Chapter 1

Introduction

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1.1 Atherosclerosis

Atherosclerosis is a condition where the arteries become narrowed and hardened due to an excessive built up of plaque around the artery wall. The disease disrupts the flow of blood around the body, posing serious cardiovascular complications. Coronary artery disease (CAD) arising from atherosclerosis is a leading cause of death and morbidity worldwide (1)..

.Under normal conditions, leukocytes in the blood do not adhere to the endothelial cells that line all blood vessels. However, injury to endothelial cells provokes an inflammatory response. The endothelial cells begin to produce cell surface adhesion molecules such as VCAM-1, causing monocytes and T-lymphocytes to adhere to the endothelium and then migrate beneath it by squeezing between the endothelial cells. Circulating monocytes and T-lymphocytes are attracted to the sites of injury by chemoattractant cytokines (chemokines)..

.The endothelial cells also change shape, and the tight junctions between endothelial cells loosen, increasing the permeability to fluid, lipids, and leukocytes. Lipoprotein particles, and especially low-density lipoprotein (LDL), enter the arterial wall and undergo oxidation. Oxidation of LDL in the arterial wall occurs as a result of its exposure to nitric oxide, macrophages, and some enzymes such as lipoxygenase. Once they have migrated into the intima, monocytes differentiate into macrophages and begin to take up oxidized LDL that has gotten into the intima. Macrophages retain the lipid they take up, and as they become more lipid-laden, they are referred to as foam cells. Eventually, the foam cell will undergo apoptosis and die, but the lipid will accumulate in the intima. Fatty streaks are the first signs of atherosclerosis that are visible without magnification. A fatty streak consists of lipid-containing foam cells in the arterial wall just beneath the endothelium. Over time, these fatty streaks can evolve into atherosclerotic plaques. **(Figure 1.1)**.

The plaque clogs up the artery, potentially causes blood clots that can result in life-threatening conditions such as heart attack, stroke and other cardiovascular diseases. Certain factors that can damage the endothelium to develop atherosclerosis and can trigger CAD include (2).

(a) Risk factors with a significant genetic component (heritability): Elevated LDL and VLDL (Very Low Density Lipoprotein) cholesterol (40%–60%); Low HDL (High Density Lipoprotein) cholesterol (45%–75%); Elevated triglycerides (40%–80%); Increased body mass index (25%–60%); Elevated systolic blood pressure (50%–70%); Elevated diastolic blood pressure (50%–65%); Elevated lipoprotein(a) levels (90%); Elevated homocysteine levels (≈45%); Type 2 diabetes mellitus (40%–80%); Elevated fibrinogen (20%–50%); Elevated C-reactive protein (≈40%); Gender; Age; Family history..

(b) Environmental risk factors: Smoking, Diet, Exercise, Infection, Fetal environment, Air pollution (particulates)..

