

Table of Contents

Chapter 1	1
1.1 Atherosclerosis.....	1
1.2 Current Treatment.....	3
1.3 Gene Delivery for Atherosclerosis: Current Status	3
1.4 Targeting of genes.....	4
1.4.1 APO A1.	4
1.4.2 APOE.....	5
1.4.3 SR-BI.....	5
1.4.4 Lecithin-cholesterol acyltransferase (LCAT).....	6
1.4.5 Cholesteryl ester transfer protein (CETP).	6
1.5 Gene Delivery Vectors.....	8
1.5.1 Liposome Based Gene Delivery.....	8
1.5.2 Lipoplexes and Polyplexes	8
1.6 Targeting Strategies to Hepatic cells and Endothelial Cells	9
1.7 Aim of the Work..	12
1.8 Rationale of the Study	13
1.9 Hypothesis.....	14
1.10 Objectives.....	14
1.11 Plan of Work.....	15
1.12 Proposed Strategy (Graphical overview)	15
1.13 References.....	18

Chapter 2.....	23
2.1 Atherosclerosis.....	23
2.1.1 Pathophysiology of the disease.....	25
2.1.2 Atherosclerosis treatment and its management.....	29
2.2 Gene therapy as an approach for atherosclerosis – Current perspective	30
2.2.1 APO A1.....	31
2.2.2 APO E.....	32
2.2.3 SR-BI	33
2.2.4 Lecithin-cholesterol acyltransferase (LCAT)	33
2.2.5 Cholesteryl ester transfer protein (CETP).....	34
2.3 Lipoplexes as gene delivery vector.....	36
2.4. Systemic gene delivery using lipid non-viral vectors - lipoplexes and other lipidic systems.....	38
2.4.1 Importance of Lipid envelope systems as nucleic acid delivery vectors.....	41
2.4.2 Structural features of lipid envelope systems of siRNA.....	47
2.4.3 Overcoming challenges.....	50
2.4.4 Structural features of complex	57
2.4.5 Cationic lipid:nucleic acid N/P ratio.....	58
2.4.6 Lipid composition of complex	59
2.5 Emerging Strategies for gene delivery.....	61
2.6 The way forward	64
2.7 Targeting liver cells	65
2.8 References.....	69

Chapter 3.....	90
3.1 Introduction.....	90
3.2 Analytical methods used for quantification of pDNA	90
3.2.1 UV Spectrophotometric Analysis of DNA	90
3.2.1.1 Material.....	91
3.2.1.2 Method.....	91
3.2.1.3 Results and discussion	91
3.2.2 Spectrofluorometric method: Quantifluor.....	94
3.2.2.1 Protocol for Quantitating dsDNA	94
3.2.2.2 Results and Discussion.....	94
3.2.3 Gel Electrophoresis of pDNA.....	96
3.2.2.1 Material.....	97
3.2.2.2 Method.....	98
3.2.2.3. Results and Discussion	98
3.3 Analytical methods used for characterization of lipids.....	101
3.3.1 TNBS assay.....	101
3.3.1.1 Materials	102
3.3.1.2 Method.....	102
3.3.1.3 Results and discussion	103
3.3.2 Sakaguchi assay	107
3.3.2.1 Materials	107
3.3.2.2 Method.....	108
3.3.2.3 Results and discussion	108
3.4 References.....	110

Chapter 4: Isolation and Characterization of pDNA
A Gene Delivery Approach for Treatment of Atherosclerosis

Chapter 4.....	111
4.1 Selection of pDNAs and their properties	111
4.1.1 Rationale for selection of ApoE3 gene.....	111
4.1.2 Properties of ApoE pDNA and protein.....	113
4.1.2.1 Vector map and pDNA description.....	113
4.1.2.2 pCMV / ampicillin plasmid complete sequence	114
4.1.2.3 ApoE3 amino acid sequence	115
4.1.2.4 Cloning sites/restriction digestion sites	115
4.1.3 Rationale for selection of eGFP gene	115
4.1.4 Properties of eGFP gene	115
4.1.4.1 Vector map and pDNA description.....	116
4.1.4.2 pCDNA3/EGFP plasmid complete sequence	116
4.1.4.3 eGFP amino acid sequence	118
4.2 Transformation of pDNA.....	119
4.2.1 Transformation of competent E. coli using magnesium chloride	119
4.2.1.1 Material	119
4.2.1.2 Method	121
4.2.1.3 Result & Discussion.....	122
4.2.2. Isolation and purification of plasmid DNA	123
4.2.2.1 Material	126
4.2.2.2 Method	127
4.2.2.3 Result & Discussion.....	129
4.3 Plasmid Digestion	130
4.3.1 Materials	130
4.3.2 Method	130
4.3.3 Result & Discussion.....	131
4.3 References.....	132

Chapter 5: Synthesis of Lipids
A Gene Delivery Approach for Treatment of Atherosclerosis

Chapter 5.....	134
5.1 Introduction.....	134
5.2 Materials and Methods.....	134
5.2.1 Synthesis of modified lipids.....	134
5.2.2 Physicochemical characteristics of the lipids and pH titration study:	138
5.3 Results and discussion	138
5.4 References.....	147

