Chapter 2

Literature Review

Chapter 2

2.1 Atherosclerosis

"Atherosclerosis is a disease in which plaque builds up inside the arteries. Arteries are blood vessels that carry oxygen-rich blood to the heart and other parts of the body. Atherosclerosis is a disease in which inside of an artery narrows due to plaque building (1). Plaque is made up of fat, cholesterol, calcium, and other substances found in the blood. Over the time, plaque hardens and narrows the arteries (2). This limits the flow of oxygen-rich blood to the organs and other parts of the body. Initially there are generally no symptoms of atherosclerosis. However, when severe it can result in coronary artery disease, stroke, heart attack, peripheral artery disease, or kidney problems depending on the arteries which are affected (1). Figure 2.1 (a) shows a normal artery with normal blood flow and (b) shows an artery with plaque. The inset image shows a cross-section of both while (C) shows steps of formation of plaque."

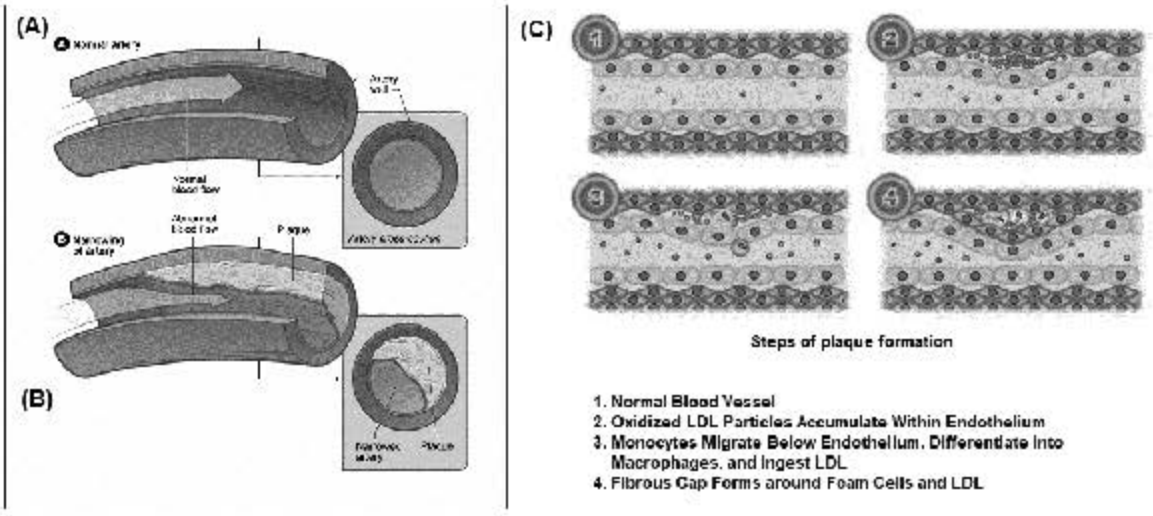

Figure 2.1 Plaque formations in atherosclerosis

Atherosclerosis can affect all large and medium-sized arteries, including the coronary, carotid, and cerebral arteries; the aorta; its branches; and major arteries of the extremities. It is the leading cause of morbidity and mortality in the US and in most developed countries. In recent years, age-related mortality attributable to atherosclerosis has been decreasing, but in 2015, cardiovascular disease (CVD), primarily coronary and cerebrovascular atherosclerosis still caused almost 15 million deaths worldwide (> 25% of all deaths (3). In the US, > 800,000 people died of CVD in 2014, corresponding to almost 1 in 3 of all deaths (4). Atherosclerosis is rapidly increasing in prevalence in developing countries, and as people in developed countries live longer, incidence will increase. Atherosclerosis is the leading cause of death worldwide."

"The cause of atherosclerosis isn't known. However, certain traits, conditions, or habits may raise the risk for the disease. These conditions are known as risk factors. Some of the risk factors are lack of physical activity, high blood pressure, obesity, smoking, oxidative stressors (eg, superoxide radicals), angiotensin II, and systemic infection, inflammation, proinflammatory cytokines, chemotactic proteins, vasoconstrictors and an unhealthy diet. Others one can't control, such as age and a family history of heart disease. Some people who have atherosclerosis have no signs or symptoms. They may not be diagnosed until after a heart attack or stroke. Prevention is generally by eating a healthy diet, exercise, not smoking, and maintaining a normal weight. Diagnosis is based upon a physical exam, electrocardiogram, and exercise stress test."

