Chapter 2
Literature Review

Begin each day like it was on purpose

-*From "Hitch"*

2.1 Osteoporosis:

Osteoporosis is a disease of bones that leads to an increased risk of fracture. It has been denoted a silent disease due to its character of occurring without symptomatic changes in the body. Worldwide estimates show that osteoporosis accounts for over 8.9 million fractures annually which turns out to be an osteoporosis related fracture every 3 seconds [1]. Osteoporosis affects 200 million women worldwide with approximately one-tenth of women aged 60 affected by osteoporosis [2]. It affects the aged people making them bedridden affecting their quality of life. Worldwide, 1 in 3 women above 50 years age and 1 in5 men above 50 years age are affected by osteoporotic fracture [3-5]. This shows that osteoporosis is a global healthcare burden.

2.1.1 Pathophysiology of the disease

In osteoporosis, the bone mineral density (BMD) gets reduced, deterioration of bone microarchitechture takes place, and the amount of various transcription factors, growth factors and cytokines etc. in bone are altered. Imbalance between bone resorption and bone formation is the underlying mechanism in all cases of osteoporosis. In normal bone, matrix remodeling of bone is constant. Bone is resorbed by osteoclast cells, after which new bone is deposited by osteoblast cells. The three main mechanisms by which osteoporosis develop are (a) an inadequate peak bone mass (the skeleton develops insufficient mass and strength during growth), (b) excessive bone resorption, and (c) inadequate formation of new bone during remodeling.

These occurs due to various hormonal level defects that lead to cascades of processes which cause increased bone resorption by osteoclasts and/or decreased bone generation by osteoblasts. Lack of estrogen (e.g. as a result of menopause) increases bone resorption, as well as decreasing the deposition of new bone that normally takes place in weight-bearing bones. In addition to estrogen, calcium metabolism plays a significant role in bone turnover, and deficiency of calcium and vitamin D leads to impaired bone deposition; in addition, the parathyroid glands react to low calcium levels by secreting parathyroid hormone (parathormone, PTH), which increases bone resorption to ensure sufficient calcium in the blood.

Main hormones that regulate bone metabolism are as follows:

Decrease bone resorption: Calcitonin, estrogen
Increase bone resorption: parathormone (PTH), glucocorticoids, thyroid hormones, high dose vit.D
Increase bone formation: Growth hormone, vit.D metabolites, androgens, insulin, low dose PTH
Decrease bone formation: Glucocorticoids

Various growth factors, cytokines and transcription factors are involved in the pathogenesis of osteoporosis. These include RANK (receptor activator of nuclear factor κβ), RunX2 (Runt related factor X2), VEGF (vascular endothelial growth factor), TNF (tumor necrosis factor), TGF (transforming growth factor), BMPs (bone morphogenetic proteins), OPG (osteoprotegerin), OTX (osterix). Many of these factors have been studied for their potential use in osteoporosis.

2.1.2 Osteoporosis treatment and its management

Osteoporosis risk can be reduced with lifestyle changes and medication; in people with osteoporosis, treatment may involve both. Lifestyle change includes diet and exercise, and preventing falls. Medication includes supplemental calcium, vitamin D, calcitonin, bisphosphonates (zaledronic acid, ibandronate, etc), bone morphogenetic proteins (BMP-2 and -7) and several others. Most of the therapies are long term therapies and require closer monitoring to avoid any adverse effects. Some of the demerits of the current therapeutics of osteoporosis are described in the **Figure 2.1** below. Effectiveness of oral calcium and vitamin D supplementation has been evaluated extensively. The analyses show that calcium supplementation alone and vitamin D supplementation alone are not effective in preventing fractures in osteoporotic patients as the combination thereof [6, 7]. Effect of intravenous calcium infusion has also been evaluated in osteoporotic women for treating osteoporosis, but it was found to be ineffective in altering bone calcium turnover in osteoporotic women. Loss of total body calcium was similar to that in untreated subjects with osteoporosis [8, 9].

Glucocorticoid-induced osteoporosis and osteoporosis related to aging are mainly outcome of reduced bone formation due to reduced number of osteoblasts. Moreover,

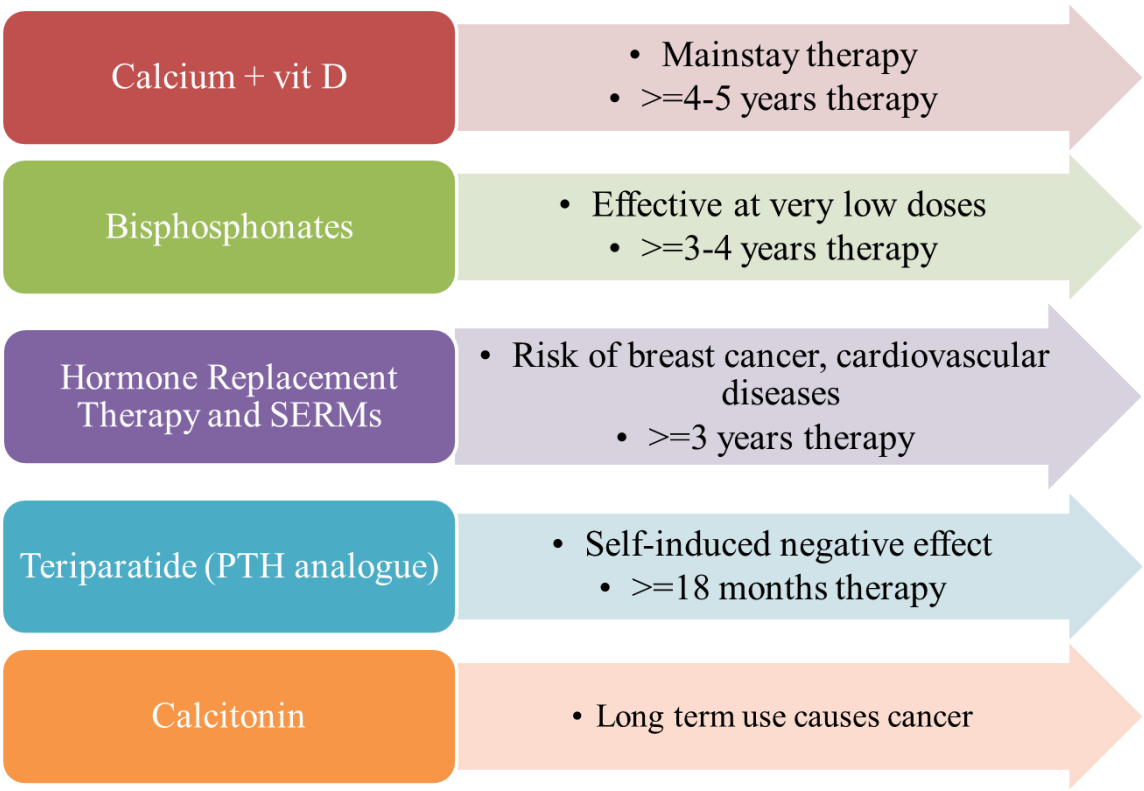

Figure 2.1 Current treatment options of osteoporosis and their drawbacks

calcium and vitamin D combination therapy has been found to be non-effective in preventing fractures in elderly (age >70 years) [10, 11]. An ideal way to prevent bone loss in such cases would be not only to reduce bone resorption, but also to promote bone formation. There is therefore an important need to develop therapeutic strategies capable of promoting bone formation in osteoporotic subjects.

