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Review Article

Vectors for Non-viral Gene Delivery - Clinical and Biomedical Applications

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Abstract

Use of viral vectors for effective delivery of therapeutic genes is frequently rebuffed by the body's adaptive immune response against viral delivery vectors. Nonviral delivery system have been used to circumvent this problem but they are encountered with problems like transient expression and inflammatory responses induced by the reaction of the innate immune system reacting against bacterial DNA. However, within past decade the nonviral gene delivery has come to age and many efforts have been made to recognize the barriers for non-viral DNA delivery as potential allies in the development of novel therapeutics. This review summarizes important aspects of nonviral gene delivery such as barriers for gene therapy, various nonviral gene delivery vectors. The controlled release of pDNA using various stimuli responsive carriers have been discussed which shows a great potential to overcome barriers for gene delivery. Various clinical trials and patents involving nonviral vectors in gene therapy were briefly mentioned as the numbers show, there is an enormous growth of interest for nonviral gene delivery vectors. Novel strategies in nonviral gene delivery such as computer assisted intracellular kinetic models with kinetic parameters, fusogenic liposomes, conjugation of nuclear localization signals, were discussed which provide an impetus for researchers to achieve goal of preparing better nonviral transfecting agents with improved efficacy, enhanced stability and minimal toxicity.

Keywords: Gene therapy; Cationic liposomes; Transfection; Polyplexes; DNA condensation; Endosomal release

Introduction

In recent years gene therapy has become one of the highly publicized areas of biomedical, pharmaceutical and biotechnological research. In Gene therapy the normal gene or genetic material is used to replace the defective gene that is responsible for the disease. Scientific breakthroughs in the field of genomics and molecular biology have revealed that almost all diseases have a genetic component. Gene therapy has been considered not only one of the most potential approach for treatment of genetic disorders but also an alternative approach to deliver proteins, as currently they are manufactured by incorporating genes in microorganisms which are cultured in the laboratory that produce required proteins which were coded by the incorporated genes. Gene therapy has been one of the most enigmatic areas of research which shows a lot of promise for treating wide spectrum of diseases but at the same time has been suffered by setbacks and has become challenge to scientific community [1].

Gene therapy is considered a unique approach in therapeutics as it can be adapted towards treatment of both inherited and acquired diseases. Gene delivery involves encapsulation of a gene of interest, which is ideally intended for delivery into the target cells. After a series of events taking place such as uptake by endocytosis, release of DNA into the cells, transcription followed by translation the protein of interest is produced. In order to achieve successful gene delivery, significant barriers must be overcome at each step of the above mentioned events to optimize gene activity while minimizing the potential inhibitory inflammatory responses.

Since the initiation of first gene therapy clinical trial fifteen years back, there were several ups and downs which staggered the field of human gene therapy. The gene therapy roller coaster started in 1990 with the treatment of the first patient in a gene therapy clinical trial for Severe Combined Immuno Deficiency (SCID) using a retroviral vector ex vivo to introduce the deficient adenosine deaminase gene in autologous T-cells [2]. This gave impetus for scientific community and nearly up to a decade the field of gene therapy blossomed with the validation of nonviral gene medicines and genetic vaccines in a plethora of animal models using plasmid- based systems. It was during that period the first nonviral gene therapy clinical trial by intratumoral injection of a cationic lipid-formulated plasmid to cancer patients [3], and the first plasmid bases vaccine clinical trial for diseases like HIV, malaria, and flu were initiated, more the a hundred gene therapy biotech companies were created worldwide [4-6]. Then a series of setbacks happened with first being happened in 1999 with the death of a patient following multiple organ failure in response to the intra hepatic infusion of an adenoviral vector. The second setback occurred in 2003 when two infants with X-linked severe combined immunodeficiency successfully treated with an ex vivo retroviral vector developed a T-cell leukemia like syndrome as a result of insertional mutagenesis [7]. In the early 2004 fortunately another clinical successes has triggered the interest of the public and stimulated the impetus and creativity of the scientific and medical community which was the world's first human gene therapy product, Gendicine®, an adenoviral vector expressing the tumor- suppressor gene p53, obtained market approval by the Chinese FDA for the