2.1.1 Pathophysiology of the disease

Atherosclerosis gives rise to cerebrovascular disease and coronary artery disease through a slowly progressing lesion formation and luminal narrowing of arteries. Upon plaque rupture and thrombosis, these most common forms of cardiovascular disease manifest as acute coronary syndrome (ACS), myocardial infarction or stroke. The underlying pathology is characterized by a chronic inflammatory process of the arterial wall that occurs at predilection sites with disturbed laminar flow, such as branch points (5). It is initiated by endothelial dysfunction and structural alterations, including the absence of a confluent luminal elastin layer and the exposure of proteoglycans (6). The earliest visible lesion of atherosclerosis is the fatty streak, which is an accumulation of lipid-laden foam cells in the intimal layer of the artery. The atherosclerotic plaque is the hallmark of atherosclerosis; it is an evolution of the fatty streak and has 3 major components; Lipids, Inflammatory and smooth muscle cells, and a connective tissue matrix that may contain thrombi in various stages of organization and calcium deposits. All stages of atherosclerosis—from initiation and growth to complication of the plaque—are considered an inflammatory response to injury mediated by specific cytokines. Endothelial injury is thought to have a primary initiating or inciting role. Figure 2.2 describes the stages of development of plaque, its progression and disruption.

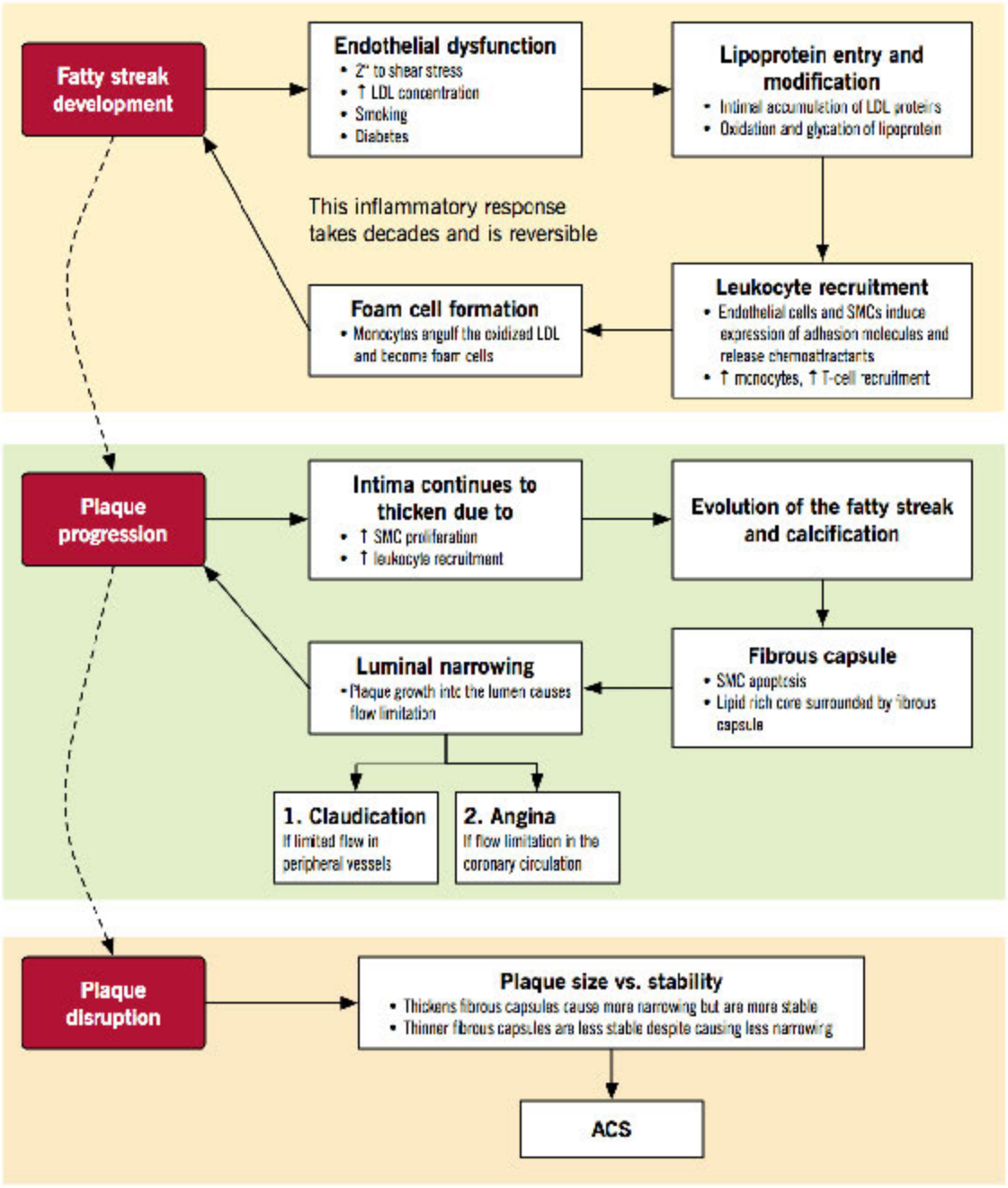

Figure 2.2 Stages of development of plaque

Atherosclerosis preferentially affects certain areas of the arterial tree. Nonlaminar or turbulent blood flow (eg, at branch points in the arterial tree) leads to endothelial dysfunction and inhibits endothelial production of nitric oxide, a potent vasodilator and anti-inflammatory molecule. Such blood flow also stimulates endothelial cells to produce adhesion molecules, which recruit and bind inflammatory cells. The net effect is endothelial binding of monocytes and T cells, migration of these cells to the subendothelial space, and initiation and perpetuation of a local vascular inflammatory response. Monocytes in the subendothelium transform into macrophages. Lipids in the blood, particularly low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL), also bind to endothelial cells and are oxidized in the subendothelium. Uptake of oxidized lipids and macrophage transformation into lipid-laden foam cells result in the typical early atherosclerotic lesions called fatty streaks. Degraded erythrocyte membranes that result from rupture of vasa vasorum and intraplaque hemorrhage may be an important additional source of lipids within plaques. Macrophages elaborate proinflammatory cytokines that recruit smooth muscle cell migration from the media and that further attract and stimulate growth of macrophages. Various factors promote smooth muscle cell replication and increase production of dense extracellular matrix. The result is a subendothelial fibrous plaque with a fibrous cap, made of intimal smooth muscle cells surrounded by connective tissue and intracellular and extracellular lipids. A process similar to bone formation causes calcification within the plaque. A link between infection and atherosclerosis has been observed, specifically an association between serologic evidence of certain infections (eg, Chlamydia pneumoniae, cytomegalovirus) and coronary artery disease (CAD). Putative mechanisms include indirect effects of chronic inflammation in the bloodstream, cross-reactive antibodies, and inflammatory effects of infectious pathogens on the arterial wall. Atherosclerotic plaques may be stable or unstable. Stable plaques regress, remain static, or grow slowly over several