2.1.3 Gene therapy as an approach for osteoporosis – Current perspective

In the past decade various gene delivery approaches have been studied for the treatment of osteoporosis. Such gene delivery approaches particularly act either by inducing or one or other growth factors, cytokines, transcription factors, other mediators or their receptors that are implicated in osteoporosis. Advancements made in the treatment of osteoporosis with gene delivery are described below with brief review of various gene delivery systems evaluated for osteoporosis treatment in animals (**Figure 2.1**).

Various cytokines, particularly interleukin-1 (IL-1) and tumor necrosis factor (TNF), have been strongly implicated in postmenopausal osteoporosis occurring due to estrogen

deficiency. Both of these cytokines are powerful inducers of bone resorption. From this information, it follows that inhibiting the biological activities of IL-1 and TNF should reduce bone loss under conditions of estrogen deficiency. Genes encoding for IL-1 receptor antagonist (IL-1Ra) or soluble form of TNF receptors would ameliorate the osteoporotic bone loss by inhibiting osteoclastic activity [12, 13].

Intravenous delivery of human osteoprotegerin (hOPG) gene using viral vectors results in systemic circulation of the OPG which in turn inhibits osteoclastic activity. The mechanism involves the binding of OPG to RANKL (receptor activator of nuclear factor κβ ligand) which prevents the binding of latter to RANK. This in turn suppresses its ability to increase bone resorption by osteoclasts [14, 15]. LIM mineralization protein (LMP) which induces the bone mineralization and expression of various osteogenic genes, BMP-2, RunX2 (Runt related transcription factor X2), OSX (Osterix) etc., and thereby promotes the osteoblast differentiation. One study has also shown that it induces bone formation more efficiently than even BMP-2 [16].

Among all gene delivery approaches, delivery of genes of bone-morphogenetic proteins has been most extensively evaluated (**Figure 2.1**). Bone morphogenetic factors (BMPs), mainly BMP-2, BMP-4, BMP-6, BMP-7 and BMP-9, are other osteogenic proteins that have been studied for bone regeneration in fractured bone healing, osteoporosis and osteopenia [17]. Recombinant human bone morphogenetic protein-2 and -7 have been recently granted United States Food and Drug Administration approval for select clinical applications in bone repair [13, 17]. These BMPs act primarily as differentiation factors, turning responsive mesenchymal cells into cartilage- and bone-forming cells. [18] While significant progress has been made in the delivery of recombinant osteogenic proteins to promote bone healing, the short half-life and instability of the protein requires the delivery of milligram quantities of factor or multiple dosages [13]. So, delivery of genes encoding for various BMPs have been investigated in various studies (**Figure 2.1**). Various transcription factors and growth factors such as VEGF, RunX2, TGF etc. have also been found to enhance the effects of various BMPs. Among various BMPs, BMP-9 has been shown to provide most robust and effective osteogenic activity in animal studies (**Figure 2.1**).

Table 2.1 Various gene delivery approaches used in osteoporosis

Gene therapy with	Encoded protein		Vector for transfection	Use of	Route of administration	Remarks	Ref.
pDNA	IL-1Ra(interleukin-1 receptor antagonist		Adenovirus	Recombinant adenovirus	Intramedullary injection		[12]
pDNA	BMP-2		Adenovirus	Recombinant adenovirus	--		[19]
pDNA	Bone morpho-genetic proteins (BMPs)	BMP-2	Adenovirus	Recombinant adenovirus and AdBMP-transduced osteoblast progenitors	Intramuscular injection (in quadriceps)	Activity inhibited by BMP-3	[20]
		BMP-3				Negative regulator of bone formation	
		BMP-6				Most robust and mature ossification, Activity inhibited by BMP-3	
		BMP-7				Activity inhibited by BMP-3	
		BMP-9				Most robust and mature ossification, Activity inhibited by BMP-3	
pDNA	BMP-2		Baculovirus	Recombinant baculovirus-transduced hMSCs (mesenchymal stem cells)	Injection into back subcutis		[21]
pDNA	BMP-2		Adenovirus with RGD tripeptide containing coat	Recombinant adenovirus-transduced hMSCs		Coat with RGD peptide enhances interaction with MSCs' surface integrins and thus enhance transfaction.	[22, 23]
pDNA	BMP-2 and RunX2		Adenovirus	Recombinant adenovirus-transduced pluripotent C3H10T1/2 cell	Subcutaneous implant	Complementary effect of RunX2 and BMP-2 on bone formation	[24]
pDNA	BMP-4 and vascular endothelial growth factor (VGEF)		Retrovirus	Recombinant retrovirus	Implantation into defect	VEGF and BMP-4 appeared to act synergistically to enhance bone healing	[25]
pDNA	BMP-9		Adenovirus	Recombinant adenovirus-transduced hMSCs	Intramuscular injection	-	[26]
pDNA and fusion	Human osteoprotegerin		Adenovirus	Adenovirus	Intravenous injection	-	[27]

Gene therapy with	Encoded protein	Vector for transfection	Use of	Route of administration	Remarks	Ref.
construct of pDNA with immune-globulin constant domain (pDNA-Fc)	(hOPG)					
pDNA	Human osteoprotegerin (hOPG)	Adenoassociated virus	Recombinant adenoassociated virus	Intravenous injection	-	[14]
pDNA	Human osteoprotegerin (hOPG)	Adenoassociated virus	Recombinant adenoassociated virus	Intramuscular injection	-	[15]
pDNA	BMP-2 and VGEF	Nonviral gene transfer	Gene transfer	Intramuscular injection	VGEF synergized the effect of BMP-2 on ossification	[28]
pDNA	BMP-7	Nanostructured calcium phosphate (NanoCaP)	Fibrin gel matrix of pDNA-NanoCaP	Intramuscular implantation	-	[29]
pDNA	BMP-2	Nanostructured calcium phosphate (NanoCaP)	Collagen Gene activated matrix of pDNA or pDNA-NanoCaP	Subcutaneous transplant or injection in bone-marrow	Modification of GAM with CaP effective in tissue regeneration at lower pDNA level	[30, 31]
pDNA	BMP-2	Nonviral gene transfer	BMP-2 gene-modified autologous MSCs or β-tricalcium phosphate	--	-	[32]
pDNA	LMP-3	Adenovirus	Recombinant adenovirus	Intramuscular injection	More efficient ectopic bone formation in-vivo than BMP-2	[16]