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treatment of head and neck squamous cell carcinoma. The large part to the setbacks was contributed by the viral vectors thus improving the safety of viral vectors has been emphasized a lot. Although viruses have been modifies to produce viral vectors that would not cause disease but would still be able to efficiently transducer the host cells, several significant issues remain unanswered related to their safety and manufacturing. Even though the number of clinical trials using nonviral gene products is small (~20%) compared to viral vectors the number of publications on nonviral gene delivery has increased exponentially over the past few years and many new delivery systems are being investigated in pre-clinical research development [8]. Viral vectors offer unique advantages as specificity and efficiency of transfection when compared with non-viral vectors. However, viral vectors suffer from disadvantages such as over expression of genes, pathogenicity and immunogenicity [9,10].

Gene delivery strategies

The challenge of specific and efficient delivery of genetic material into the diseased sites and to particular cell populations is the challenge that is being addressed using a variety of viral and nonviral delivery systems which have distinct advantages and disadvantages. The nonviral vectors (synthetic vectors) are generally reputed to lack of efficiency while offering flexibility and safety. The suitability of any gene delivery system should always be matched with the clinical situation, the specific disease and the chosen therapeutic strategy.

Conceptually nucleic acid based therapies take two different approaches first the delivery of plasmid DNA or related constructs to express the gene of interest which result in the increased activity of the target and second the expression of oligomeric genetic material such as antisense oligonucleotide, siRNA or DNAzyme which result in reduction of target activity. None of the current vector systems is able to clearly satisfy the various diverse needs and it is therefore important to appreciate the strengths and weaknesses of synthetic vector systems in the appropriate therapeutic context [11]. Following are some strategies of gene delivery.

Barriers to gene delivery and pharmacokinetics of gene delivery in cells

In order that a DNA to transfect the cell it has to overcome barriers such as surviving the extracellular environment and entering the target cell type, cytosolic delivery from the endosomes, traversing to the nucleus from the cytoplasm and finally dissociation of the DNA from carriers for transcription.The extracellular environment presents the major hurdle the foreign would have to overcome. The nuclease capable of digesting unprotected DNA into fragments and eventually incapacitating its ability to express the encoded protein. One of the major approaches of the DNA overcoming degradative enzymes is to condense it into a compact form such that sites vulnerable to cleavages could be protected. This condensation is based on the electrostatic interactions between the anionic nucleic acid and the positive charges of the synthetic vector which complex and condense the NA into nanoparticles. Several poly cations such as Polyethylenimine (PEI), Poly-L-Lysine (PLL), cationic lipids, dendrimers, etc have been used for this purpose. Based on the synthetic vectors used the resulting particle were termed as polyplex, lipoplex and dendriplex respectively [11].

Synthetic vectors, regardless of the route of entry which could either be via receptor mediated endocytosis or by pinocytosis, they would be brought into the early endosomes, which would either fuse with other endocytic vesicles, more commonly late endosomes that exocytose internalized products. The sudden lowering of PH to 5 within the microenvironment of the late endosomes triggers the process of fusion between early and late endosomes. Late endosomes finally fuse with the lysosomes where degradation takes place due to acidic environment and degrading enzymes. Thus for a successful gene transfer to take place polyplexes have to escape from the endosomes before they fuse with lysosomes. To increase endoosmolytic property several strategies have been proposed one of them is to conjugate polycation with Mellitin, a major component of bee venom known to lyse cell membranes, a 25kDa PEI was conjugated with mellitin which showed augmented levels of endosomal release and nuclear transport [12].

In order that a delivered DNA to be functional it has to be transported into the nucleus where it could be transcribed into mRNA and ultimately translated into protein. This entry form cytoplasm to nucleus is governed by the nuclear membrane. The nuclear pore complex mediates the transport of molecules. This complex allows passive passage of small molecules but severely limits passage of larger molecules of more than 50kD across membrane [13,14].

Dissociation of carriers form plasmid is an essential process for the efficient transcription. To demonstrate these plasmids where micro injected into the nucleus and into the cytoplasm were compared, the plasmid injected into the nucleus gave rise to higher expression levels [15].