decades until they may cause stenosis or occlusion. Unstable plaques are vulnerable to spontaneous erosion, fissure, or rupture, causing acute thrombosis, occlusion, and infarction long before they cause hemodynamically significant stenosis. Most clinical events result from unstable plaques, which do not appear severe on angiography; thus, plaque stabilization may be a way to reduce morbidity and mortality. The strength of the fibrous cap and its resistance to rupture depend on the relative balance of collagen deposition and degradation. Plaque rupture involves secretion of metalloproteinases, cathepsins, and collagenases by activated macrophages in the plaque. These enzymes digest the fibrous cap, particularly at the edges, causing the cap to thin and ultimately rupture. T cells in the plaque contribute by secreting cytokines. Cytokines inhibit smooth muscle cells from synthesizing and depositing collagen, which normally reinforces the plaque. Once the plaque ruptures, plaque contents are exposed to circulating blood, triggering thrombosis; macrophages also stimulate thrombosis because they contain tissue factor, which promotes thrombin generation in vivo. One of 5 outcomes may occur:.

- .The resultant thrombus may organize and be incorporated into the plaque, changing the plaque's shape and causing its rapid growth.
- The thrombus may rapidly occlude the vascular lumen and precipitate an acute ischemic event.
- The thrombus may embolize.
- The plaque may fill with blood, balloon out, and immediately occlude the artery.
- Plaque contents (rather than thrombus) may embolize, occluding vessels downstream..

. Plaque stability depends on multiple factors, including plaque composition (relative proportion of lipids, inflammatory cells, smooth muscle cells, connective tissue, and

thrombus), wall stress (cap fatigue), size and location of the core, and configuration of the plaque in relation to blood flow. By contributing to rapid growth and lipid deposition, intraplaque hemorrhage may play an important role in transforming stable into unstable plaques. In general, unstable coronary artery plaques have a high macrophage content, a thick lipid core, and a thin fibrous cap; they narrow the vessel lumen by < 50% and tend to rupture unpredictably. Unstable carotid artery plaques have the same composition but typically cause problems through severe stenosis and occlusion or deposition of platelet thrombi, which embolize rather than rupture. Low-risk plaques have a thicker cap and contain fewer lipids; they often narrow the vessel lumen by > 50% and may produce predictable exercise-induced stable angina. Clinical consequences of plaque rupture in coronary arteries depend not only on plaque anatomy but also on relative balance of procoagulant and anticoagulant activity in the blood and on the vulnerability of the myocardium to arrhythmias..

