

Folate Receptor Targeted Gene Delivery Using Cationic Liposomes as Nonviral Vectors

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Abstract. Gene therapy is a promising strategy for the treatment of human diseases rooted in genetic disorders such as cancer. The success of gene therapy however depends on the efficient delivery of therapeutic genes into target cells in vitro and in vivo. Liposomes have shown the potential to be ligand-conjugated and receptor targeted. Our aim is to develop and test a lipid-based system for efficient targeted gene delivery to the folate receptors that are overexpressed in a broad spectrum of malignant tumors viz. Hence it represents an attractive target for selective delivery of anticancer agents to folate receptor expressing tumors. Novel cationic liposomes were formulated with and without the targeting ligand, folate. Folate conjugated liposomes were prepared using the cationic cholesterol derivative *N,N*-dimethylaminopropylamidodisuccinylcholesterylformyl-hydrazide (MSO9), the helper-lipid, dioleoylphosphatidylethanolamine (DOPE), and distearoylphosphatidylethanolamine (DSPEPEG₂₀₀₀) which was conjugated to folate. DNA-binding and protection abilities of all liposomes have been confirmed by band shift assays, dye displacement assays, and nuclease protection assays. The complexes were evaluated in an in vitro system for cytotoxicity using the MTT assay, and finally gene regulation using the luciferase reporter gene assay. Relatively low cytotoxicities were observed and encouraging gene expression levels were noted. This research will have a significant impact in the targeting of genes or drugs to cancer cells in vivo.

Keywords: Gene Therapy; Non-viral Vectors; Lipid Nanoparticles; Folate Receptor.

1. Introduction

Gene therapy involves the delivery of a specific gene (DNA) to the targeted cells thus combating the disease at the level of its origin. Successful Gene therapy relies on devising methods for efficient transport of nucleic acids through the cell membrane into the nucleus [1]. Targeted gene delivery systems have been used to increase the efficiency of drug/gene delivery to specific tissues as well as to optimize the minimum effective dose of the drug and its side effects. Cationic liposomes are good non-viral vectors, since they readily form complexes with DNA via electrostatic interactions [2]. Folic acid is involved in essential one carbon transfer reactions that are important in DNA synthesis and replication, cell division, growth and survival, particularly for rapidly dividing cells. Conjugates of folic acid can be taken up by cancer cells via receptor-mediated endocytosis, thus providing a mechanism for targeted delivery to FR+ cells [3].

2. Methods

Cationic liposomes were prepared using the method [4], with or without the conjugated lipid DSPE-PEG-FA. Briefly, MSO9 and the helper lipid DOPE were dissolved in CHCl₃ and deposited as a thin film on the inner wall of the test tube by evaporation of the solvent in vacuo. The dried lipid film was rehydrated overnight at 4 °C in a solution containing 20 mM HEPES and 150 mM NaCl (pH 7.5). The resulting liposome suspension was briefly vortexed and sonicated. Size and structure of cationic liposomes and lipoplexes were established by Zetasizing, and cryoTEM. Lipoplex formation and DNA protection abilities were studied by band shift, nuclease protection and ethidium bromide assays. Growth inhibition studies of the complexes were determined using the MTT assay and luciferase gene expression levels were assayed using the Luciferase Reporter gene assays (Promega) and expressed as RLU/mg protein.

3. Results and Discussion

3.1. Gel retardation assay

Agarose gel electrophoresis of cationic lipid:DNA complexes was used to assess the relative amounts of DNA that were free or incorporated into the complex as a function of the lipid:DNA ratio. DNA in a lipid complex did not migrate out of the well. This was most likely the result of the charge neutralization. The Fig.1 shows that the amount of uncomplexed, or free DNA decreased as the ratio of lipid:DNA was increased.