2.2. Lipoplexes as gene delivery vector

Among various vectors researched for gene delivery, those used in osteoporosis include viral vectors mainly adenoviral vector, adenoassociated viral vector, baculoviral vector and retroviral vector. Though providing very efficient transfection ability, viral vectors bear a lot of disadvantages mainly higher oncogenic, inflammatory and immunogenic potential and also virus insert their genome into host genome in random pattern restricting functioning of host genes. This is changing the scenario of gene delivery from viral based delivery to non-viral gene delivery. However, recently a few instances have been reported where non-viral gene delivery have been used *in vivo* preclinically. These vectors include lipid-nucleic acid complexes. This opens a possibility to develop and use liposomal vector (synonymously used terms are lipoplexes, lipid-DNA complex, liposomes etc.) for gene delivery in osteoporosis.

Lipoplexes have become the most used gene delivery vector for in vitro gene delivery to cells and have been successfully evaluated *in vivo* in animals for treatment of various genetic conditions such as cancer, osteoporosis, Alzheimer's disease, Parkinson's disease, multiple sclerosis, viral infections, cardiovascular diseases and many more [33].

Among the non-viral gene delivery systems, naked DNA and lipofection has been used in clinical trials with 5.9% of clinical trials employing lipofection as a gene delivery system [34]. With increasing attention on the nanotechnology based gene delivery systems and advancing understanding of viral and non-viral gene delivery systems, lipoplexes based gene delivery are projected to be used most used gene delivery system.

Liposome mediated gene transfer occurs by endocytosis where liposomes can bind to cell membrane and get engulfed into the cells. Endocytosed liposome-DNA complexes can release DNA into cytosol [35-37]. Cytosolic release is often promoted by the helper lipids which have fusion capabilities i.e. DOPE or the lipids which have capability to destruct endosomal wall. Additionally, enhanced transfection can be rendered by the lipids which provide buffering effect. DNA released can migrate to nucleus. Additionally, transfection can also follow the direct cytosolic uptake through direct fusion to cell membrane of the lipoplexes [35-37]. Lipoplexes offer inherited low toxicity characteristics of biocompatible

bilayer structure. Moreover, lipoplexes can be modified in order to provide advantages such as a) ability to target various organs by modifying liposome surface by attaching appropriate ligands, b) reduced immunogenic response, c) differential release characteristics and d) protection of the complexed gene [35]. Few shortcomings of the liposomal gene delivery systems are cellular toxicities, low transfection efficiency, uptake by reticuloendothelial system cells, low target organ delivery, low protection of DNA against *in vivo* milieu etc. Following sections will discuss in detail on the aforementioned challenges with special focus on systemic delivery of gene therapeutics.

2.3. Systemic gene delivery using lipid vectors-lipoplexes and other lipidic systems

Over the past two decade, 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 the 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 [38]. Further, the plasmid DNAs are usually large and of the order of several kilo base pairs against RNAs which are often 19-21 base pairs long. These renders DNA different in the

molar charges which makes them require different condensation (complexation) chemistry for development of sTable 2.and effective delivery vector using cationic lipids/polymers. It is reported that RNA, owing to its stiff structure and low charge density, forms loose complexes with cationic vector as compared to plasmid DNA [39, 40]. However, delivery of DNA is also challenging and involves factors such as the site of its action. Unlike RNA, their therapeutic site is inside the nucleus which demands vectors differing in their intracellular trafficking and necessitates the thorough evaluation of DNA delivery systems for their therapeutic activity. Mentioned differences in physicochemical and biological properties of DNA and RNA make it mandatory to make a meticulous choice of therapeutic gene i.e. DNA or RNA and choose and optimize a delivery vector suiTable 2.for needs.

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 [41]. 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 [42-52], cationic proteins/peptides [44, 53-61], aptamer conjugation [62-65], antibody conjugation [60, 66, 67], dendrimers [68] 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.

2.3.2 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 Natherlands), 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. However, PEI based systems often pose problem of toxicity [45, 69-71]. This has been attributed to their high charge density [72] and non-biocompatibility due to their non-degradable nature [73]. 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 [45, 46]. 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 [47-49], 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. Lipid based systems have one to several advantages over these delivery systems as well. Polypeptides provide better cell uptake [74], however instability of nucleic acid-peptide complex in physiological conditions pose a problem [75]. In contrast, lipid based systems have been found to form sTable 2.complexes through covalent modification of polymer with lipids like stearoyl chains or cholesterol with enhancement of transfection and/or reduction of toxicity [44, 56, 76].

Another additional advantage of lipid based systems is that one has a vast range of choice of lipids (**Table 2.2**) 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. 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.

Table 2.2 Choice of lipids for gene delivery

Cationic Lipids	Feature(s)
Monovalent cationic lipids	
DOTAP	Contains quaternary ammonium group with ester linkage, Most widely used cationic lipid
DOTMA	Contains quaternary ammonium group with ether linkage, First demonstrated cationic lipoid for transfection of plasmid DNA
DORI	One of methyl group of Quaternary ammonium group of DOTAP replaced with β-hydroxymethyl group, Increases integrity and stability of bilayer structure
DORIE	One of methyl group of Quaternary ammonium group of DOTMA replaced with β-hydroxymethyl group, Increases integrity and stability of bilayer structure
DDAB	Contains quaternary ammonium group
CTAB	
Stearyl amine	Contains primary amine group
Cholesterol based monovalent cationic lipids	
DC-Chol	Tertiary amine group linked with cholesterol and Degradable carbamate linkage
AC-chol	Primary amine group linked with cholesterol
MC-chol	Secondary amine group linked with cholesterol
TC-chol	Quaternary ammonium group linked with cholesterol
Multivalent cationic lipids	
DOSPA	Contains quaternary ammonium group and lipoamine, High transfection efficiency but high toxicity
DOGS	Lipoamine, helper lipids not required to achieve high transfection
GAP-DLRIE	Contains a primary amine, Quaternary ammonium and dodecyl tail, Higher transfection ability with low toxicity
MVL-5	Pentavalent cationic lipid with carboxamidoethyl benzamide structure