2.1.2 Atherosclerosis treatment and its management

Atherosclerosis management involves various facets. Lifestyle Changes focuses on weight management, physical activity and a healthy diet. Medications include administration of various lipid lowering drugs such as Satins (HMG CoA Reductase inhibitors), Fibrates, Bile acid binding resins and several others including nicotinic acid, probucol etc. Severe cases of atherosclerosis may be treated by surgical procedures, such as angioplasty or coronary artery bypass grafting (CABG). However, the oral antihyperlipidemic therapy needs to be continued on a daily basis for maintenance of the blood cholesterol level.

Treatment involves aggressive modification of risk factors to slow progression and induce regression of existing plaques. Lowering LDL to below a certain target is no longer recommended, and "the lower the better" approach is currently favored. Lifestyle changes

include diet modification, smoking cessation, and regular participation in physical activity. Drugs to treat dyslipidemia, hypertension, and diabetes are often required. These lifestyle changes and drugs directly or indirectly improve endothelial function, reduce inflammation, and improve clinical outcome. Statins can decrease atherosclerosis-related morbidity and mortality even when serum cholesterol is normal or slightly high. Antiplatelet drugs help all patients with atherosclerosis. Patients with coronary artery disease may benefit additionally from ACE inhibitors and beta-blockers..

2.2 Gene therapy as an approach for atherosclerosis – Current perspective

New pro- and anti-inflammatory pathways linking lipid and inflammation biology have been discovered, and genetic profiling studies have unveiled variations involved in human CAD. The growing understanding of the inflammatory processes and mediators has uncovered an intriguing diversity of targetable mechanisms that can be exploited to complement lipid-lowering therapies (7).

Although considerable progress has been made in the prevention and treatment of atherosclerotic cardiovascular disease, new therapeutic strategies are still needed. Atherosclerosis is a systemic disease and represents an attractive target for the development of somatic gene transfer intended to modulate systemic factors with the goal of inhibiting disease progression. This approach should be differentiated from localized vascular gene delivery to isolated atherosclerotic lesions such as that intended to prevent restenosis.

Systemic gene therapy for atherosclerosis can involve either: 1) gene replacement therapy in patients with defined genetic disorder causing premature atherosclerosis, or 2)

overexpression of proteins which directly or indirectly inhibit atherosclerosis or stabilize vulnerable lesions (8)..

In the past decade various gene delivery approaches have been studied for the treatment of atherosclerosis. Such gene delivery approaches act particularly by inducing or overexpressing receptors that lead to increase in LDL metabolism. Advancements made in the treatment of atherosclerosis with gene delivery are described below. Various gene delivery approaches explored for atherosclerosis is summarised in table 2.1.

2.2.1 APO A1

.The ApoA1 gene provides instructions for making a protein called apolipoprotein A-I (ApoA-I). ApoA-I is a component of HDL. HDL is a molecule that transports cholesterol and certain fats (phospholipids) through the bloodstream from the body's tissues to the liver. Once in the liver, cholesterol and phospholipids are redistributed to other tissues or removed from the body. ApoA-I attaches to cell membranes and promotes the movement of cholesterol and phospholipids from inside the cell to the outer surface. Once outside the cell, these substances combine with apoA-I to form HDL. ApoA-I also triggers a reaction called cholesterol esterification that converts cholesterol to a form that can be fully integrated into HDL and transported through the bloodstream. Epidemiological data indicate a strong inverse association between HDL cholesterol levels and atherosclerotic disease. Genetic syndromes of high HDL are associated with longevity and a decreased incidence of coronary heart disease (9). The concept that intervention to raise HDL cholesterol levels could be a method of treating or preventing atherosclerosis is attractive. The gene can be transported to

endothelial cell by using specific viral or non-viral vector leading to its expression at the damaged endothelium cell..