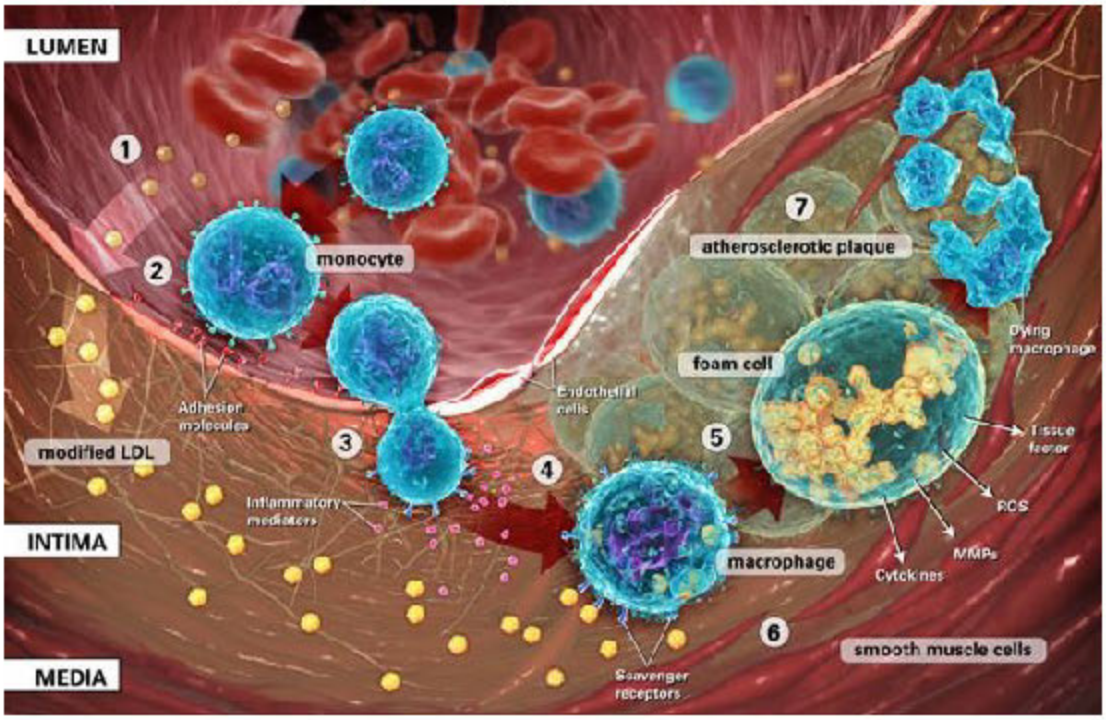


Figure 1.1 Pathogenesis of Atherosclerosis

1.2 Current Treatment

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 z 2002 showed an incidence of 0.15 deaths from rhabdomyolysis per 1.0 million statin prescriptions (3) (4)..

1.3 Gene Delivery for Atherosclerosis: Current Status

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 (5).

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.(6) These approaches may involve

administration of therapeutic pDNA that may increase the production of deficient proteins or siRNA that inhibit the overexpression of faulty genes (7)..

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..

A hepatocellular-targeted, atheroprotective gene therapy as an approach to the prevention and treatment of atherosclerosis is being sought. 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. Targeted gene therapy has substantial theoretical advantages over systemic drug and gene therapies for atherosclerosis in that it can deliver therapeutic gene precisely to the site of vascular disease. This approach maximizes local therapeutic effects and minimizes systemic side effects.(8).

1.4 Targeting of genes

1.4.1 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..

1.4.2 APOE

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).

1.4.3 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 (12)..

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..

1.4.4 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 (6).

1.4.5 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.(13).

Table 1.1 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 through ET receptors	(14)
pDNA	The peptide SIGYPLP, targeted gene delivery specifically to endothelial cells with a significantly enhanced efficiency over nontargeted adenovirus	Adeno virus	Intraperitoneal	Feasibility of using small, novel peptides isolated via phage display to target gene delivery specifically and efficiently to HUVECs.	(15)
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	(16)
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	(8)
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.	(17)

1.5 Gene Delivery Vectors

Among various vectors researched for gene delivery, those used in atherosclerosis include viral vectors mainly adenoviral vector, adeno-associated viral vector and retroviral vector (Table 1.1). 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."

"The non-viral carrier studied in atherosclerosis till date includes nano-constructs. Different carriers have been used for targeting of drug, such as immunoglobulin, serum proteins, synthetic polymers, liposome, microspheres, erythrocytes and niosomes. Some are discussed herein:.

1.5.1 Liposome Based Gene Delivery

Liposomes are generally formed by the self-assembly of dissolved lipid molecules, each of which contains a hydrophilic head group and hydrophobic tails. These lipids take on associations which yield entropically favorable states of low free energy. Cationic lipids commonly attain a positive charge through one or more amines present in the polar head group. The presence of positively charged amines facilitates binding with anions such as those found in DNA..

1.5.2 Lipoplexes and Polyplexes

.To improve the delivery of the new DNA into the cell, the DNA must be protected from damage and its entry into the cell must be facilitated. New molecules, lipoplexes and polyplexes, have been created that have the ability to protect the DNA from undesirable degradation during the transfection process..

Plasmid DNA can be covered with lipids in an organized structure like a micelle or a liposome is termed Lipoplex. There are three types of lipids, anionic (negatively charged), neutral, or cationic (positively charged)..

