA must escape from the endosome in order to avoid degradation, traverse the cytoplasm intact and in turn cross the nuclear membrane to gain entry to the nucleus (Figure 3b).

2.2.1 Endosomal Escape

Some polyplexes can facilitate endosomal escape. Cationic polyplexes prepared from cationic polymers such as poly (ethylenimine), by virtue of their high level of amino groups are believed to act by buffering the acidic contents of these vacuoles. The accompanying increase in the pH prevents the action of the degradative enzymes and eventually enables a rupture of the endosome by an increase in the ionic and latterly water content of the endosome. The ionic content of the endosome is increased by an energy-driven influx of chloride ions and protons in response to the intraendosomal buffering by the polymer. This hypothesized mechanism of action by polyamines is termed **the proton sponge hypothesis**. Peptides, although not strictly polymers, are also able to promote endosomal escape of gene transfer systems. Fusogenic peptides undergo a pH-triggered conformational change on entering the endosomes, which leads to membrane destabilization and the release of entrapped DNA. Examples of endosomolytic peptides are the viral peptides such as the amino-terminal domain of the influenza virus hemagglutinin HA2 subunit and the synthetic mimics of amphiphilic anionic viral peptides.

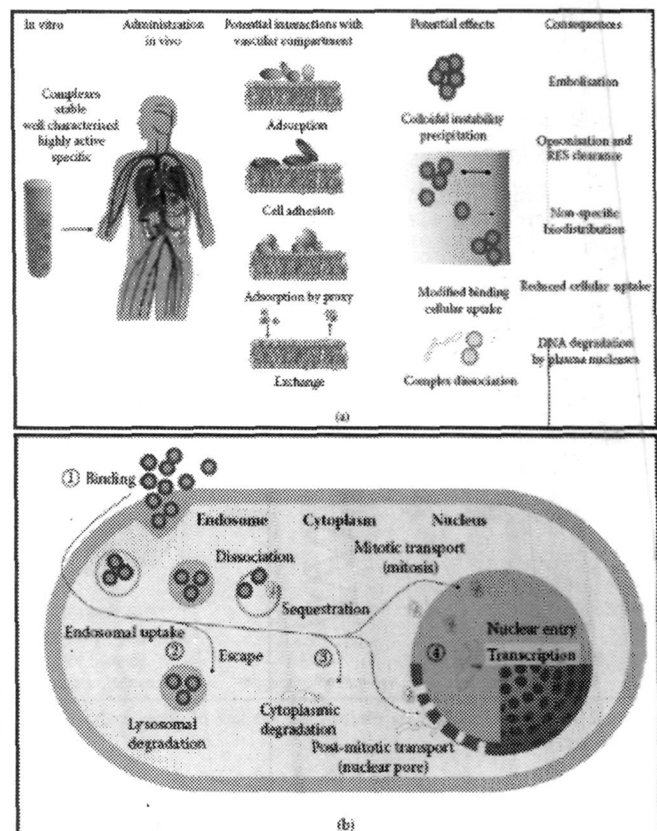
2.2.2 Stability in the Cytoplasm

After escaping from the endosomes, DNA may be subjected to the onslaught of cytoplasmic nucleases. Microinjected plasmid DNA injected into the cytoplasm undergoes rapid degradation by cytoplasmic nucleases with an apparent half-life of 50 to 90 min. The cytoplasm is a hostile environment for the exogenous gene. It is possible that polymeric gene delivery systems which protect DNA from nuclease degradation may protect DNA from intra-cytoplasmic degradation.

2.2.3 Nuclear Import

Assuming that DNA remains stable within the cytoplasm, there is still a requirement for DNA to enter the nucleus for transcription to occur, and breaching the nuclear barrier is undoubtedly the most formidable challenge of all. The transport of DNA from the cytoplasmic medium into the nucleus is limited by the presence of the nuclear envelope. In dividing eukaryotic cells, nucleocytoplasmic transfer of DNA can occur when the nuclear envelope breaks down during mitosis. Cells in the nondividing phase, however, are normally resistant to nucleocytoplasmic transfer of plasmid DNA. In nondividing cells, the nucleocytoplasmic exchange of molecules occurs through the nuclear pore complexes (NPC) that span the nuclear envelope. Hence, the nuclear envelope acts as a molecular sieve, enabling small aqueous molecules of up to 9 nm in diameter (<17-kDa) to diffuse freely through the NPC. However, larger molecules of up to 25 nm such as plasmid DNA and larger DNA fragments undergo a sequence-specific active transport process involving multiple cellular components. There is very little direct evidence that polymers actually assist the translocation of DNA or oligonucleotides to the nucleus. More common strategies include exploiting the nucleocytoplasmic transport machinery by modifying the plasmid DNA with specific sequences so that it can be recognized by cellular factors as a nuclear import substrate. A common example of such modification is to attach a DNA nuclear localization signal (NLS) to the plasmid construct, aiding the recognition of the plasmid DNA constructs by transcription factors with subsequent nuclear import of the resultant complex. Furthermore, oligopeptide sequences known as NLS peptides, which direct transport to the nucleus, may be linked to plasmid DNA by the use of electrostatic attractions or covalent bonds. These NLS peptides are mainly composed of positively charged oligopeptides made up of sequences of lysine or arginine residues.