Helper lipids	
DOPE	Inverted hexagonal phase promoting lipid acting as membrane destabiliser
Glyceryl monooleate	Double gyroid cubic phase forming lipid higher fusogenic capacity and transfection
DSPE m-PEG2000	Improves serum stability and prolongs blood circulation
Cholesterol	provides structural rigidity
DOPC, HSPC, DPPC	Lamellar structure promoting lipids
DPPG, DPPS, DSPG	Anionic lipids for reducing toxicity of lipoplexes
Special purpose lipids	
Tristearin, Precirol ATO 5, Tricaprin, GMS, Cholesteryl oleate, triglycerides	For solid lipid nanoparticles
Soyabean oil, lipiodol, Squalene, Oleic acid,	For nanoemulsion based formulations

ABBREVIATIONS:DOTAP-- Dioleoyl-trimethylammoniumpropane, DOTMA-- *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium chloride, DORIE-- 1,2-dioleoyloxypropyl-3-N,N'-dimethyl-N'-hydroxyethyl ammonium bromide, DOSPA-- N,N-dimethyl-N-[2-sperminecarboxamido)ethyl]- 2,3-bis(dioleyloxy)-1-propanimium pentachloride, DOGS- dioctadylamidoglycylspermine, GAP-DLRIE --N-(3-aminopropyl)-N,N-dimethyl- 2,3-bis(dodecyloxy)-1-propaminium bromide, DOPE-- Dioleoyl-glycerophosphoethanolamine , DOPC-Dioleoyl –phosphatidylcholine, HSPC-- Hydrogenated soya phosphatidylcholine, DPPC--Dipalmitoyl phosphatidylcholine, DSPG – Distaroyl-phosphatidylglycerol, DPPG – Dipalmitoyl-phosphatidyl glycerol, DPPS – Dipalmitoyl-phosphatidylglycerol, DSPE-PEG_{2000}: 1,2-Distearoyl phosphatidylethanolamine-methylpolyethyleneglycol conjugate-2000, DC-chol-- 3b-[N-(N',N'-dimethylaminoethyl)carbamoyl) cholesterol), DORI (1,2-dioleoyloxypropyl-3- dimethyl-hydroxyethyl ammonium chloride), DDAB --dioctadecyldimethylammonium bromide

A number of lipid based formulations have been devised till date. Structural features of such lipid envelope systems and challenges associated with their systemic delivery are described here.

2.3.3 Structural features of lipid envelope systems of siRNA

Cationic lipids have been explored in delivery of siRNA and DNA since long back. Use of cationic lipids and helper lipids make the basic compositional differences that exist in the formulations of lipoplexes. A wide choice of lipid components is available for

formulating cationic lipid systems for delivery of siRNA **(Table 2.2**). A large variety of cationic lipids ranging from stearylamine and DOTAP to complex multivalent MVL5 (+5) are available for formulating lipoplexes.

Different formulation strategies have been developed using cationic lipids to make structurally diverse group of nanoparticulate systems. The most commonly employed systems for gene delivery evaluated using cationic lipids are phospholipid based systems which in particular are liposomal systems which exhibit lamellar structure which holds therapeutic gene on the surfaces of the lamella or inside the aqueous core of the liposomes. Additionally, other systems include inverted hexagonal micelles, micelles, solid lipid nanoparticles, lipid emulsions etc. All these formulations bear different structural features in terms of complexation with siRNA depending on their composition. The differences in their structural features and their physicochemical properties are discussed below.

The most lipoplex formulations reported in the literature bear most common lamellar phase of the lipids [77]. This may range from small unilamellar vesicles to large multilamellar vesicles. However, small angle X-ray diffraction work on DOTAP based vesicles has shown different structural arrangements taking place depending on the type of other neutral helper lipids used and concentrations of different lipids in complex (**Figure 2.2**). Namely, lamellar structure (L_{α}^{NA}) and inverted hexagonal structure (H_{II}^{NA}) have been observed for the cationic lipid-nucleic acid complexes [78, 79]. Incorporation of DOPE, due to its molecular shape, promotes the inverted hexagonal phase (promoting inverted micelle formation) [77]. Increasing concentration of DOPE in DOTAP/DOPE liposomes showed lamellar to inverted hexagonal phase transition with complete lamellar structure at mole fraction of 0.1, mixed phases made of lamellar and inverted hexagonal phases at mole fraction of 0.5 and complete inverted hexagonal phase at 0.8 [80]. However, incorporation of lipids other than DOPE (i.e. DOPC, Cholesterol, HSPC etc.) lamellar phase is promoted. DOPC forms lamellar phases at all molar fractions (0.1 to 0.9) with DOTAP [81]. A third phase which is related to the cylindrical micelles arranged in a honeycomb lattice have also been observed with the lipids with very large and highly charged head-group i.e. MVL5 which favor formation of micelles [82]. Additionally, study have been performed on DOTAP/GMO (glyceryl monooleate) siRNA complexes which have shown to form lamellar,

inverted hexagonal as well as a third type, cubic phases. This Table 2.cationic double gyroid cubic phase $Q_{II}^{G,NA}$ which enable highly ordered siRNA incorporation in complex leading to bicontinuous 3D cubic structure was formed at mole fractions of GMO of 0.75 to 0.975. Different structural features of these cationic lipid complexes affects the transfection efficiency, toxicity behavior and extent of non-specific silencing due to complexes [78, 79, 81, 82]. These diverse structural features of lipid siRNA complexes make it a prime requisite that use of neutral lipids incorporated in complex formation should be chosen wisely and evaluated to have insight on the vital parameters of evaluation of siRNA delivery.