Complexes of polymers with DNA are called polyplexes. Most polyplexes consist of cationic polymers and their production is regulated by ionic interactions. One large difference between the methods of action of polyplexes and lipoplexes is that polyplexes cannot release their DNA load into the cytoplasm, so co-transfection with endosome-lytic agents (to lyse the endosome that is made during endocytosis, the process by which the polyplex enters the cell) such as inactivated adenovirus must occur. However, this isn't always the case, polymers such as polyethylenimine have their own method of endosome disruption as does chitosan and trimethylchitosan..

A solution of cationic lipids, often formed with neutral helper lipids, can be mixed with DNA to form a positively charged complex termed a lipoplex. Well-characterized and widely used commercial reagents for cationic lipid transfection include N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) , [1,2-bis(oleoyloxy)-3-(trimethylammonio)propane] (DOTAP), 3β[N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and dioctadecylamidoglycylspermine (DOGS). Dioleoylphosphatidylethanolamine (DOPE), a neutral lipid, is often used in conjunction with cationic lipids because of its membrane destabilizing effects at low pH, which aids in endolysosomal escape. (18).

Few other gene delivery vectors are enlisted herein and includes niosomes, nanoparticles and hybrid lipo-polymeric particles (not discussed)..

1.6 Targeting Strategies to Hepatic cells and Endothelial Cells

a) Targeting liver cells:

On the surface of hepatocytes there are glycosyl receptors which may mediate endocytosis of liposome-associated substances by means of ligand receptor reactions. Unilamellar liposomes, which contain negatively charged monosialoganglioside (GM,) in their lipid bilayer, as well as 60% galactose residues (especially P-D-galactopyranosyl) on their surface, have an accelerated rate of hepatocyte uptake. On the other hand, a-mannosylated liposomes are preferentially incorporated by liver

nonparenchymal cells, predominantly by RES cells, on which there are mannose receptors. A number of investigators have used mannose-bound liposomes to target the liver and believe that the liposomes are taken up by macrophages via mannose receptors on their surface. However, a recent report by Matsuo et al. (19) showed that hepatic uptake of acetylmannoside- modified multilamellar vesicles (Man-MLV) were inhibited by preheating the serum or by treatment with anti-rat complement C, antiserum. The results suggested that hepatic incorporation of Man- MLV was primarily mediated by a complement receptor rather than by mannose receptors on Kupffer cells in vivo. The mechanisms of preferential liposome uptake need to be further elucidated. Liposomes, which contain dipalmitoylphosphatidylcholine (DPPC) and lactosylceramide, are small enough (less than 100 nm) to pass through the endothelial fenestrae, and are more rigid than the fluid (soft) liposomes which consist of dimyristoylphosphatidylcholine (DMPC). DPPC-liposomes also have better hepatocyte uptake, and are stable in serum, but have a shorter half-life in serum. These findings imply that the transfer of the targeted liposomes into hepatocytes by a galactose-specific receptor may also be affected by the membrane fluidity of the liposomes (20). DPPC-liposomes are more rigid than those consisting of DMPC and EPC, and they have a higher blood clearance rate because of ready uptake by RES cells. These findings indicate that the membrane fluidity of the liposomes in vivo is a crucial factor for their preferential liver uptake (21). A number of galactosyl ligands for liposomes have been synthesized and their effects on liposome accumulation in rat liver were compared. Among these ligands, [8-(hexadecyloctadecanoylamido)-3,6- dioxaotyl-P-D-galactoside was found to have the longest circulating time and the highest rate of liver accumulation. It was also shown that the length or lipophilicity of the anchor (aliphatic moiety) is important for introduction and incorporation of the ligands. The branched anchor in the galactosyl ligands that have been synthesized is most preferable for recognition of the galactosyl residues by the receptors. This suggested that the cluster effect could be achieved by ligands without forming a cluster structure on the liposomes in vitro (22). Interestingly, stearyl glycyrrhizin (GLOSt)-bound liposomes were accumulated in the

liver at a several-fold higher concentration than control liposomes (23). A high liver accumulation of liposomes was also observed by the incorporation of a high proportion (60-80%) of nonionic surfactant (HC060) into liposomes (24)..
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,)
- Labelling liposomes with galactose or N-acetylgalactosamine residues, such as, P-D-galactopyranosyl or lactosylceramide
- Addition of stearyl glycyrrhizin
- Increasing- liposome rigidity by addition of dipalmitoylphosphatidylcholine (DPPC).

b) Targeting Endothelial cells:

Apart from targeting to liver cells, several targeting strategies have been designed for endothelial cells also and are listed in Table 1.2 below:.