2.2.2 APO E

Apolipoprotein E (ApoE) is a critical ligand for the clearance of chylomicron and VLDL remnant lipoproteins(10). ApoE is synthesized by many tissues, but the apoE in plasma is derived largely from the liver. Genetic deficiency of ApoE results in substantially elevated levels of lipoprotein remnants and is associated with an increased risk of premature atherosclerotic disease (11). Systemic delivery of APO E along with siRNA to elevate APO E levels in genetic deficiency or expression of gene at the site by use of vectors are promising approaches. Human apolipoprotein E (apoE) is a 34-kDa multifunctional protein that plays a key role in lipoprotein metabolism and inhibits the development of atherosclerosis (12). Deficiency of apoE in mice results in hypercholesterolemia and atherosclerosis on a chow diet (13). Apolipoprotein (apo) E, which is present in plasma lipoproteins that carry dietary and liver-derived cholesterol, plays a protective role in atherosclerosis (14). ApoE plays a requisite role in remnant lipoprotein clearance by the liver, and although hepatic LDL receptors can clear both LDL and apoE-containing lipoproteins, LDL receptor– related protein-mediated clearance of remnants is dependent on apoE (15). Compared with wild-type mice, apoE-deficient mice have high levels of plasma cholesterol as a result of this impaired clearance of cholesterol-enriched lipoproteins (16). Moreover, these apoE-deficient mice develop complex atherosclerotic lesions that are a direct result of the plasma accumulation of cholesterol-rich lipoproteins. Addition of apoE to apoE-deficient mice (by either expression of apoE Transgenes (17), intravenous injection of synthetic mimics of apoE (18) or administration of adenovirus to achieve hepatic expression of apoE) reduces plasma

cholesterol levels and provides protection against the progression of atherosclerosis (19). Thus, apoE plays a requisite role in maintenance of plasma cholesterol levels..

2.2.3 SR-BI

.The class B scavenger receptor, SR-BI, is the first HDL receptor to be well defined at a molecular level and is a mediator of selective cholesterol uptake in vitro. It is expressed most abundantly in steroidogenic tissues, where it is coordinately regulated with steroidogenesis by adrenocorticotropic hormone (ACTH), human chorionic gonadotropin (hCG) and oestrogen, and in the liver, where its expression in rats is suppressed by oestrogen. Adenovirus-mediated, hepatic overexpression of SR-BI in mice on both sinusoidal and canalicular surfaces of hepatocytes results in the virtual disappearance of plasma HDL and a substantial increase in biliary cholesterol. SR-BI may directly mediate these effects by increasing hepatic HDL cholesterol uptake or by increasing cholesterol secretion into bile, or both (20). These results indicate that SR-BI may be important in hepatic HDL metabolism, in determining plasma HDL concentrations, and in controlling cholesterol concentrations in bile, and thus may influence the development and progression of atherosclerosis..

2.2.4 Lecithin-cholesterol acyltransferase (LCAT)

By converting unesterified to esterified cholesterol, LCAT is believed to facilitate the process of "reverse cholesterol transport". A first-generation adenovirus has been used to achieve somatic gene transfer and expression of human LCAT in transgenic mice expressing human apoA-I, and resulted in a substantial increase in HDL cholesterol and human apoA-I

levels. It is unclear whether gene therapy to raise LCAT activity would be beneficial in terms of reducing atherosclerosis (8).

2.2.5 Cholesteryl ester transfer protein (CETP)

It is a hydrophobic plasma glycoprotein that mediates the transfer and exchange of cholesteryl ester (CE) and triglyceride (TG) between plasma lipoproteins, and also plays an important role in HDL metabolism (21).

Table 2.1 Various Gene Delivery Approaches used in Atherosclerosis

Gene therapy with	**Mechanism/target**	**Vector for transfection**	**Route of administration**	**Remarks**	**Ref.**
Antisense oligodeoxynucleotides (AS-ODNs)	Declined concentration of mRNA in the nuclei of cells after transfection.	N,N-dipalmitylglycylapolipoprotein E	-	This approach may enable gene regulation in vivo and could be used to regulate vascular tone and constriction	(22)

				through ET receptors	
pDNA	The peptide SIGYPLP, targeted gene delivery specifically to endothelial cells with a significantly enhanced efficiency over nontargeted adenovirus	Adeno virus	Intraperit oneal	feasibility of using small, novel peptides isolated via phage display to target gene delivery specifically and efficiently to HUVECs	(23)
pDNA	LDL receptor protein modulation by AAV serotypes coding for the human LDL receptor	Adeno associated virus	Injection (in portal vein)	Complete normalization of serum lipids	(24)
pDNA	APO A1 expression stimulating reverse cholesterol transport	helper-dependent adenovirus (HDAd)	Infusion into the carotid artery	HDAd provided prolonged, stable expression of a therapeutic transgene in the artery wall	(25)

pDNA	AAV2/7 and AAV2/8 vectors mediated hepato-specific expression of apoE	Adeno associated virus	Intravenous injection	Prevented atherosclerosis after 1 year of sustained expression.	(26)

2.3 Lipoplexes as gene delivery vector

Among various vectors researched for gene delivery, those used in atherosclerosis 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 atherosclerosis.

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, atherosclerosis, Alzheimer's disease, Parkinson's disease, multiple sclerosis, viral infections, cardiovascular diseases and many more (27).