Pharmacokinetic considerations in optimization of intracellular trafficking

In order to optimize intracellular trafficking, it is necessary to balance various processes related to the rate-limiting intracellular barriers. The increased efficiency of one process may reduce that of others. For example tight condensation of pDNA, so as to produce small complex, enhances cellular uptake by endocytosis, but excess condensation inhibits transcription. A computer-assisted intracellular kinetic model with kinetic parameters (i.e. first order rate constant: time-^{1}) determined using quantitative data is a useful tool to analyze, simulate and optimize transgene expression. An integrated kinetic model for cellular uptake, endosomal release, nuclear binding, nuclear translocation, dissociation, and protein synthesis with first-order mass action kinetics was proposed by varga et al. 2001and demonstrated the utility of kinetic modeling for optimization [16].

Physical chemistry of DNA carrier complexes

The study of condensation of giant DNA molecules has been studied actively not only in order to develop non-viral gene therapies but also to understand self- regulation of genetic activity in living cells [17]. Extensive studies on the drastic change involving the confirmation of DNA known as DNA condensation have been carried due to that fact that DNA molecules in viral caspids, bacterial nucleoids, and nuclei of eukaryotes occupy a volume 10^{-4} to 10^{-6} times less then when free in aqueous solution [18]. Due to the limitations in available experimental approaches to analyze DNA condensation, such as light scattering, sedimentation, viscometry, linear dichroism, circular dichroism, and UV spectrometry, most studies dealing with DNA condensation have not clearly distinguished between

transitions occurring in individual DNA molecules and those involving aggregation/ precipitation of numbers of DNA molecules [19]. The important aspect in relation to the manner of transition is the morphological changes in the compact state. As illustrated in Figure 1, when the transition is diffuse or continuous, the final compact state exhibits a spherical morphology with liquid drop like properties, whereas when the transition is discrete, an ordered packed state, generating toroid and rod morphologies, results [20]. DNA is a highly charged polymer with rather condensed arrays of highly acidic phosphate groups. Small counter – cations, such as sodium and potassium in physiological conditions are present at concentrations above mM. Thus it is important to consider the degree of dissociation of the sodium or potassium salt of the phosphate moieties along DNA chain [18]. According to the counter ion condensation theory, about 70% of the negative charge of DNA is neutralized because of the condensation of the counter ion. When a DNA molecule is tightly packed, almost all of the negative charge should be neutralized, accompanied by the enhancement of counter-ion condensation. Thus the volume part of the compact DNA is fully neutralized whereas negative charge remains on the surface [21]. An interesting finding in this aspect is that multivalent cations such as spermidine and spermine induces the folding transitions of DNA and this phenomenon is inhibited by the monovalent ions. Thus at higher salt concentrations, larger amounts of multivalent cations are necessary to induce the compaction. In some cases like folding transition induced in crowding environment, the presence of salt is a necessary condition, i.e., salt is a promoting factor for compaction [21].

Based on the physicochemical insights into the folding transition of DNA, it has now become possible to propose a novel hypothesis regarding the relationship of the higher-order structure of DNA and its biological activity. As the compaction is completed and DNA molecule took an order for packing, there is complete inhibition of enzymatic action, such as transcription. However, when the folded structure is a swollen globule, enzymes can access DNA molecules. In fact, folded DNA from circular plasmid exhibits high enzyme sensitivity. It is highly expected that precise knowledge of DNA compaction will contribute to the development of non-viral vectors, and also to a full understanding of the mechanism of self-regulation of genetic activity in living cells.

Non-viral gene delivery vectors

Non- viral techniques of gene transfer represent a simple and more importantly, safer alternative to viral vectors. Thanks to their relatively simple quantitative production and their low host immunogenicity, nonviral vectors are attractive tools in gene therapy. In the past, the low levels of transfection and expression of a gene in host cells were among the viral vectors greatest disadvantages. However, ongoing studies and the development of new vectors that show transfection efficiency just like that of viral vectors point towards their great potential [22].