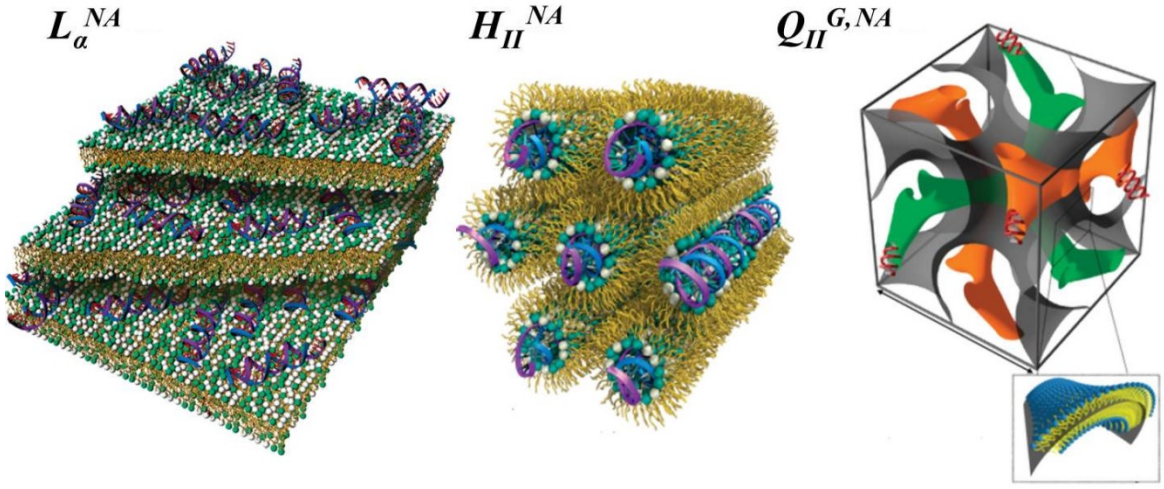

Figure 2.2 Schematic presentation of different phases of cationic lipid complexes with NA (nucleic acid) observed with different neutral lipids at different molar fractions. Lamellar phase (L_α^{NA}): NA is adhered to in a monolayer fashion on the surface of the bilayered lamella, inverted hexagonal phase (H_{II}^{NA}) : NA molecules surrounded by inverted cylindrical micelles and gyroid cubic phase ($Q_{II}^{G,NA}$) : water channels (shown in orange and green)containing NA molecules surrounded by lipid bilayers (shown as grey sheet which represents the lipid bilayer as shown in enlarged inset)

Structure of another such lipid RNA formulation has been simulated through Gromacs v.4 software using MARTINI force field and was shown to contain electron-dense core rather than a dense aqueous core characteristic to liposomal vesicle structure [83]. ^{31}P-NMR also confirmed that the core contained inverted micelles of ionizable cationic amino lipid complexed with RNA covered by a coat of PEG-lipid coat. The view was further supported by the demonstration of the dense core of particles through cryo-TEM studies

[84]. This unique structure was thought to be dependent on the manufacturing process of nanoparticle formation which leads to rapid mixing of RNA with cationic species forming nucleating complex and further increase in polarity of the system which formed a coat of PEGylated lipid coat around the system [83, 84]. This system completely protects the RNA from nuclease mediated degradation due to complexation of RNA inside the core of the lipids.

Other cationic lipid based formulations i.e. solid lipid nanoparticles (SLN), lipid emulsions have not been studied extensively in gene delivery and correlation between their structural features and transfection efficiency is yet to be established. However, as described earlier, structural differences might play important role in the transfection and toxicity profiles of nucleic acid complexes, and hence, it is required that studies be performed in this direction which will allow researchers to optimize such formulations with better outcomes. Researchers have hypothesized the structures of these formulations as shown in **Figure 2.3**. SLN prepared for nucleic acid delivery may either bear two structures depending on the preparation methodology employed. SLN prepared by solvent evaporation using tristearin as solid lipid and DOTAP-RNA complex have proposed to entrap the complex inside the solid lipid core surrounded by the surfactants [85]. Another RNA SLN based system showed a solid lipid core (with paclitaxel) surrounded by cationic lipid coat which was complexed with RNA [86]. A few examples are there of nanoemulsions with nucleic acid ionically attached on the cationic coat made of cationic lipids like (DOTAP, DODAB and/or DOTMA) and non-ionic surfactant surrounding the oil core (**Figure 2.4**) [87-89]. One nanoemulsion system employed oleic acid based cationic surfactant obtained by conjugation of cationic amino acids like lysine, arginine and histidine [90]. Such systems can offer an inexpensive alternative to cationic phospholipid based systems due to their low or no requirement of cationic phospholipids. Such delivery systems can be further explored for nucleic delivery as well.

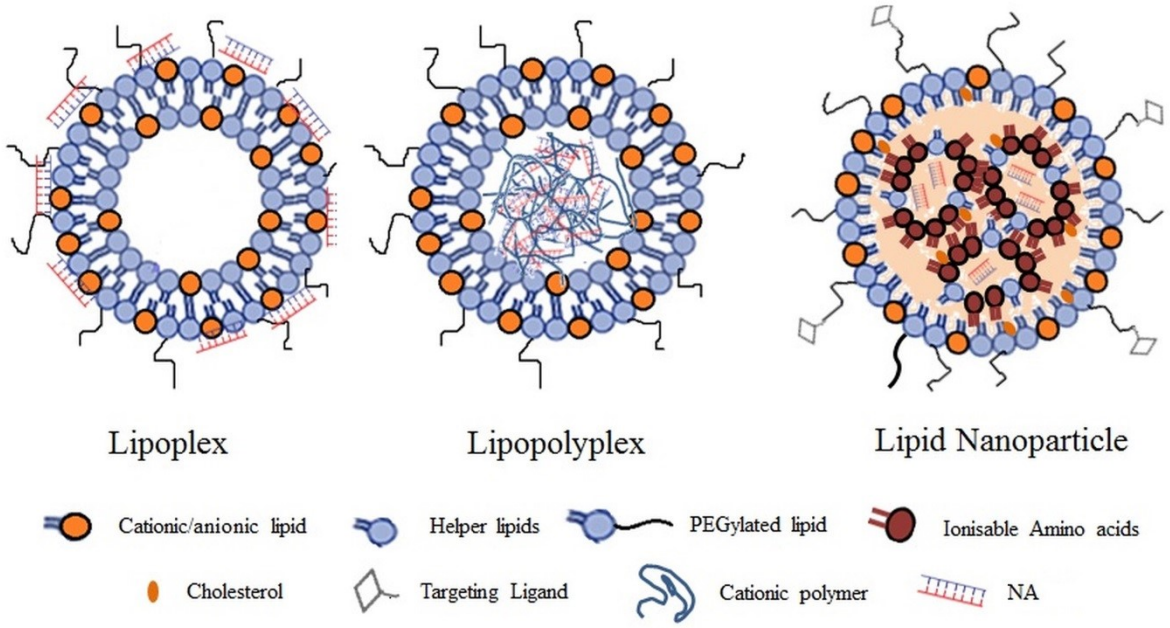

Figure 2.3 Schematic structures of lipoplex, lipopolyplex and lipid nanoparticle based NA delivery systems