Figure 3 Barriers to the delivery of therapeutic genes: (a) extracellular, (b) intracellular [Ref. 1]



3. Overcoming Barriers to Gene Transfer

Desirable elements of a vector would include: a polymer (cationic) sequence for DNA condensation, a stealth-type coating to evade detection by the macrophages of the reticuloendothelial system-preferably one that may be shed at the site of cell entry, a colloidal stabilizing entity to prevent colloidal instability in the blood and accumulation in lung capillaries, ligands facilitating cell-specific entry or the site-specific uncovering of a cationic surface to facilitate cell entry, an endosomolytic component that could also be a polycation and finally NLS. Although synthetic viruses such as the ideal system described above do not exist at present, various polymers have been evaluated for their ability to protect and deliver genes across the barriers outlined here.

4. Poly (L-lysine)

The first cationic polymer-based gene delivery vehicle was poly (L-lysine). In this pioneering effort, poly (L-lysine) was conjugated to asialoorosomucoid for targeted gene delivery to liver hepatocytes. Since then a number of targeting ligands have been used to enable tissue- or pathology-specific gene expression, e.g., galactose for hepatocyte targeting, artery-wall-binding peptide for targeting the arteries and both transferrin and folate for tumor targeting. On its own, poly (L-lysine), although able to efficiently bind DNA, is not an effective gene transfer agent with adenovirus particles, histidyl residues, or lysomotropic agents such as chloroquine being required for gene transfer. Additionally poly (L-lysine) - based polyplexes are associated with their own intrinsic cytotoxicity and although this characteristic may be alleviated somewhat by glycosylation or by converting the polymer to an amphiphile; unnecessary cytotoxicity baggage associated with an intrinsic poor activity makes this polymer an unlikely first-choice material.

5. Poly (ethylenimine)

Poly (ethylenimine) (PEI; Figure 1) is one of the most efficient cationic polymer gene-transfer systems available and although it has not yet been approved for clinical use, PEI anticancer gene formulations are able to achieve tumor regression through a combination of gene transfer and an intrinsic antiproliferative activity. PEI's *in vivo* gene-transfer ability has been proven on both local (intraventricular, intratracheal, and intratumoural) and systemic administration. PEI exists in a number of molecular weight formats (0.42 to 800 kDa) and transfection efficiency is highest at a molecular weight of between 12 and 70 kDa the most commonly used PEI molecular weight being 22 to 25 kDa. Both linear and branched formats of the polymer exist (Figure 1.1); the linear molecule is a more efficient gene-transfer agent than the branched one. Toxicity is undoubtedly modulated by reducing the quantity of protonable amine groups per molecule either through the attachment of poly (ethylene oxide) chains to PEI or by the methylation of secondary and tertiary amines to give quaternary ammonium groups. However, an improved biocompatibility with these two methods usually comes at the expense of a reduction in activity. Every third atom of the PEI molecule is nitrogen, these amine groups are important for DNA binding and enabling the therapeutic transgene escape from the endosome on uptake into the cell. The proton sponge hypothesis (see subsection 4.2) has been put forward to explain the mechanism by which PEI enables escape of the exogenous gene from the endosome subsequent to cellular uptake. But one of the problems associated with the systemic (intravenous) administration of cationic lipids and cationic polymers such as PEI is the fact that gene transfection occurs predominantly in the lung endothelium. This passive targeting to the lung is believed to follow aggregation of the polyplexes in the blood and their entrapment within the lung capillaries (see subsection 4.1). Although transfection of the lung endothelial cells may sometimes be welcome, it is sometimes necessary to achieve gene expression in other areas such as tumors located at sites remote from the lung or the site of injection. To reduce the likelihood of passive lung targeting, poly (ethylene oxide) has been grafted on to PEI. Conjugation of poly (ethylene oxide) units to PEI reduces the surface charge of the polyplexes and prevents their aggregation and localization within the lung capillaries. However, although poly(ethylene oxide) chains improve the colloidal stability of these particulate formulations by providing a steric hindrance to particle aggregation, this strategy is often accompanied by diminished polyplex gene-transfer activity largely due to poor cellular uptake of the poly(ethylene oxide)-covered polyplex by cells. It is thus necessary to apply a poly (ethylene oxide) coating as well as a ligand, promoting receptor mediated uptake, to counteract the poor cellular uptake problems (Figure 4). Such a strategy has been used to produce a tumor necrosis factor-alpha gene medicine, with the iron transporter transferrin serving as the receptor-mediated uptake ligand. This targeted gene medicine was able to produce gene expression in tumors distant to the injection site and ultimately delay tumor progression. A variety of other PEI derivatives have been employed in an effort to improve the gene transfer ability of PEI polyplexes. In summary, PEI is an efficient gene-transfer polymer that has proved its mettle; however, it appears that the cytotoxicity of this molecule is yet to be ameliorated to the extent that it may be considered as a suitable candidate in the clinical development of gene therapeutics.