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 (28). 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 (29-31). Cytosolic release is often promoted by the helper lipids which have fusion capabilities 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 (29-31). 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 (29). 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.

2.4. Systemic gene delivery using lipid non-viral 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 (32). 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 formulations. 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 (33, 34). 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.

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 (35). 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 (36-46), cationic proteins/peptides (38, 47-55), aptamer conjugation (56-59), antibody conjugation (54, 60, 61), dendrimers (62) 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.

2.4.1 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 (39, 63-65). This has been attributed to their high charge density (66) and non-biocompatibility due to their non-degradable nature (67). 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 (39, 40). 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 (41-43), 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 (68), however instability of nucleic acid-peptide complex in physiological conditions pose a problem (69). 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 (38, 50, 70).

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. 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.

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.

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	For solid lipid nanoparticles

oleate, triglycerides	
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-phosphatidylethanolamine , 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

2.4.2 Structural features of lipid envelope systems of siRNA

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.

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 **Error! Reference source not found.**. 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 (71). Another RNA SLN based system showed a solid lipid core (with paclitaxel) surrounded by cationic lipid coat which was complexed with RNA (72). 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**) (73-75). One nanoemulsion system employed oleic acid based cationic surfactant obtained by conjugation of cationic amino acids like lysine, arginine and histidine (76). 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.

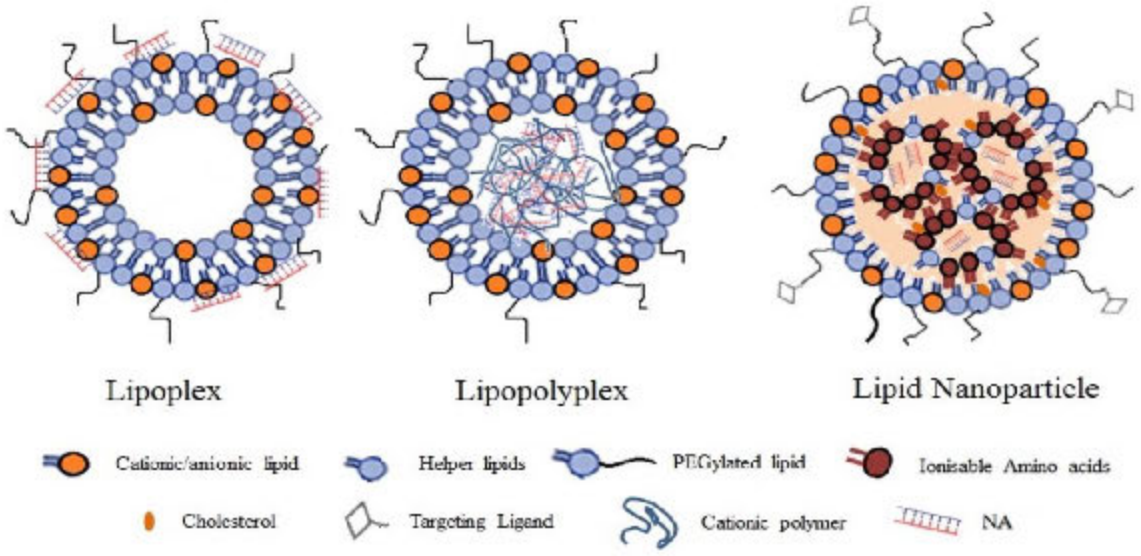

Figure 2.3 Schematic structures of lipoplex, lipopolyplex and lipid nanoparticle based delivery systems

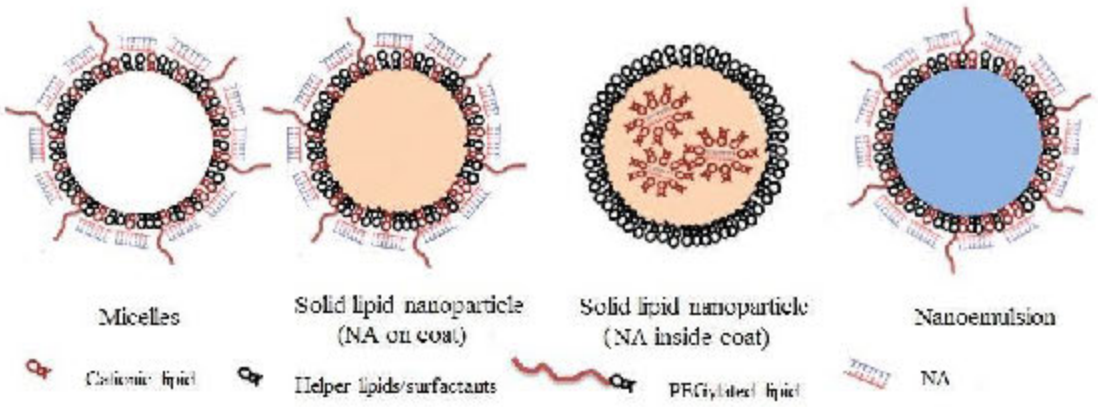

Figure 2.4 Schematic structures of micelles, solid lipid nanoparticle and nanoemulsion based delivery systems

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 (77). 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 (77). 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.