Naked plasmid DNA

The simplest technique of non-viral gene transfer is the use of so called naked DNA. A series of approaches for naked plasmid DNA based gene delivery strategies have been reported in recent years like, Naked plasmid DNA transfer method wherein a cytotoxic T-lymphocyte antigen 4- immunoglobulin (CTLA4-Ig) gene was delivered using a naked plasmid DNA [23]. Naked DNA was used for antiangiogenic therapy where the fetal liver kinase-1 gene was delivered [24]. One more interesting area that is the use of naked plasmid DNA gene delivery as electro gene therapy which is done after injection of naked plasmid DNA and delivery of electric pulses directly to the tissue the expression of gene of interest can be obtained [25], because of its inherent simplicity naked DNA is an attractive non-viral vector and moreover its ease of production in bacteria and manipulation using standard recombinant DNA techniques substantiates its use as non-viral gene delivery system. The other important advantage in using naked DNA gene delivery system is its ability to show very little dissemination and transfection at distant sites following delivery and also can be administered several times as it does not show any antibody response against itself [26].

Cationic lipids

Cationic liposomes are an important class of compounds suitable for carrying negatively charged DNA. There are at present several commercial transfection reagents that are based on cationic lipids like DOTMA(Lipofectin), DOTAP, DOSPA, DOSPER, DDAB, DODAC, Neophectin (PCL-2), DMRIE, DC-Chol, DOGS (Transfectam). However use of these reagents in vivo is plagued by their inherent toxicities.

Cationic lipids consist of a positively charged head group, a hydrophobic tail and a linker connecting the head to the tail group. The charged head groups are usually quaternary amines, tails are saturated or unsaturated alkyl chains or cholesteryl groups. Plasmid DNA can be covered by lipids into organized structures such as liposomes or micelles. This complex of DNA with lipids is called a lipoplex. Cationic liposomes in contrast to neutral and anionic liposomes, which need DNA implementation into the vehicle, cationic liposomes naturally, create complexes with negatively charged DNA. Their positive charge, moreover, allows interactions with the negatively charged cell membrane and thus penetration into the cell is permitted [27].

There are numerous reports on the use of various cationic lipids as non-viral gene delivery vectors. The use of cationic liposomes has made great strides between the initial report by Felgner et al. in 1987, and their use in the world's first human gene therapy clinical trial by Nabeletal [28]. Recent reports revealed the ability of lipoplex gene transfer into epithelial cells of the respiratory tract, which supports their usage in therapy of respiratory diseases and cystic fibrosis. Their expression in all main organs, mainly in lungs, was observed after intravenous administration of lipoplexes [29]. The development of Allovectin-7 was one of the most promising approaches of cationic

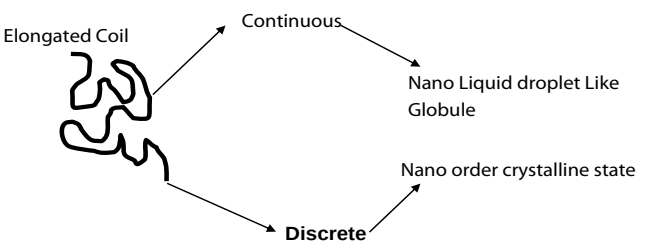

Figure 1: Transition of DNA.

lipids, which consists of a DNA plasmid harboring the genes for the allogenic MHC class I protein, HLA-B7 and beta-2 microglobulin and complexed with cationic lipid mixture. This DMRIE/DOPE lipid mixture facilitates the DNA uptake to tumor cells.Allovectin-7, therefore, provides several potential immuno stimulating functions and has been subjected to phase I clinical trials [30]. Another important area in which cationic liposomes are used in recent times is siRNA delivery. In a recent study by sato et al. siRNA complexed with galactosylated cationic liposomes for liver parenchymal cell selective delivery of siRNA has shown that siRNA did not undergo nuclease digestion and urinary excretion and moreover was delivered efficiently to the liver and was detected in parenchymal cells rather than liver non parenchymal cells. The endogenous gene (ubc13 gene) expression in the liver was inhibited up to 80% when complexes of ubc13-siRNA and galactosylated liposomes were administered to mice [31]. Though cationic liposomes have been extensively used as transfection agents *in vitro*. There, *in vivo* success is plagued by toxicity. In a recent study it was found that the mechanism behind the toxicity of cationic liposomes is largely induction of apoptosis. A cDNA micro array study showed that up regulation of 45 genes related to apoptosis, transcription regulation and immune response was due to lipofection [32]. Owing to their specificity, and ease of production cationic liposomes have carved niche among the non-viral gene delivery vectors. However their reaction is need for some issues to be addressed such as toxicity, in vivo stability of cationic liposomes.