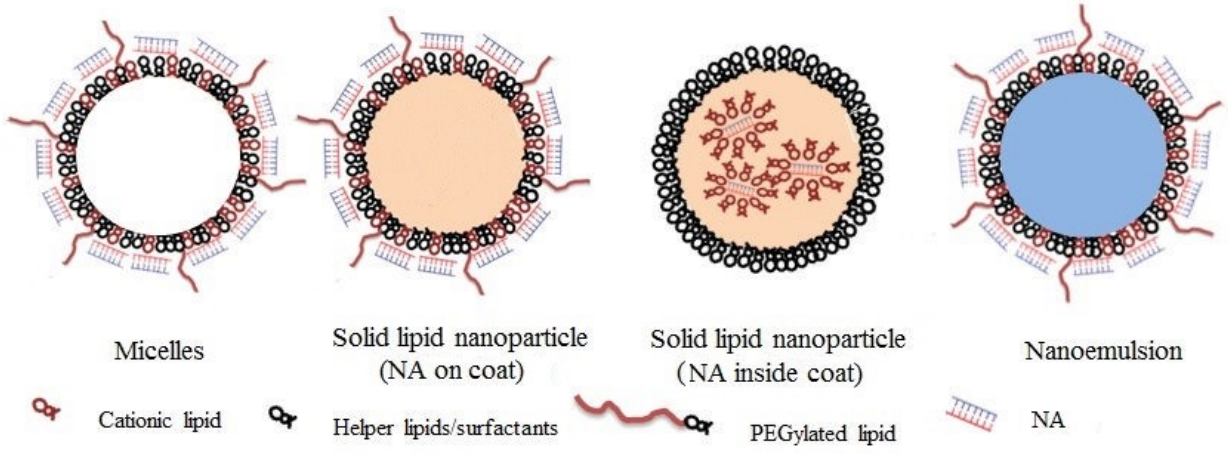

Figure 2.4 Schematic structures of micelles, solid lipid nanoparticle and nanoemulsion based NA delivery systems

Another important feature of gene delivery systems is the particle size. Lipid based nuceic acid delivery systems have shown a wide range of particle sizes ranging from few tens to several hundred nanometers. As reviewed by Ma et al., there is conflicting literature available on particle size requirements for maximal transfection through lipoplexes [91]. However,

generalization have been made that larger complexes provide more contact surface with cell membrane promoting endocytosis, fusion and subsequent transfection efficiency. However, with cells which are not engaged in active endocytosis, smaller particle size may be effective [91]. Now, looking at the constraint of systemic delivery, the particle size is the major governor of tissue distribution. For systemic delivery, optimal particle size is reported to be less than 200 nm. This particle size is found to be effective not only in cancer where leaky vasculature promotes accumulation of complexes in tumours, however, other conditions as well due to their lower uptake by RES. Most of the reports available on lipid based gene delivery systems have reported particle sizes of only few hundred nanometers.

2.3.4 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.

2.3.4.1 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 [92, 93]. Even though said to be prolonged, knockdown of target gene lasts only for few days to few weeks [93-97]. 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 [98-100]. 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 [100, 101]. 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 [102]. 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 [101]. 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-PEG_{2000}) in DOTAP/DOPE liposomes completely abolished toxicity. This is attributed to reduced binding to cell membrane and subsequent cell uptake [101]. 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 [103]. 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 [104]. Pore formation in RBC membrane leads of erythrocyte haemolysis. This tendency is also reduced through incorporation of PEGylated lipids like (DSPE-PEG_{2000}) in the lipid component [105]. 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 [105-107]. 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 [80]. 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 [80]. 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 [79, 80]. 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 [106, 107]. 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 [105]. 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 [80, 108]. 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 [80, 108].

Cationic head-groups of lipids can also interact with cellular enzymes like protein kinase-C causing cell toxicity [109]. This tendency is higher with cholesterol derivatives containing cationic moieties due to their steroid backbone [110]. 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 [111, 112]. Additionally, stearyl amine liposomes have also been shown to induce cell apoptosis [113, 114]. 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 [112-114]. 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.

2.3.4.2 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 [108]. 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 [115, 116]. 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 [117-119]. 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 [120-123]. Complement activation in part can be reduced by proper optimization of cationic lipid:nucleic

acid ratio [120, 124]. 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 [125]. PEGylation, by preventing opsonisation and also by creating a highly hydrated sheath around the lipid carriers, hinders the macrophage uptake [125]. Formulation containing DOPE has also been shown to be profusely bound to serum proteins (albumin in particular) in mice [123]. 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 [123].

2.3.4.3 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. 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 [126-128]. 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 [128]. 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 [129], integrins for angiogenic vessels of cancer [130, 131] and transferrin receptors for brain targeting [55] and tumour targeting [132] may be utilized for targeted delivery of nucleic acids. Also, one can select ligands from a range of growth factors, peptides, proteins, antibodies and lipoproteins etc. [133].

2.3.5 Enhancing transfection efficiency (cellular uptake and endosomal escape)

Successful gene delivery to the target cells requires the vectors to carry their cargo into the cells which is crucial for transfection efficiency of gene based therapeutics. Cellular barriers and trafficking can be of prime significance for cellular uptake and effective transfection into the cells. Initially, it was proposed that cellular uptake of cationic lipoplexes takes place through direct cellular membrane fusion, however, studies have now confirmed that cellular uptake pathway of cationic lipid vectors is majorly endocytosis mediated [134]. Endocytosis has been shown to take place through a variety of mechanisms ranging from macropinocytosis, phagocytosis, clathrin mediated endocytosis, caveolae mediated endocytosis and receptor mediated endocytosis [134]. Endocytosed material follows the pathway of early endosome, late endosome and then endolysozomes. However, for gene delivery systems, it is necessary to ensure release of nucleic acid in cytosol before endolysozome forms, as lysosomal enzymes lead to degradation of gene leading to therapeutic failure. So, it is essential to understand the internalization and cellular uptake mechanism of gene-carrier complex through the cell membrane and the factors which impact on the endocytosis and cellular release of therapeutic gene. Several key parameters have been identified which play role in the transfection by lipid-nucleic acid complexes and include structural differences in complexes, cationic lipid:nucleic acid charge ratio, complex membrane charge density, target ligand attachment etc.

