Systemic gene delivery using lipid envelope systems and its potential in overcoming challenges
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Nonviral vectors which offer a safer and versatile alternative to viral vectors have been developed to overcome problems caused by viral carriers. However, their transfection efficacy or level of expression is substantially lower than viral vectors. Among various nonviral gene vectors, lipid envelop systems are an ideal platform for the incorporation of safety and efficacy into a single delivery system. Emerging strategies for gene delivery using lipid-based delivery systems mainly aim at improving the transfection efficiency and potency while reducing toxicity, achieving prolonged release, cell-specific targeting, co-delivery of drug and gene. Earlier efforts to improve the transfection efficiency while overcoming the toxicity led to the need for preparing conjugates of lipids with polyamines. In this review, we highlight current lipidic vectors that have been developed for gene therapy, challenges, and their solutions.

Keywords: Lipid envelop, gene delivery, chronic diseases.

Introduction
Over the past two decades, gene delivery has transitioned the therapeutic arena of the diseases with over 1800 gene delivery clinical trials ongoing or conducted for a wide array of genetic diseases. Among the two broad gene or nucleic acid delivery approaches i.e. delivery DNA or delivery of RNA, DNA delivery deals with the delivery of therapeutic gene which either corrects the lacking expression of the required protein in the body by inserting the corrective gene in the host cell genome or induces the expression of the protein providing additional pool of the therapeutic protein in the body which elicits a specific therapeutic activity. The latter approach of delivery of RNA to the cells deals with suppressing the expression of the faulty gene or the overexpressed gene which is dysfunctional leading to the inception or exaggeration of a disease. Among these approaches, delivery of the therapeutic DNA to cells has been the most widely accepted technique as reflected by their highest number in the registered clinical trials worldwide. Though not much different in the composition, the physicochemical differences between DNA and RNA makes DNA more robust for its use. Although similar in structure comprising sugar-phosphate backbone connected with nitrogen bases arranged in a double-stranded helical structure, there are some crucial differences which forbid researchers in concluding about the ability of a vector in delivering both DNA and RNA. Unlike DNA, siRNA contains ribose sugar instead of deoxyribose. The ribose ring contains 2'-hydroxy group which makes RNA more susceptible to hydrolysis by serum nucleases than DNA [1]. The course of action of a DNA after administration requires to follow a specific path. The cellular delivery is
the first and prime important part of it. The cellular delivery deals with the cellular uptake and cytoplasmic release of the nucleic acid. Once in the cytoplasm, it uses cellular machinery to reach inside the nucleus where the nuclear enzymatic pool help translate the therapeutic protein from the inserted gene. However, practical applications is severely limited by the extracellular barriers such as high hydrolytic instability of nucleic acids due to susceptibility to degradation by nucleases and clearance mechanisms as well as intracellular barriers like endosomal degradation and cytosolic release of DNA [2]. Henceforth, the discussion will be carried out in terms of nucleic acids except for specific mentions.

Therapeutic gene delivery with DNA is employed in two approaches. One of which is direct in vivo administration of the therapeutic gene delivery system. And the other one is transfection of the cells in vitro using the gene delivery system and injecting the transformed cells directly into the target site. With advancement in the nanotechnology based delivery systems, the focus is growing in the direction of developing delivery systems that can be used for in vivo administration to address the target organs where it is difficult to inject externally transformed cells. Out of various routes of administration available for delivery of nucleic acids, the intravenous route is the most exploited due to its connectivity with every organ of the body. The intravenous route is apt for nanosized delivery systems, as they can be easily carried by vascular hydrodynamics. Therefore, systemic delivery of nucleic acids invokes use of various vectors which could be viral or polymer and lipid based nanocarrier systems. Out of these, latter non-viral vector systems have emerged as potential delivery vectors due to their negligible propensity for infection and immunostimulation.