Polymeric gene carriers

Synthetic polycationic polymers have gained wide attention as non-viral vectors for gene delivery. A number of reviews and book issues have already been published which illustrates various mechanisms through which they act and also various biochemical and therapeutic aspects of these systems. Polyplexes form these polymers form spontaneously as a result of electrostatic interaction between phosphate groups of DNA and oppositely charged groups of polycationic polymer [8,33-35]. However, a detailed description of various polycationic polymers used in gene delivery is out of scope of this review various polymers which are proved to be better transfecting agents can be discussed. To start with PEI (polyethyleneimine) is more appropriate as it has set a gold standard for nonviral gene delivery. Their ability, to condense large DNA molecules and eventually leading to homogenous spherical molecules of 100nm size or less as, they are capable of transfecting into cells efficiently for both *in vitro* and *in vivo*. In addition to above mentioned advantages PEI offers more protection against nucleases degradation than other polycations like poly-L-lysine owing to their high charge density and efficient complexation. However the major limiting factor for using PEI is their toxicity because of high cationic density [33]. The other synthetic polymers showing promisingresults in gene delivery are poly-L-lysine. It is one of the first polymers to be studied for nonviral gene delivery because of its peptidic nature i.e.it is biodegradable and hence it is more suitable for *in vivo* use [36]. Imidazole containing polymers have been reported to have efficient transfection properties. In various approaches -amino groups of poly-Lysine were modified with histidine or other imidazole-containing structures proved to be better transfecting agents than the unmodified poly-L-lysine [37-39]. Polyaminomethacrylates are one more class of synthetic class of poly cations which has shown to be efficient vectors for gene delivery *in vitro* and *in vivo* [40]. Poly(2-dimethylamino)ethyl methacrylate[p(DMAEMA)], the most studied of this class, was synthesized by free radical polymerization of 2-(dimethylamino) ethyl methacrylate in water using ammonium peroxydisulphate as initiator [40]. Similar to PEI this resulted in a high molecular weight linear polymer which would condense large amounts of DNA and provide a physical barrier to nuclease digestion [40]. In one study the surface groups of p(DMAEMA) were modified so as to introduce hydrolysable groups on to the polymer surface which would enhance the DNA release from the corresponding polyplexes [41]. When p(DMAEMA)-based polyplexes were coated with folate PEG conjugate there was a sharp reduction in zeta potential and small increase in size. The interesting thing, about these folate PEG conjugated polymers, is that they have shown increased transfection of OVCAR-3 cells *in vitro* and hence are promising for targeted gene delivery [42]. Transfection and cytotoxicity studies were carried on amino methacrylate polymer where quaternary amine groups are connected to uncharged hydrophilic polymer of similar structure which is poly(N-hydroxypropylmethacrylamide)-b-poly(trimethy laminomethylmethacrylate) (PHPMA-b-PTMAEM). It was found that while toxicity has not been changed much but the transfection efficiency has been increased with the addition of PHPMA block [43].

Dendrimers as DNA carriers

Dendrimers are highly branched three dimensional macromolecules with well-defined structures constructed around a multifunctional core. They have novel structural properties such as a single molecular weight, a large number of controllable peripheral functionalities and a tendency to adopt a globular shape. There are two synthetic approaches used in preparation of dendrimers: the convergent approach and the divergent approach [44].

Earlier reports have shown that dendrimers with their modified surfaces utilize the basic cellular physiological mechanisms for better transfection of nucleic acids. Dendrimers have largely proved themselves as efficient carriers for nonviral gene delivery .This is substantiated by the fact that even though the utilization of dendrimers, as gene delivery carriers, is few decades old there are already commercial products available as gene transfecting agents. Polyfect® and Superfect® are two commercially available. Activated dendrimers, as gene transfecting agents where in Polyfect®, has intact dendrimer and Superfect® has fractured dendrimer as their component [45]. These dendrimers have a primary amine groups on their surface which binds to the DNA and also aid in condensing DNA into nanoscale molecules and eventually enhance the cellular uptake of DNA molecules. While the tertiary amine groups, which are buried in the deep core of dendrimers, act as proton sponge in endosomes and enhance the release of DNA in cytoplasm. The PAMAM dendrimers have gained wide attention of all dendrimers owing to their safety, non immunogenecity and their ability to function as highly efficient cationic polymer vectors for delivering genetic material into cells. These dendrimers have demonstrated more efficiency and safety than cationic liposomes or other cationic polymers for *in vivo*-gene transfer [46]. Currently, there are no clinical trials using the dendrimers, despite of the large number of *in vitro* and *in vivo* studies showing the potential applications of DNA complexes with dendrimers. The most important reason being the

Table 1: List of some vectors used in gene therapy clinical trials during the period 1996-2004.