2.3.5.1 Structural features of complex

Nucleic acid complexes made of DOTAP/DOPE, DOTAP/DOPC and MVL/DOPC have shown different transfection efficiencies *in vitro* [80, 135]. This has been attributed to the structural differences in the complexes described earlier and hence, aforesaid structural differences between the complexes can be related to their transfection efficiencies as well as toxicities. Replacement of DOPC with DOPE has not improved the transfection efficiencies, however, at the amount required for efficient transfection they were found to be toxic. The

inverted micellar phase promoted by DOPE has been shown to be playing role in fusogenicity of DOPE based systems. Additionally, even the systems formulated using other lipids along with cationic lipid and DOPE, which have exhibited lamellar liposomal structures may undergo transition to inverted hexagonal phase when ionization of DOPE takes place in acidic environment of endosomes triggering the phase transition, membrane fusion and membrane rupture events leading to cytosolic release of nucleic acid. However, with some formulations such phase transition requires additional mechanism. Cationic lipid based lamellar formulations show transition to hexagonal phase in presence of anionic phospholipid vesicles [136]. One study employing Saint-2/DOPE and Saint-2/DPPE lipoplexes demonstrated that DOPE based systems exhibit hexagonal phases even in absence of anionic phospholipids while DPPE based vesicles require the presence of anionic phospholipids for such transformation and subsequent fusion [137]. This demonstrated that different phosphatidylethanolamines exhibit differential ability to mediate nucleic acid release in cytosol. Similar structural behaviour has been observed with glycerylmonooleate/DOTAP based complexes which exhibit a distinct gyroid cubic phase which has been shown to improve the transfection efficiency [81]. Hence, one need to take into the structural features of the lipid based systems in order to get best outcome in terms of transfection.

2.3.5.2 Cationic lipid:nucleic acid N/P ratio

Studies have reported the effects of N/P ratio (charge ratio, cationic nitrogen of lipid/Phosphate of nucleic acid) with transfection efficiency for DNA and siRNA molecules.. Study has shown that for efficient RNA transfection, higher N/P ratio is required as compared to DNA transfection [108]. This has been attributed to reduced stability of complexes at lower ratios due to small siRNA molecules. Though cationic lipid-DNA complexes and cationic lipid-RNA complexes are structurally similar, there exists difference in local ordering of RNA and DNA in the lipoplex [80]. In lipoplexes, DNA exist as a rigid structure, in contrast siRNA exist in a liquid like phase. A considerably large amount of lipid is required to attain the charge ratio to achieve effective silencing efficiency using siRNA [80]. This is due to higher degree of freedom of siRNA as compared to DNA leading to higher energy barrier for complex formation. Secondly, lower adhesion energy of siRNA per unit length than for DNA because short chains of siRNA, unlike DNA, doesn't contain bound counterions which release on complexation contributing the half the adhesion energy [79, 80, 138, 139]. Additionally, ionic repulsion

between siRNA molecules in complex is larger than that between DNA molecules causing problems of packing of siRNA in complexes. All these factors lead to instability problems and transfection issues with siRNA complexes at low charge ratios and makes DNA as a better therapeutic choice for gene delivery if choice is possible.

Transfection studies with varying head-group charges indicated that in transfection efficiencies, initially there is an increase in the transfection efficiency with increasing N/P ratio [140]. The transfection reaches a plateau at a point after which further increase in N/P ratio doesn't confer more transfection efficiency to the complex. However, one thing which is noteworthy is that N/P ratio at which plateau occurred was different for lipids with different headgroups i.e. for lipoplexes with singly charged cationic lipid (DOTAP) transfection efficiency was not further enhanced after N/P ratio of 3, while with other dendritic lipids with head-group charge of +4, +8, and +16 N/P ratio of almost 4.5 was required for reaching plateau.

2.3.5.3 Lipid composition of complex

The lipid composition of membrane is the second factor which affects the efficiency of gene transfection. Types as well as content of neutral lipids in cationic lipid membranes affect the transfection efficiencies of complexes. Incorporation of DOPE has been shown in several studies to enhance the transfection efficiency. The mechanism of the DOPE mediated enhancement of transfection is reported to be due to membrane fusing capability of DOPE causing endosomal escape of nucleic acid cargo inside the cell [141, 142]. Other lipids like sphingomyelin and cholesterol also play important role in fusion [143].

Additionally, incorporation of the neutral lipids in the membrane influences the membrane charge density i.e. charge per unit area which can be related to the cationic nature of the membrane and transfection efficiency [105, 144]. However, membrane charge densities may be different for liposomes made at same cationic lipid:neutal lipid ratio using two different cationic lipids with varying head-group charges i.e. DOTAP vs. DOGS or DOTAP vs. MVL5 etc. conversely, it may be noted that two liposomes made using different mole ratios of cationic lipid to neutral lipid might show similar membrane charge density even when the lipids have different head-groups used. The effect of different lipid composition on transfection efficiency is given in **Figure 2.5**. There is a trend showing increase in transfection efficiency with increasing molar fraction of cationic lipids in complexes. When membrane charge densities of lamellar

phases of different cationic lipids with DOPC were plotted against transfection efficiencies it showed, regardless of the head-group charge of cationic lipid, a bell shaped curve showing an initial rise and then decline with a peak at the membrane charge density (17 x 10^{-3} e/Å) with maximum transfection efficiency. While the same plot for DOTAP/DOPE formulation showed no change in transfection efficiency on changing the membrane charge density. This results also confirmed the effect of lamellar phases (cationic lipid:DOPC) and hexagonal phases (DOTAP/DOPE) on transfection efficiencies. Hence, in order to achieve maximum transfection efficiency, formulation should be optimized to have correct membrane charge density and also correct lipid composition i.e. DOPE vs. DOPC or any other neutral lipids. Also, it may be noted that use of DOPE excludes the need of optimizing the formulation charge density and serves as a better choice for transfection. Additionally, multivalent cationic lipids have shown better results for specific gene silencing as compared to non-specific gene silencing.