Viral vectors like adenovirus, adeno-associated virus, retrovirus, though having high transfection efficiency, have been besmirched by limitations like immunogenicity, toxicity, oncogenicity of the virus and scale up issues. These limitations refocused the direction of research towards development of non-viral vectors having transfection efficiency approaching that of viral vectors. Several non-viral vectors have been evaluated for systemic delivery of siRNA which range from most widely used liposomes and other lipid systems, polyethyleneimine [3-13], cationic proteins/peptides [5, 14-22], aptamer conjugation [23-26], antibody conjugation [21, 27, 28], dendrimers [29] etc.

Amongst the non-viral vectors, the lipid-based delivery systems are considered the most promising due to their more biocompatibility as compared to other cationic systems. However, since all delivery vectors involve different principles of transfection, the development of each vector has to be studies distinctly. This review focuses on the role of lipid-based delivery systems for widely used systemic route of nucleic acid delivery. It highlights their uniqueness right from their physicochemical features to molecular mechanics of cell uptake and transfection efficiency as compared to other delivery systems. It also spotlights the challenges being faced in the current development, objectives of newer strategies for delivery and clinical scenario of lipid-based systems for systemic delivery of genes.

**Importance of Lipid envelope systems as nucleic acid delivery vectors**

Due to the structural similarity between the liposomes and cell membrane as well as tolerability of lipids, lipid-based vectors for delivery of genes make a logical choice due to their possible good interaction with cell surface. Cationic lipids have been used for more than decades now in gene delivery, with DOTAP being the most popular choice. Several commercially available transfection agents for gene delivery which include reagents of Lipofectamine® Series [Invitrogen, USA], Oligofectamine™ [Invitrogen, USA], RNAiFect™ [Qiagen, The Netherlands], X-tremeGENE® [Roche Molecular Biochemicals, USA], MVL5 [pentavalent cationic lipid from Avanti Polar Lipids, USA], DOTAP [Roche Molecular Biochemicals, USA], siPORT™NeoFX™ [Invitrogen, USA] and GeneSilencer® [Genlantis, USA] are all cationic lipid based vectors for gene delivery.

Lipid based systems also stand out due to their advantages over polymer based systems in several ways. PEI is considered a gold standard for gene delivery and is being studied extensively [30]. However, PEI based systems often pose problem of toxicity [6, 31-33]. This has been attributed to their high charge density [34] and non-biocompatibility due to their non-degradable nature [35]. Though toxicity has been the issue with the cationic lipids also, reports indicate improved transfection and/or reduced toxicity through use of liposomal coating of PEI polyplexes [6, 7]. Additionally, lipid based systems have shown high transfection efficiency due to rapid release of
therapeutic gene in cytosol after endosomal escape owing to their ease of metabolism in the cytosolic environment and property of endosomal membrane fusion which leads to direct cytosolic release of nucleic acid. However, studies have reasoned out hindered release of nucleic acids from PEI polyplexes in cytosol as compared to cationic lipid based systems [8-10], even though PEI provides good endosomolytic effect due to proton sponge phenomenon.

Other cationic polymers like chitosan, peptides and dendrimers have been explored recently however, they have not yet gained popularity as PEI or other lipid based systemic gene delivery systems [36]. Lipid based systems have one to several advantages over these delivery systems as well. Polypeptides provide better cell uptake [37], however instability of nucleic acid-peptide complex in physiological conditions pose a problem [38]. In contrast, lipid based systems have been found to form complexes through covalent modification of polymer with lipids like stearoyl chains or cholesterol with enhancement of transfection and/or reduction of toxicity [5, 17, 39].

Another additional advantage of lipid-based systems is that one has a vast range of choice of lipids. which can be selected and optimized for their amounts in the lipid composition of the delivery system depending on the cell types, toxicity issues, frequency of administration, targeting requirements, etc. to get optimal balance between transfection and toxicity. Also, modification of lipids is a relatively easy task for attachment of ligands or other functional moieties due to variety of easy and scalable conjugation chemistry available i.e. streptavidine-biotin conjugation, EDC/NHS conjugation. Maleimide-thiol conjugation etc. It is noteworthy that such modifications can be utilized for several purposes. Conjugation of targeting ligands to lipids allows modifying the surface of the liposomes for targeted delivery to cells [40, 41]. Cationic lipid vectors catering the needs of enhanced transfection and low toxicity can be synthesized through attachment of cationic polymeric, peptidic or other moieties. Hydrophilic chains and protein moieties can be attached to the lipids and modified lipids can be incorporated to provide long circulation and low cytotoxicity. Additionally, surface chemistry of the liposomes/any lipid envelope system can be modified using different amounts of the desired lipids.