Vector	% Accounting for clinical trials*
Retrovirus	27
Adenovirus	26
Naked/plasmid DNA	15
Lipofection	8.6
Herpes simplex virus	3
Adeno associated virus	2.5

*The data was obtained from www.clinicaltrails.gov

low transfection efficiency *in vitro* and *in vivo*. Thus, more rational and intelligent molecular design of dendrimers and their conjugates is needed in order to improve transfection efficiency and to minimize cytotoxicity and side effects.

Synthetic peptides for nonviral gene delivery

Peptides, which are specifically designed for gene delivery such as cell penetrating peptides are being under investigation as novel nonviral carriers for gene delivery. Cell-Penetrating Peptides (CPPs) are characterized by their ability to become internalized by mammalian cells and also assist in co transport of the macromolecules to the cellular interior. The actual mechanism, by which cell penetrating peptides get internalized into the mammalian cells by penetrating through lipid bilayer, is yet to be envisaged properly (Ref.??). However, initial reports say that it is due to passive passage over the membrane based on a temperature and energy dependent physicochemical mechanism, Later it was suggested that it is due to classic endocytosis pathway the CPPs are being internalized into the cells. Recent studies say that multiple pathways are involved as exact mechanism, is still needed to be elucidated. It was reported that in almost more than 50% of cases it is the Tat peptide that has been used for delivery of various molecules into the cell [47]. The bioactivity of RGD (arginyl-glycyl-aspartic acid tripeptide motif) is having a long history of inhibition of angiogenesis.. RGD peptide and its analogues showed several bioactivities, such as the induction of apoptosis by the endothelial cells and the inhibition of angiogenesis. Hart et al. [48] reported that application of RGD peptide in gene delivery. They used a peptide $[K]_{16}$ RGD peptide containing RGD motif and DNA binding domain of 16 lysine residues as gene carrier. They have demonstrated that efficiency was enhanced by the addition of the RGD motif to $[K]_{16}$ peptide [48]. Peptides are not only used individually but also have been used in conjugation with other cationic polymers for gene delivery. In a study Polyethyleneimine (PEI) was modified with RGD peptide. The *in vitro* transfection data have shown that the expression of PEI-RGD was 10 to 100 fold higher in integrin expressing epithelial (HeLa) and fibroblast (MRC5) cells than that of PEI without the RGD motif [49]. The mechanism by which RGD peptides act as transfecting agents is their recognition by integrins, a family of cell surface proteins. The molecular science of bioactive peptides has been progressed. There should be enhanced efforts on the basic research of peptides such as their physical characteristics, their detailed structure-function relationships, their mechanism of their biological activity, and further screening of functional peptides using peptide library. This will provide a large number of tools for use in gene delivery.

Oligonucleotide carrier based on β- 1,3- Glucans

Schizophyllan (SPG) is member of the β-1,3- glucans that is produced as a cell wall polysaccharide by fungi. SPG forms a macromolecular complex in DMSO and water by forming triple-helix structure. The novel features of this complex are its remarkable stability (large binding constant) and considerable water solubility under physiological conditions. The complex automatically dissociated at $P^H < 6.0$, because protonation of the nucleotide base induces conformational changes, which cause dissociation of the complex. Sakurai et al. 2003 has demonstrated the potential of modified SPG as an oligonucleotide carrier in antisense and CpG DNAs. They have modified SPG with spermine, RGD peptide, octarginine (R8) and cholesterol. The results demonstrated 20 to 55 fold increase in transfection when compared to the naked CpG DNA [50].The most distinguishing feature of SPG based-carrier in comparison, with other cation types, is that the driving force of the complexation is hydrogen bonds instead of electrostatic forces. Hence the total charge of the SPG/nucleotide complex is negative and there is no DNA-compaction problem in this system.