2.4.3 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.

2.4.3.1 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 (78, 79). Even though said to be prolonged, knockdown of target gene lasts only for few days to few weeks (79-83). 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 (84-86). 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 (86, 87). 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 (88). 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 (87). 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 (87). 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 (89). 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 (90). 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 (91). 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 (91-93). 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 (94). 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 (94). 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 (94, 95). 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 (92, 93). 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 (91). 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 (94, 96). 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 (94, 96).

Cationic head-groups of lipids can also interact with cellular enzymes like protein kinase-C causing cell toxicity (97). This tendency is higher with cholesterol derivatives containing cationic moieties due to their steroid backbone (98). 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 (99, 100). Additionally, stearyl amine liposomes have also been shown to induce cell apoptosis (101, 102). 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 (100-102). 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.

2.4.3.2 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 (96). 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 (103, 104). 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 (105-107). 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 (108-111). Complement activation in part can be reduced by proper optimization of cationic lipid:nucleic acid ratio (108, 112). 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 (113). PEGylation, by preventing opsonisation and also by creating a highly hydrated sheath around the lipid carriers, hinders the macrophage uptake (113). Formulation containing DOPE has also been shown to be profusely bound to serum proteins (albumin in particular) in mice (111). 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 (111).

2.4.3.2 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 (114-116). 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 (116). 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 (117), integrins for angiogenic vessels of cancer (118, 119) and transferrin receptors for brain targeting (49) and tumour targeting (120) 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. (121).

2.4.3.3 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 (122). 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 (122). 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.

2.4.4 Structural features of complex

Nucleic acid complexes made of DOTAP/DOPE, DOTAP/DOPC and MVL/DOPC have shown different transfection efficiencies *in vitro* (94, 123). 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 (124). 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 (125). 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 (126). Hence, one need to take into the structural features of the lipid based systems in order to get best outcome in terms of transfection.

2.4.5 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 (96). 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 (94). 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 (94). 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 (94, 95, 127, 128). 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 (129). 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.

2.4.6 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 (130, 131). Other lipids like sphingomyelin and cholesterol also play important role in fusion (132).

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 (91, 133). 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 trend generally shows 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 (17×10^{-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.

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.

2.5 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 (134-136). 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) (137). 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, PEG coating is required for preventing interactions with serum components and subsequent aggregation which lead to rapid systemic clearance through RES (138). However, PEGylation performed to improve circulation times inhibits both uptake and endosomal escape and is undesirable after cellular internalization (139). 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 (140). 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 (141, 142). 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 (143). 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 (144).

One of the most promising lipid based vectors for systemic delivery of gene are SNALPs (Stable 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 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 (145). SNALP have been studied intravenously in animal models of dyslipidemia and viral infections like hepatitis B (HBV), and Ebola (Zaire) (146). 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 nanoparticle into a cationic charged transfection-competent entity (147). 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 (148). 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 (149). 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 (150).

2.6 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.

2.7 Targeting liver cells

Normally most of the drugs achieve high hepatic concentration, still their targeting is necessary because liver is the major organ in the body equipped for uptake, detoxification, metabolic transformation, and excretion of xenobiotics into bile by means of carrier-mediated mechanism. As a consequence, most of the drugs are rapidly cleared from the blood and display high first pass clearances by the liver. However, it should be realized that the total hepatic uptake predominantly depends on hepatocytes, whereas Kupffer cells largely contribute to hepatic uptake of particulate material. Therefore, the drugs that enter the liver as such or in the form of covalent carrier conjugates will not necessarily reach the required cell type. Moreover, if drugs are accumulated in the liver, their residence time in the organ is influenced by the factors discussed under macrophages interaction with delivery system and pharmacokinetic consideration. Thus, the challenge is to obtain selective accumulation of drugs in one specific cell type and to sustain intracellular levels for longer period..

Some of the suggested strategies for hepatocyte targeting are:

- Reducing liposome size (less than 100 nm)
- Increasing negative charge of liposomes by incorporation of monosialoganglioside (GM_1)
- Labelling liposomes with galactose or N-acetylgalactosamine residues, such as, P-D-galactopyranosyl or lactosylceramide
- Addition of stearyl glycyrrhizin

The target cells/receptors within the liver for treatment and possible entry mechanisms in these cells have been identified (Table 2.2).