**Overcoming challenges**

For effective treatment with gene therapeutics, lipid vector devised should ensure [i] delivery to correct cells of the correct tissues [ii] delivery to large number of target cells [iii] release in the cytosol and [iv] activation to silencing complex. In order to achieve these goals, several challenges and barriers need to be overcome.

**Overcoming toxicity**

One of the major issues of concern in case of systemic delivery of nucleic acids through cationic lipid vectors is the toxicity. This problem needs more attention in case of siRNA delivery than DNA delivery. siRNA activity is dependent on the cell division and hence, highly dividing cells show short duration gene silencing using siRNA while non-proliferating and slow-dividing cells or growth arrested cells show prolonged duration gene silencing [42, 43]. Even though said to be prolonged, knockdown of target gene lasts only for few days to few weeks [43-47]. Toxicity issues with such short-term activity of siRNA may become more concerning in case of diseases with high cell proliferation rate i.e. cancer and with chronic diseases which necessitate frequent administration of cationic lipid-based systems of siRNA. However, in case of delivery of DNA, cells’ capability to retain transfected DNA remains higher and hence, the tissue toxicities, though of concern, would be less making DNA delivery as gene therapy more feasible. Systemically administered cationic vectors may pose toxicity issues to the cells which are directly in exposure to these vectors i.e. RBCs, macrophages, monocytes, neutrophils, etc. which mediate several inflammatory cascades [48-50]. Uptake of cationic liposomes/lipoplexes by RES macrophages modulate the release of IL-6, IL-12, TNF-α, IFN-γ, NO and other proinflammatory mediators and immune cell activation inducing inflammatory cascade [50, 51]. Inflammatory toxicity, liver toxicity or haematological and serological changes have been reported on intravenously administered lipid-based DNA formulations. However, only inflammatory reactions in macrophages and moderate leukopenia have been associated with cationic lipids [52]. In particular, cationic liposomes formulated using cationic lipids [DOTAP, DSTAP, DPTAP, DMTAP and DDAB] have been shown to act preferentially on phagocytic macrophages than non-phagocytic cells [51]. The toxicity shown by cationic lipids were further enhanced by incorporation of DOPE in the formulation. Incorporation of DSPE instead of DOPE reduced the toxicity towards macrophages and use of PEGylated
lipid [DPPE-PEG2000] in DOTAP/DOPE liposomes completely abolished toxicity. This is attributed to reduced binding to cell membrane and subsequent cell uptake [51]. Also, proteins like albumin and transferrin have been shown to reduce the interactions with cells. Incorporation of DC-Chol in formulation has shown to form aggregates that tend to accumulate in capillaries of pulmonary region [53]. Avoiding of such lipids may be beneficial in case where very frequent administrations are required.

Cationic lipids have been shown to induce cytotoxicity to RBCs. They induce pore formation in RBC membrane which is further promoted by incorporation of fusogenic lipids like DOPE [54]. Pore formation in RBC membrane leads of erythrocyte haemolysis. This tendency is also reduced through incorporation of PEGylated lipids like [DSPE-PEG2000] in the lipid component [55]. Also, incorporation of HSPC and/or Cholesterol in the formulation of liposomes reduces the surface charge density of DOTAP/DOPE liposomes leading to reduced hemolysis[55-57]. Toxicity to RBCs has also been extrapolated to other cells of the body.