Biological and chemical hybrid vectors

In 1998 Nakanishi et al. proposed the concept of "hybrid vector". The term hybrid vector sometimes refers to the nonviral vectors containing some biological moieties and sometimes viral vectors combined with various synthetic materials. They proposed that, firstly, hybrid vectors have a virus like structure and function both for preserving their genetic materials and for efficiently delivering them into the target cells. Secondly, the hybrid vectors contain either DNA produced in a microorganism defective in DNA recombination or RNA, which in itself, lacks the recombination mechanism. They have prepared "Fusogenic Liposome" (FL) which is referred to as in generic meaning lipid vesicles (liposomes) capable of fusing with the cell membrane. The FL's are generated by the primary fusion of Sendai virus particles with simple liposomes at 37°C. These FLs encapsulate the contents of liposome and deliver them efficiently into cells by secondary fusion with the cell membrane. Thus inactivated Sendai virus and liposome encapsulating plasmid DNA can be used to generate FL capable of delivering the encapsulated DNA into cells efficiently and harmlessly [51]. In the preparation of FL's if unilamellar liposomes with a diameter of 200-250 nm are used, the resultant FLs have function and structure similar to that of intact Sendai virus particles [51].

It was reported that FL functions most effectively as a vector for novel cancer gene therapy when used in combination with tumor specific cytotoxic suicide genes. Horimoto et al. [52] have done an *in vivo* study where fusigenic liposome complexes containing Mammalian Degenerin (MDEG)-G430F mutant driven by the CEA promoter were injected intraperitoneally into CEA-producing gastric cancer cells in a mouse peritoneal dissemination model [52]. The development of a hybrid vector is a challenging task and may require long period of time to be modulated. Successful application of these vectors will benefit patients with various incurable diseases.

Miscellaneous

Endovascular microcoils are widely used in interventional procedures for the treatment of cerebral aneurysms. John et al. 2002 have reported the use of an endovascular micorcoil as gene delivery for the first time. Endovascular microcoil occlusion of cerebral aneurysms often uses platinum Gugliemi Detachable Coils (GDCs). In their study they have formulated gene delivery system onto the

Table 2: Clinical trials involving naked plasmid DNA.

	Indication	Phase	Gene	Route
US-519	Pancreatic cancer	II	GM-GCSF*	Intradermal
US-475	Non-Small Cell Lung Cancer	II	Transforming Growth Factor-α	Subcutaneous
US-378	Squamous cell carcinoma of head and neck	II	Interferon Gamma/Interleukin-2	Intratumoral
US-254	Melanoma	II	Melanoma antigen gp100	Intradermal
UK-071	Ano-genital neoplasia	II	Human papilloma virus(HPV) E6 and E7	Intramuscular
US-645	Peripheral Artery disease	II	Fibroblast growth factor (FGF)	Intramuscular
US-567	Ischemic Myocardium	II	Vascular endothelial growth factor(VEGF)	Intramyocardial
XX-002	Refractory angina pectoris	II	VEGF	Intramyocardial
UK-084	Tetanus	II	HIV-1 Env	Intramuscular
US-595	Cervical cancer	II	Human pailloma virus (HPV) 16 E7	Intramuscular
US-541	Stage IV Breast cancer	II	NY-ESO-1	Intralymphonodal
PL-003	Glioblastoma, meningoma	II	Insulin like growth factor-1 (IGF-1)	Subcutaneous

Table 3: List of patents involved in non-viral gene delivery vectors during the period 1996-2004.

Patent Number	Vector	Gene
US-20050164964	Chitosan containing branching groups	pLUC(Plasmid encoded Luciferase)
US-20030166593	Hep B envelop protein with cardiac cell targeting sequence	SERCA-2
US-20050287110	Cationic graft-coplymer	pLuc
US-20030100113	ODN-Nuclear localization signal conjugate	pLuc
US-6620617	Various biodegradable, bioadhesive and biocompatible polymers	Gene encoding adenosine deaminase.
US-20030096280	Biotinyalteddendrimer	β-galactosidase
US-6113946	DendrimerPolycation	pLuc-4
US-20060204472	Diaminobutane poly(propylene imine) dendrimer	pGFP
US-20040204377	PAMAM dendrimer	Cy3-SS/AS Duplex siRNA
US-6133243	Liposomal adenoviral DNA complexes	Tumor suppressor gene p53