Hepatocytes are functional units responsible for most of the metabolic and secretory activities of the liver. Small size delivery system, that is, 150 nm, avoid capture by Kupffer cells and can diffuse out of the sinusoids through the fenestrations and reach the hepatocytes plates. These cells can take up colloidal carrier system through pinocytosis and receptor-mediated endocytosis. Improved delivery (enhanced localization) to the parenchyma is achieved with small size delivery system, that is, ≤50 nm, that can diffuse deeper in the space (151), (152). For a therapeutic moiety to exert its desired effect, it needs to be in physical contact with its physiological target such as a receptor present on liver cells. Site-specific drug delivery ensures that such interactions take place only in the desired anatomical location of the liver and for that delivery system should be recognized selectively by the receptor present on liver cell such as asialoglycoprotein. Specific targeting of hepatocyte receptors can also be achieved. The most commonly exploited target is the asialoglycoprotein receptor (ASGP-R) that recognizes carbohydrates (mainly galactose and N-acetylgalactosamine) with variable affinity (153).

Table 2.2 Receptors present on various hepatic cell and may be used for drug targeting (154)

Hepatocytes	Kupffer cells	Endothelial cell	Hepatic stellate cells
Asialoglycoprotein receptor (ASGP-R)	Mannose/N-acetyl glucose amine R	Mannose/N-acetyl glucose amine R	M6P/IGF II R
HDL-R	Galactose particle R	Scavenger R (Class AI and AII)	α_2 macroglobulin R
LDL-R	Galactose specific R	Fc R immune complexes	Ferritin R
IgA-R	Fc R (immune complexes, opsonized material)	Matrix compound (hyaluronan fibronectin, denatured collagen PIIINP)	Uroplasminogen R
Scavenger R (Class BI)	Scavenger R (Class AI, BI, BII, MARCO CD36 and macrosialin)		Thrombin R
Transferrin R	LDL R matrix compounds (fibronectin)		RBP R matrix compounds (integrin, collagen type VI, fibronectin CD_{44})
Insulin R	Complement R (C3b and C1q) LPS R α_2 macroglobulin R		

* R: Receptor.

A major goal for gene therapy is to obtain targeted vectors that transfer genes efficiently to specific cell types. The liver possesses a variety of characteristics that make this organ very attractive for gene therapy. Atheroprotective gene therapy involves localized expression of therapeutic genes in the hepatocytes. Expression of these therapeutic genes is aimed at counteracting the fundamental processes that drive atherosclerosis, including lipid accumulation in the vascular intima and inflammatory cell recruitment. Vascular gene therapy has substantial theoretical advantages over systemic drug and gene therapies for atherosclerosis in that it can deliver therapeutics precisely to

the site of origin (i.e., hepatocytes) (25). Apolipoprotein E (APOE) is a multifunctional plasma protein mainly acting in lipid metabolism. Human APOE is polymorphic with three major isoforms (APOE2, APOE3 and APOE4). Up to 75% of the body's APOE is produced

by the liver (155). Hepatic targeting of the liposomes is proposed for achieving specific expression of APOE plasmid in the hepatocytes.

Drug carriers with specific ligands for the corresponding receptors on the cell surface are useful for targeted drug delivery. Specific targeting to the liver has been achieved by using ligands that bind the specific receptor such as asialoglycoprotein receptor (ASGPr), which is uniquely presented on the surface of hepatocytes in large numbers with high-binding affinity (156). It is the most commonly exploited target that recognizes carbohydrates (mainly galactose and N-acetylgalactosamine) with variable affinity. Among various ligands investigated so far, galactose has been shown to be a promising targeting ligand to hepatocytes (liver parenchymal cells) because the cells possess a large number of the asialoglycoprotein receptors that recognize the galactose units on the oligosaccharide chains of glycoproteins or on chemically galactosylated drug carriers (157). The receptor-ligand interaction is known to show a significant "cluster effect" in which a multivalent interaction results in extremely strong binding of ligand to the receptors (158). This receptor is responsible for the clearance of glycoproteins with desialylated galactose or acetylgalactosamine residues from the circulation by receptor-mediated endocytosis. Galactosylated surface is an attractive substrate for hepatocyte culture because of the specific interaction between the galactose ligand and the asialoglycoprotein receptor on hepatocytes. The density of galactose is one of the important parameters for the hepatocyte attachment as it is a major determinant of the hepatocyte attachment, morphology, and functions (157)..

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