Table 1.2 Targeting Endothelial Cell

Targeting receptor	**Targeting ligand**	**Remarks**	**Ref.**
p32	Atherosclerotic plaque–homing peptide, LyP-1 (peptide sequence CGNKRTRGC)	LyP-1 accumulated in the plaque interior, predominantly in macrophages	(25)
Fibrin and fibronectin clots	Atherosclerotic plaque–homing peptide, CREKA peptide	Homes on the surface of the atherosclerotic lesion	(25)
Stabilin 2	S2P (Peptide sequence CRTLTVRKC)	Transmembrane protein expressed in	(26)

		atherosclerotic arteries endothelial cells, macrophages and smooth muscle cells	
Endothelin receptors	Endothelin antagonistic peptides and antibodies	Overexpression on endothelium of atherosclerotic vessels	(27)
αvβ3 integrin	Integrin (cRGD peptides)	Angiogenic expansion of vasa vasorum	(28)
ICAM-1	LFA-1 peptide, LFA-1 antagonistic antibodies	Expression in atherosclerosis prone endothelial cells	(29)
VCAM-1	Ile-Leu-Asp-Val sequence peptides	Expression in atherosclerosis prone endothelial cells	(29-31)

1.7 Aim of the Work

The aim of the current project was to develop a delivery system for APOE gene for targeting to liver. A general constraint in delivery of naked plasmid is their short term limited expression and rapid degradation in the blood stream. Hence, for achieving longer circulation and duration of action, carrier systems such as liposomes and nanoparticles are generally employed. Liposomes are preferred due to the biodegradability and biocompatibility. Herein, cationic liposomes were formulated followed by preparation of lipoplexes. The lipoplexes were imparted receptor specificity to liver cells by conjugation them with galactose moiety as ligand. Thus, this approach was aimed for investigating a safe, effective and targeted gene delivery system for management of atherosclerosis..

1.8 Rationale of the Study

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) (8). Hepatic targeting of the liposomes is proposed for achieving specific expression of APOE plasmid in the hepatocytes. APOE was selected as it critical ligand in the plasma clearance of triglyceride- and cholesterol-rich lipoproteins (chylomicron remnants, VLDL, intermediate density lipoproteins, and a subclass of HDL)..

One of the essential feature that confers interaction of liposomal delivery system with cells is the ionic character. For efficient interaction and internalization, the liposomes are prepared with cationic lipids. However, many of these cationic lipids have the disadvantage of being high cytotoxicity leading to cell death. Hence, these cationic lipids are modified to decrease their cytotoxicity and improving biocompatibility. Herein, cationic lipid has been incorporated in the liposomes at minimal level, so as to minimize cytotoxic potential and a neutral lipid (DSPE) was modified to impart the system with endosomal escape capacity. Most studies have focused on cholesterol (Chol) as a lipophilic anchor moiety, because Chol derivatives can be easily synthesized (32). However, it is very easy for Chol to fall out from the liposome membrane if the hydrophilic head group is too large, whereas distearoylphosphatidylethanolamine (DSPE) anchor may be located deeper in the liposome membrane with its two long aliphatic chains (2 × 17-CH_2−), thus steadily inserting into the walls of lipid bilayer structures (33) (34)..

Hepatocytes, the liver parenchymal cells, constitute 60%–80% of the mass of the liver tissue, and liver diseases mainly develop from the hepatocytes (35). Although hepatocytes are the main functional cells of the liver, high uptake of compounds into other liver cell types, such as Kupffer cells, often leads to serious

degradation of them, which in some cases destroys their therapeutic activity (36). However, hepatocyte targeting is often equated with liver targeting, and total liver uptake of a compound is measured without proper identification of the cell type. This has induced the necessity of the development of cell-specific delivery carriers, through surface modification, which are usually transferred via a receptor-mediated endocytosis system. Asialoglycoprotein receptors (ASGP-Rs) are exclusively expressed on the membranes of hepatocytes, providing active membrane-bound sites, and have been used as the target receptors for drug delivery to the hepatocytes (37) (38). ASGP-Rs main function is to recognize, bind, and internalize ASGPs that contain terminal galactose (Gal) or N-acetylgalactosamine (GalNAc) residues (39). Thus, for targeting purpose of the delivery system to the liver cells, galactose was selected for conjugating to liposomal surface as anchor..