6. Chitosan

Chitosan (Figure 1) is a carbohydrate polymer, derived from chitin; the latter a by-product of the shellfish industry and was first reported as a gene delivery tool in the mid-1990s. Chitosan is also one of the few agents, that is capable of gene transfer via the oral route. Chitosan is composed of *N*-acetyl-D-glucosamine and D-glucosamine monomers linked by *b* (1, 4) glycosidic bonds. The presence of amino groups in chitosan gives it the ability to condense DNA. Chitosan is attractive as a gene delivery tool because of its good biocompatibility profile when compared to cationic liposomes, polyamine polymers and polyamine dendrimers. Chitosan is not as amine dense as poly (ethylenimine) or poly (L-lysine) (Figure 1) and this property could be responsible for its good biocompatibility and also possibly for its relatively poor gene-transfer ability. Its favorable biocompatibility has prompted researchers to find ways to optimize gene transfer with this agent. Controlling molecular weight appears to be the key; an optimum gene-transfer activity lies between a degree of polymerization of 7 and 635. Additionally, increasing the charge density by incorporating a permanent positive charge in the molecule in the form of a trimethyl quaternary ammonium group appears to offer some marginal benefit and the incorporation of targeting groups such as galactose improves targeting to hepatocytes.

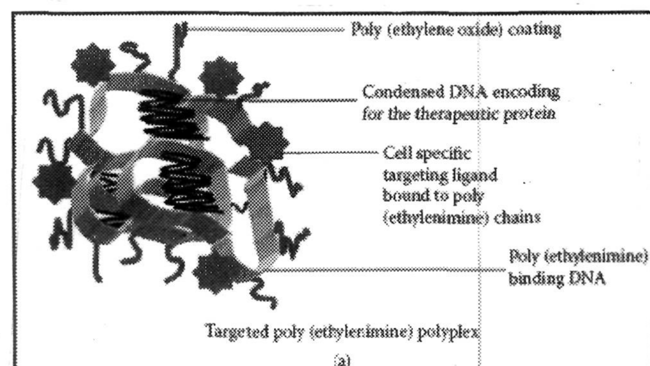


Figure 4 (a) Gene expression targeting after administration of a therapeutic gene may be achieved by coating polyplexes with covalently bound poly (ethylene oxide) and the use of targeting ligands [Ref. 2].

Urocanic acid groups have aided gene transfer, the latter possibly by aiding endosomal escape. However, chitosan, although able to achieve gene transfer to some extent, lacks the required level of efficiency that would be needed to allow it to be developed for the clinical delivery of genes.

7. Other Delivery Polymers

The cationic polymers discussed earlier have so far served as key players in the gene delivery field, with poly(L-lysine) being the most studied of the poly(amino acid) class. Other polyamino acids with protonable nitrogen atoms, necessary for DNA binding, have also been studied; these include poly (L-histidine) and poly (L-ornithine) derivatives. In addition, various other polymers have been studied as gene-transfer systems. Among these are a group of biodegradable polymers, e.g., poly [α-(4-aminobutyl)-L-glycolic acid], designed to degrade subsequent to having performed their delivery function. This strategy is aimed at minimizing the cytotoxicity associated with the accumulation of such agents within the cell and hence is aimed at improving the biocompatibility of the gene-transfer agent. Other polymers that have been developed are thermo sensitive ones designed to form a gel *in situ* on administration and thus provide a depot system capable of sustained DNA delivery.

8. Conclusion

In conclusion, a number of polymers by virtue of possessing a cationic charge at physiological pH have been found to be suitable candidates for the transfer of genes across the various biological barriers outlined above. Although PEI and a few others have demonstrated the ability to bring about a therapeutic response in preclinical models, the search for efficient and safe gene delivery systems is far from over. We have achieved a modicum of success with these first-generation agents, although not to the degree warranted for the initiation of a clinical development program. The promise offered by gene therapy is so great that it is not possible to abandon hope just yet. An ideal gene delivery system has to be able to shuttle the gene safely to the nuclei of its target tissue with the traveling gene having limited encounters with degradative influences. We know that a cationic macromolecule can achieve this, and so long as its own inherent toxicity can be curtailed, it is likely that the first class of synthetic gene delivery agent that makes the clinical development journey may be a cationic macromolecule with targeting ability.

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