Toxicity issues of NA based lipid formulations may be due either to the nucleic acid itself or to the cationic lipid vector. Though siRNA molecules are specific in their activity, they may act on other cells causing off target adverse events. So goes for the DNA delivery systems, where wrong integration of the therapeutic gene in the host genome may alter the activity of gene where it gets inserted. However, similar in vitro cytotoxicity behaviour have been shown by nucleic acid complexes and liposomes alone indicating that only lipid type and concentration of different lipids in liposomes influence the toxicity behaviour [58]. Additionally, the toxicity mediated by lipoplexes have been shown to be dependent on the cationic lipid:nucleic acid charge ratio and composition of lipid in bilayer [58]. Reduced toxicity have been observed with vectors having high number of cationic head groups than singly charged cationic lipids due to reduced charge ratio required for transfection. Though inclusion of DOPE has a positive influence on the transfection efficiency of lipoplexes, it has exhibited more cytotoxicity to the cells as compared to lipoplexes prepared with DOTAP/DOPC [58, 59]. Replacement of DOPE with DOPC may be employed to reduce the toxicity of the lipid complexes. Incorporation of HSPC and/or cholesterol in liposomes also reduces the surface charge density of liposomes formulated only with DOTAP/DOPE [56, 57]. However, incorporation of cholesterol has been shown to be more effective in charge separation in cationic liposomes due to better interdigitation capability of cholesterol as compared to HSPC [55]. Also, reduced toxicity of PEGylated lipid carriers over non-PEGylated carriers has been reported. Studies with lipids with head-group charge ranging from +1 to +16 have shown that higher cationic lipid:nucleic acid charge ratios are required for efficient transfection, however, it has shown toxicity to the cells [58, 60]. This is attributed to the number of cationic lipid molecules in the complex rather than the charge density of the complex suggesting that dendritic lipids with higher head-group charge may be beneficial to obtain maximal transfection without causing significant toxicity [58, 60].

Cationic head-groups of lipids can also interact with cellular enzymes like protein kinase-C causing cell toxicity[61]. This tendency is higher with cholesterol derivatives containing cationic moieties due to their steroid backbone [62]. Avoiding such lipids in the lipoplex formulation may help to formulate a less toxic version for gene delivery. Commercially available cationic lipids, lipofectamine, lipofectin and oligofectamine have been shown to cause alteration in expression of several genes which ultimately caused marked increase in tendency of cells to enter early cell apoptosis [63, 64]. Additionally, stearyl amine liposomes have also been shown to induce cell apoptosis[65], [66]. The underlying mechanisms are attributed to the generation of reactive oxygen species as ectopic activity of superoxide dismutase and glutathione reductase and addition of ROS scavenger N-acetylcysteine reduced the apoptosis due to cationic liposomes [64, 67, 68]. This indicates that use of cationic lipids may inadvertently raise safety concerns and hence, should not be overlooked in RNA and DNA delivery experiments where interference in/masking of desired genotypic or phenotypic endpoints might occur. Though no strategies have been devised yet for overcoming apoptotic cell toxicity of cationic lipids, work on strategies which can reduce ROS generation or scavenge ROS may provide solutions to these toxicity issues.

**Overcoming loss of nucleic acid in systemic circulation**

In order to get maximum output from nucleic acid therapeutics, overcoming loss of activity of nucleic acid in systemic circulation is the first step. Though
intravenous delivery of gene delivery vectors affords a potential and attractive way for nucleic acid delivery, the applicability of route faces several confounding challenges and vector has to ensure delivery to the correct cells in correct amounts. Short length of RNA has been shown to pose stability issues even in in vitro cultures causing low transfection at lower cationic lipid/nucleic charge ratios which were efficient for DNA delivery [60]. Thus, in order to maintain stability of complex in hostile environment of systemic circulation, higher charge ratios are required.

RNA molecules themselves are below the molecular weight threshold limits of renal filtration which leads to their rapid elimination from the systemic circulation. Additionally, presence of nucleases in serum causes degradation of nucleic acids if administered intravenously in naked form [69, 70]. Though for DNA molecules, kidney clearance of whole DNA molecule becomes a less preferred pathway of elimination; degradation in serum by serum nucleases causes rapid loss of DNA. Lipidic vector systems protect nucleic acids from such renal clearance and nuclease based degradation. However, they also have their own demerits causing loss of nucleic acid in systemic circulation. Such systemic loss of nucleic acid from lipid envelope systems is attributed to several factors which range from RES uptake, binding to negatively charged serum components, degradation by serum nucleases etc.