1.9 Hypothesis:

It is hypothesized that delivery of APOE gene to hepatocytes will increase the local expression of protein in the cell and would stimulate the cholesterol efflux pathway that will prevent further deposition of cholesterol at plaque sites along with removal of cholesterol from the plaque sites leading to effective remission in the disease..

1.10 Objectives

Liposomes have emerged as one of the safest delivery drug delivery, however, requirement of cationic liposomes which enhance the toxicity of liposomes on cells is inevitable. One of the essential property for these carrier system is to impart capability for endosomal escape so that the payload can be released inside the cells without being endocytosyzed and cleared by the RES. It is required that they escape from endosome as soon as possible due to reducing pH of endosomes through late endosomes and endolysozomes. Hence, synthesis of modified lipids which will provide buffering effect inside endosomes and will provide sufficient time for cytosolic release is sought.. To achieve these goal DSPE has been modified to change

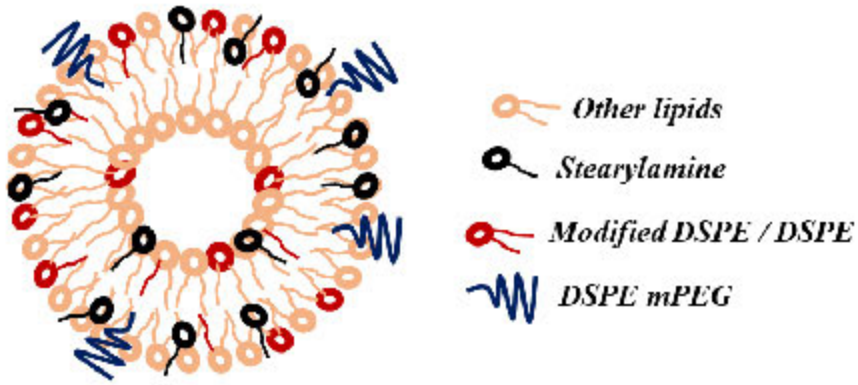
the head-group chemistry using amino acids/dipeptide i.e. histidine, arginine and carnosine. Such modification involving amino acid is a novel strategy to couple two functional moieties without substantially increasing the overall molecular weight due to linker. These modified lipids will be used in preparation of cationic liposomes containing stearylamine as cationic lipid. The pDNA will be complexed with these liposomes to prepare lipoplexes. Further, to achieve targeted delivery to hepatocytes, galactose ligand was conjugated to lipoplexes..

1.11 Plan of work

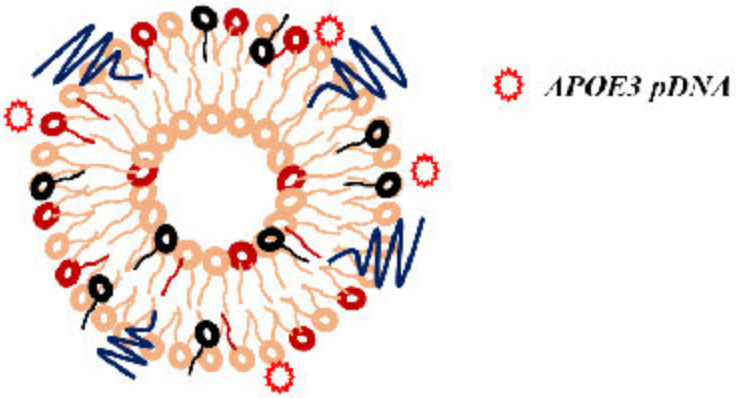
a) Literature review
b) Isolation and characterization of plasmid
c) Synthesis of novel lipids and their characterization
d) Preparation of the cationic liposomes and preparation of lipoplexes, along with their characterization
e) *In vitro* performance studies
f) Formulation of targeted lipoplexes and their evaluation
g) *In vivo* performance studies: Atheroprotective activity in atherosclerotic animal model.
h) Stability study of the optimized formulations.
i) Summarizing and concluding the research work