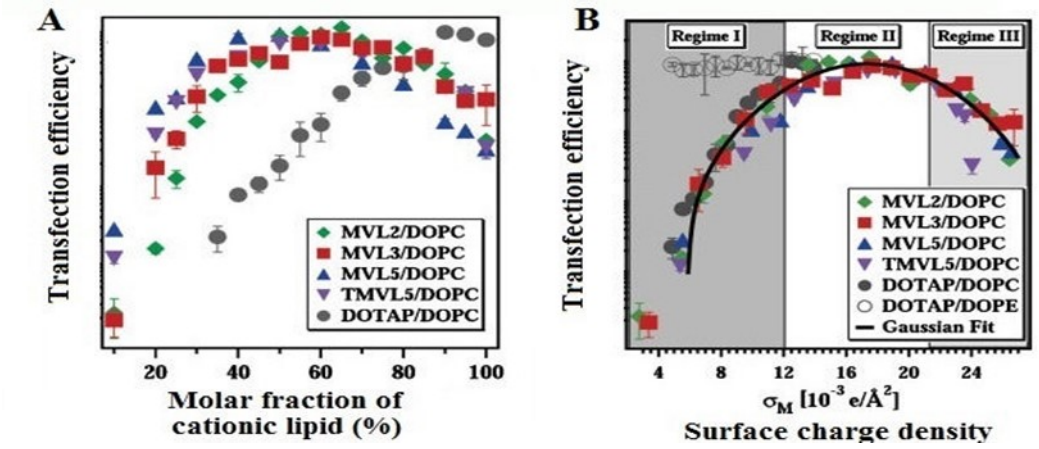

Figure 2.5 (A) Transfection efficiency as a function of mole fraction of DOPC and cationic lipids (B) Transfection efficiency as a function of membrane charge density. Color legends: Different lipids evaluated are shown in different colour legends i.e. DOTAP – monovalent (grey circles for DOTAP-DOPC system and open grey circles for DOTAP-DOPE system), MVL2 - divalent (green diamonds), MVL3 - trivalent (red squares), MVL5 - pentavalent (blue triangles) and TMVL5 - pentavalent (purple inverted triangles). Gaussian fit to the DOPC systems is shown as a bell shaped curve which is divided in three regimes of transfection efficiency by grey and white shading.

Additionally, cellular uptake has not only been found to depend on the cationic lipids in the complexes but also the attachment of the ligands on the surface of the complexes. Surface modification of the complexes with ligands enhances cellular uptake of complexes through receptor mediated uptake. Thus, attachment of receptor targeted ligand enhances accumulation of

nucleic acid complexes in target tissue as well as provide better transfection efficiency through endocytosis mediated uptake which have been confirmed in the human trials as well.

2.3.6 Emerging Strategies for gene delivery

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 [145-147]. Polycation liposomes (PCL) prepared so were thought to provide advantage of both liposomes and polycations for systemic siRNA delivery. Recently, Asai et al. have proposed systemic siRNA delivery using liposomes made of dicetyl phosphate-tetraethylenepentamine (DCP-TEPA) [148]. PCL were prepared suing DCP-TEPA, DOPE, DPPC and cholesterol and were loaded with siRNA. They reported that short polycations such as TEPA, unlike polyethyleneimine, are stably presented on PCL surface; and therefore, do not interfere with advantages of PEGylation such as RES escape and log circulation half-life after systemic administration. For ideal systemic delivery, a sTable 2.PEG coating is required for preventing interactions with serum components and subsequent aggregation which lead to rapid systemic clearance through RES [149]. However, PEGylation performed to improve circulation times inhibits both uptake and endosomal escape and is undesirable after cellular internalization [150]. To overcome this, Hatakeyama et al. developed a PEG-Peptide-DOPE (PPD) which can get rid of PEG after cleavage in matrix metalloprotease environment of tumour cells and also used fusogenic GALA peptide to enhance transfection [151]. The content of GALA and PPD was optimized to get synergistic functions of both GALA and PPD and a molar ratio of 1:1 was able to restore the transfection efficiency of system lost due to PEGylation.

Realizing the fact that gene delivery is not about overcoming a range of extracellular barriers but also overcoming the intracellular challenges as described earlier such as endosomal escape and cytosolic release, efforts are being directed to control the intracellular trafficking of delivery systems. Multifunctional envelope type nanodevices (MEND) have been proposed to have better endosomal escape capacity than any other lipid based vectors. MEND contains nucleic acid condensed into core particle which is surrounded by a lipid

envelope. MEND with permanently cationic lipids like DOTAP or pH sensitive lipids such as YSK05 have been studied [152, 153]. MEND containing pH sensitive lipid having an apparent pKa 6.4 to 6.6 becomes cationic in endosome and fuses with anionic endosomal membranes through phase transition to inverted hexagonal phase.

Another way to enhance transfection is to conjugate lipids with cell penetrating peptide (CPP) such as TAT peptide, oligoarginine, penetratin and low molecular weight protamine [154]. Recently, Tomohiro et al. conjugated lipid such as DOPE with CPP derived from protamine which acted both as a CPP and gene carrier maintaining stability. The cell uptake studies showed that lipid nanoparticles without CPP were poorly internalized into B16F10 murine melanoma cells which suggests that lipids modified with protamine derived CPP are facilitators of nucleic acid internalization and can be used to boost the transfection efficiency of lipid based nucleic acid carriers [155].

One of the most promising lipid based vectors for systemic delivery of gene are SNALPs (STable 2.Nucleic Acid-Lipid Particles). The uniqueness of SNALP lies in the fact that they contain the nucleic acid enclosed by a lipid lamella of cationic and other helper lipids. The core makes it highly sTable 2.to nuclease degradation and aids in cellular uptake, while the fusogenic lipids facilitate endosomal release. The PEG coating makes it highly bioavailable by protecting the particles *in vivo* to escape rapid systemic clearance [156]. SNALP have been studied intravenously in animal models of dyslipidemia and viral infections like hepatitis B (HBV), and Ebola (Zaire) [157]. Ambegia et al. reported that a PEG-lipid conjugate in envelope can provide the advantage dissociation of PEG-lipid conjugate from the SNALP after reaching the site of action converting the sTable 2.nanoparticle into a cationic charged transfection-competent entity [158]. The content of cationic lipids in SNALP is generally lower than that of PEGylated liposomes, e.g. 5 – 10 % by mole for gene and still lower for siRNA. Systemic administration of SNALP-siRNA in HBV infected mice displayed a long plasma half-life [159]. Three Daily dosing of 3 mg/kg/day of siRNA showed prominent and long lasting reduction in serum HBV DNA levels (one log unit for >7 days) and further reductions up to 6 weeks on weekly dosing indicating long circulation characteristics of SNALP.

Very recently, a new strategy has been devised using cell penetrating peptide of which lysine residues are caged by a photolabile protective group which helps specific uptake of siRNA liposomes by cancer cells through tumour localized exposure of near infrared-NIR light [160]. The infrared exposure on tumour area causes cleavage of the photolabile protective groups and the cationic charge of CPP is exposed which in turn enhances cellular interaction and uptake giving efficient anticancer activity. Additionally, this targeting strategy has been augmented by incorporation of asparagine-glycine-arginine peptide which renders liposomes to preferentially accumulate in tumour tissue *in vivo* followed by NIR mediated CPP uncaging and interaction with other cells [161].

2.3.7 The way forward

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. Cationic liposomes, SNALPs, lipid conjugates, lipidoids and ionizable lipids appear to be most promising for intracellular delivery of gene therapeutics. The evidences from clinical trials are pointing out safety issues and inadequate potency issues which need attention for future developments.

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