surface of a GDC using immobilized anti-adenoviral antibodies to tether the vector, thereby delivering transgenes to modify cells in the arterial wall and invading the coil localized thrombus. They have used a collagen-based coating for endovascular coils that could be used for covalently linking anti adenoviral antibodies. These antibodies were used to tether replication deficient adenovirus (Ad-GFP encoding Green fluorescence protein) or Ad-LacZ(encoding β-galactosidase). The cell culture studies performed on rat arterial smooth muscle cells (A10) shown that GFP-positive smooth muscle cells were detected on platinum surface and LacZ positive cells were detected on the polyglycolic acid coil surface [53].

Clinical trials involving nonviral gene delivery systems

Many clinical trials of gene and antisense therapy are currently underway. Of the entire nonviral gene delivery systems only two approaches: a) naked plasmid-DNA delivery and b) cationic liposomes have been achieved the state of clinical trials. Cationic polymers have only been used in animal models and have not advanced into clinical trials due to various problems. Vectors used in gene therapy clinical trials are given with their relative percentages in the following (Table 1). Allovectin-7,that is a gene transfer product consisting of the gene encoding the allogenic MHC class I protein Human Leukocyte Antigen (HLA)-B7 heavy chain and β2- micorglobulin gene that is complexed with a cationic lipid mixture, was subjected to a phase II trial on patients with metastatic melanoma.

The first clinical trial of IFN-β gene therapy was started by patients with malignant glioma in April 2000 by yoshida et al. They have used cationic multilamellar liposomes consisting of N-(α-trimethylammonioacetyl)-disodecyl-D-glutamate chloride (TMAG), Dilaurolylphosphatidylcholine (DLPC), and DOPE. In some patients after treatment tumor growth ceased with little change in size over 10 weeks [54]. Various clinical trials are underway involving naked plasmid DNA especially in the gene therapy of cardiovascular diseases. The following (Table 2) gives more information about various clinical trials involving naked plasmid DNA delivery. Apart from several nonviral delivery systems involved in various clinical trials and great number of non-viral delivery vectors have been patented. The following (Table 3) gives more information about some of the patents involving non-viral gene delivery vectors.

Conclusion

The completion of human genome project has heralded a new era of gene discovery relevant to disease. The genetic treatments of diseases will require efficient and robust gene delivery methodologies. Nonviral gene therapy though reached stage of clinical trials by virtue of liposomal gene delivery technology still remains immature technology. The most challenging work in nonviral gene therapy will be overcoming the full range of rate limiting steps, from extracellular physical and chemical barriers to intracellular steps of endocytosis, endoosmolysis, and nuclear import. New polymeric or lipidic

materials that can bond and condense DNA and have low cytotoxicity and very high endocytosis efficiency are still needed. The studies in trying to synthesize functional polymers had revealed some exciting new developments. Such as, researchers in Washington university has reported that their polymer micelles with Shell Cross linked Knedel like (SCK) nanospheres could more efficiently compact DNA and protect it from enzyme digestion than traditional lipids and polymers [55]. Many endocytic pathways involving peptide assistant delivery techniques could be explored. For example, the TAT peptide, RGD peptide and MPS peptides are very efficient in introducing genes into the cells.

Use of magnetic nanoparticles is another attractive non-viral gene delivery strategy because of advantage such as high surface area, controllable size, facile surface modification and excellent magnetic properties. Surface engineered magnetic nanoaprticles were widely used for therapeutic gene delivery in various diseases such as cancer [56]. For efficient use of nonviral vectors for gene delivery it is important to explore various endocytic pathways and means to utilize those pathways for efficient gene delivery. The most important problems that have plagued the use of nonviral gene vectors are toxicity and specificity. Both of these drawbacks, in my opinion can be overcome if delivery vectors would be designed in such a way that vector has an endogenous biological component for specificity and to reduce toxicity. Development of fusogenic liposomes have shown a ray of hope in this aspect. The focus should be in that direction so as to achieve the goal for development of ideal nonviral gene delivery vector.

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