1.12 Proposed Strategy (Graphical overview)

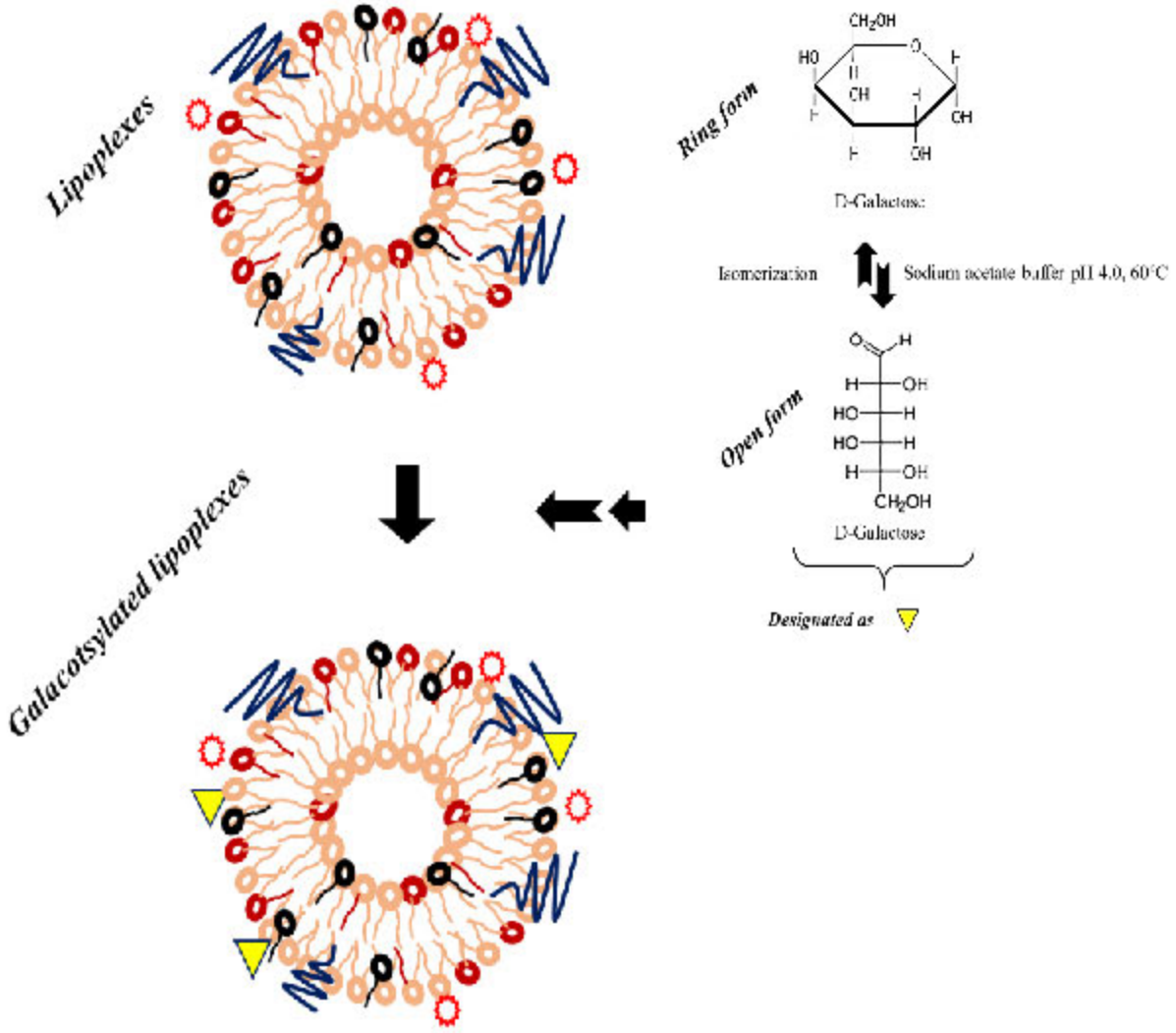
1. Preparation of Cationic liposomes



2. Preparation of Lipoplexes



3. Preparation of Targeted Lipoplexes



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