Chapter 6: Formulation Development
A Gene Delivery Approach for Treatment of Atherosclerosis

Chapter 6.....	148
6.1 Development of lipoplexes:	148
6.1.1 Stearyl amine	148
6.1.2 DOTAP	149
6.1.3 Synthesized lipids	150
6.2 Preparation of liposomes	150
6.2.1 Preparation of Stearyl amine-DSPE based liposomes	150
6.2.2 Preparation of lipoplexes:	150
6.2.3 Optimization of Parameters	151
6.2.4 Lyophilization of Lipoplexes.....	151
6.2.5 Physicochemical characterization of liposomes and lipoplexes.....	153
6.2.5.1 Complexation Efficiency	153
i. EtBr intercalation assay using gel electrophoresis	153
ii. UV spectrophotometric determination after Centrifugation.....	153
ii. Spectrofluorometric determination after Centrifugation	153
6.2.5.2 Particle size and zeta potential analysis.....	154
6.2.5.3 Cryo-Transmission Electron Microscopy (Cryo-TEM).....	154
6.2.5.4 Assay	155
6.2.5.5 Residual Water content:	156
6.2.5.6 Statistical Analysis.....	156
6.3 Results and Discussion.....	157
6.3.1 Preparation of stearyl amine-DSPE based liposomes.....	157
6.3.1.1 Optimization of process parameters.....	157
6.3.1.2 Optimization of formulation components	160
6.3.1.3 Preliminary Screening of Formulations: DSPE lipid incorporated.....	169
6.3.2 Preparation of DSPE based liposomes.....	169
6.3.2.1 Formulation Optimization using DOE.....	178
6.3.2.2 Preparation of liposomes of different modified lipids	204
6.3.3 Preparation of lipoplexes	207
6.3.3.1 Optimization of Process Parameters	207
6.3.3.2 Optimization of Formulation Parameters.....	214
6.3.3.3 Physicochemical characterization of the lipoplexes	222
6.3.4 Lyophilization of lipoplexes.....	226
6.4 References	230

Chapter 7: In Vitro Characterization
A Gene Delivery Approach for Treatment of Atherosclerosis

Chapter 7.....	232
7.1 <i>In vitro</i> physicochemical characterization	232
7.2 Stability of lipoplexes in presence of electrolyte (NaCl).....	232
7.2.1 Method	233
7.2.2 Results and Discussion	234
7.3 Serum Stability Study	242
7.3.1 Methods.....	242
7.3.2 Result and discussion.....	243
7.4 References.....	248

Chapter 8: Cell line studies
A Gene Delivery Approach for Treatment of Atherosclerosis

Chapter 8.....	250
8.1 Introduction.....	250
8.2 Materials and Instruments.....	251
8.3 Methods.....	252
General Methods and Preparations	252
8.4 Cytotoxicity studies	253
8.4.1 Haemolysis Study	254
8.4.1.1 Method	254
8.4.1.2 Results and Discussion	255
8.4.2 MTT assay - on HEPG2 cells	257
8.4.2.2 Methods.....	258
8.4.2.3 Results and Discussion	263
8.5 GFP expression studies	268
8.5.1 Flow cytometry	268
8.5.1.1 Method	269
8.5.1.2 Results and discussion:	270
8.5.2 Confocal microscopy	274
8.5.2.1 Method	274
8.5.2.2 Results and discussion:	275
8.6 References.....	279

Chapter 9: Development and Evaluation of Targeted Lipoplexes
A Gene Delivery Approach for Treatment of Atherosclerosis

Chapter 9	281
9.1 Development and evaluation of targeted lipoplexes	281
9.1.1 Preparation of ligand anchored cationic liposomes containing modified lipids:	281
9.1.2: Preparation of ligand anchored cationic liposomes:.....	281
9.2 References	288

Chapter 10.....	289
10.1. Acute Toxicity Study	289
10.1.1 Method.....	290
10.1.1.1 Selection of animals species.....	290
10.1.1.2. Housing and feeding conditions.....	290
10.1.1.3. Preparation of animals	290
10.1.1.4. Preparation of doses	291
10.1.1.5. Procedures	291
10.1.2 Results and discussion	293
10.2 <i>In vivo</i> performance study	296
10.2.1 Diet induced atherogenesis	296
10.2.1.1 Method	297
10.2.2 Estimation of total cholesterol:.....	298
10.2.3 Estimation of HDL cholesterol:.....	299
10.2.4 Estimation of Triglycerides:	301
10.2.5 Estimation of LDL Cholesterol:	302
10.2.6 Histopathology of atherosclerotic lesions:	302
10.2.7 Results and discussion:	303
10.3 References	307

Chapter 11	309
11.1 Stability Studies.....	309
11.1.1 Method.....	310
11.1.2 Results and discussion.....	310
11.2 References	313

Chapter 12: Summary and Conclusion
A Gene Delivery Approach for Treatment of Atherosclerosis

Chapter 12.....	315
Summary and Conclusion:	315