Apart from inflammatory reactions described earlier, macrophage uptake also contributes to the loss of therapeutic nucleic acid in systemic circulation affecting therapeutic outcome. Uptake of cationic lipid vectors take place through non-specific ionic interaction with negatively charged cell surface constituents like chondroitin sulphate, dermatan sulphate and heparin sulphate proteoglycans and integrins and subsequent endocytosis [71-73]. Along with this, systemically administered cationic lipid vectors of nucleic acids show very low transfection partly due to their interaction with components of blood i.e. serum proteins like albumin, antibodies, complements and other negatively charged serum components [74-77]. Complement activation in part can be reduced by proper optimization of cationic lipid:nucleic acid ratio [74, 78]. As mentioned earlier, binding to serum proteins can be reduced through incorporation of PEGylated lipids in the lipid bilayer which provide a steric barrier around the liposomes hindering the closer approach of negatively charged serum components [79]. PEGylation, by preventing opsonisation and also by creating a highly hydrated sheath around the lipid carriers, hinders the macrophage uptake [79]. Formulation containing DOPE has also been shown to be profusely bound to serum proteins [albumin in particular] in mice [77]. Replacement of DOPE with cholesterol has reduced the association with serum proteins. Additionally, incorporation of cholesterol has also improved the transfection efficiency and reduced the total amount of cationic lipid required for maximal transfection [77].

Overcoming unwanted distribution

The second step after reducing the RES uptake and protecting lipid systems from serum components is to prevent unwanted distribution to non-target tissues. therapeutic RNA molecules are very specific and selective in their actions on mRNA. However, they can silent genes with slight variations in the sequences. Even, it has been reported that long double stranded RNA molecules cause antiviral interferon response as well as global protein expression shutdown. In case of DNA, the expression of the protein at the target site will be very much efficient in disease alleviation than to induce its expression at a remote place in the body which ultimately will be distributed to the whole body through systemic circulation making less concentration available at the target organ. Thus, it is of prime importance that nucleic acid complexes reach the target cells[80]. This might lead to several off-target effects as well as loss of therapeutic activity will be there due to unwanted distribution of nucleic acid molecules to non-target cells [81-83]. Additionally, such unwanted distribution on systemic administration accounts for very low levels of nucleic acids in the target cells, which will increase the dose requirements ultimately contributing to the toxicity due vector.

These concerns necessitate that systemic nucleic acid delivery systems be targeted to specific cells. However, though targeting ensures accumulation in the target organ, the formulation needs to remain in circulation for longer periods to ensure the targeting or the distribution to target organ to become strong. One approach is the surface conjugation of shielding moieties like PEG that mask the surface charge of cationic lipid vectors and can reduce the unwanted uptake in non-target cells [83]. However, to ensure delivery to target cells, these formulations need to be modified with ligands for receptors identified to be overexpressed or specifically
expressed by these cells. To quote a few examples, epidermal growth factor receptors for tumour tissue targeting [84], integrins for angiogenic vessels of cancer [85, 86] and transferrin receptors for brain targeting [16] and tumour targeting [87] may be utilized for targeted delivery of nucleic acids[88]. Also, one can select ligands from a range of growth factors, peptides, proteins, antibodies and lipoproteins etc. [89].

Conclusion
The physiological barriers in successful delivery of genes are making the clinical promises of gene therapy elusive ones. Therefore, it is essential that a sound scientific rationale is laid for future developments of lipid-based gene delivery systems for its delivery through potential intravenous administration to hasten its clinical applications. The development has to be rationalized to address individual challenges posed by extracellular barriers like serum stability, long circulation life, non-specific distribution, low cell uptake and toxicity as well as intracellular barriers such as endosomal escape and cytosolic delivery. Conventional liposomes and lipid-based formulations, though optimized to address these barriers, often lack in addressing one of these completely. So, efforts are being focused to develop newer lipid-based systems which overcome these barriers.

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