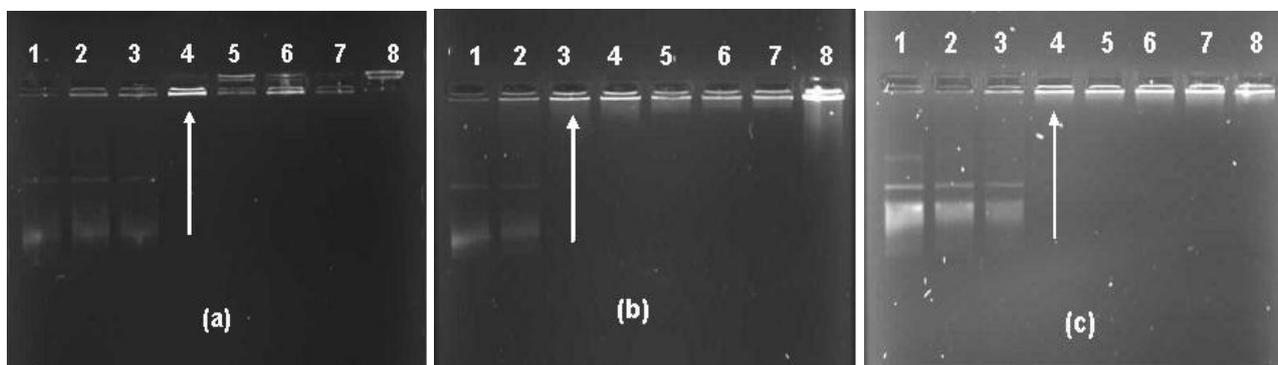


Fig 1: Gel retardation assays. Cationic liposomes were complexed with pDNA at various weight ratios. The weight ratio of cationic lipid/pDNA (a, b, c) was 1:1, 2:1, 3:1, 4:1, 5:1, 6:1,7:1 (lanes 2, 3, 4, 5, 6, 7 and 8, respectively). Lane 1, 0.5 μ g plasmid DNA only.

3.2. Cytotoxicity assay

For in vitro toxicity study cells (HeLa, HEK293) were incubated with three formulations (plain and PEG coated and folic acid conjugated liposomes) for 48 h in 48-well microtitre plates. Control cells were taken without formulations and incubated with medium. Cell viability assay was performed using MTT assay. Percent cell viability was determined using control as 100%. Results obtained were, the pegylated liposomes were slightly toxic to the cells as compared to plain and FA-targeted liposomes (see Fig.2).

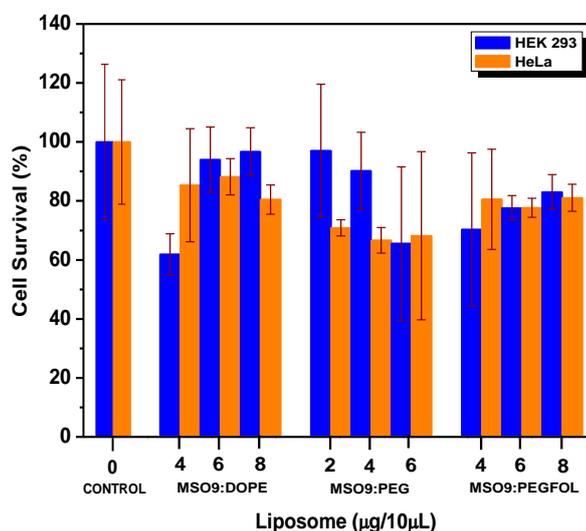


Fig 2: In vitro Growth inhibition studies of liposome:pCMV-luc DNA complexes in HEK293, HeLa cell lines. Incubation mixtures (10 μ L) contained 0.5 μ g of plasmid DNA. Varying amounts of liposome from its suboptimal to supraoptimal ratios were assayed. Control: untreated cells. Data are presented as means \pm SD (n= 3).

3.3. Zeta sizing

The relevance of the parameter 'particle size' in gene delivery by non viral vectors is known. The particle size of the vector influences the internalization pathway of particles through the cell membrane. The preferred particle size would be 100-200 nm, in theory. This point is specially required for in vivo gene delivery in order to be small enough to allow systematic delivery [5]. Particle sizes of complexes were

measured by dynamic light scattering in the absence or presence of folate ligand and the folate modification did change the sizes of liposomes. The average particle sizes of cationic liposomes (MSO9) used in this study were 196 nm (untargeted), 121 nm (untargeted, pegylated), 168 nm (pegylated, targeted) (see Table 1). The reason for the smaller pegylated liposomes could be the repulsion feature of PEG molecules that prevent the liposomal aggregation.

Table 1: Particle sizes of liposomes and lipoplexes at their end point retardation ratios.

Formulation	Lipid/DNA charge ratio	Liposome		Lipoplex	
		Size (nm)	PDI	Size (nm)	PDI
MSO9:DOPE	3:1	196	0.22	695	0.57
MSO9:DOPE:DSPEPEG 2000	2:1	121	0.23	106	0.21
MSO9:DOPE:DSPEPEG 2000:DSPEPEGFOL	3:1	168	0.33	191	0.47

3.4. Transfection assay

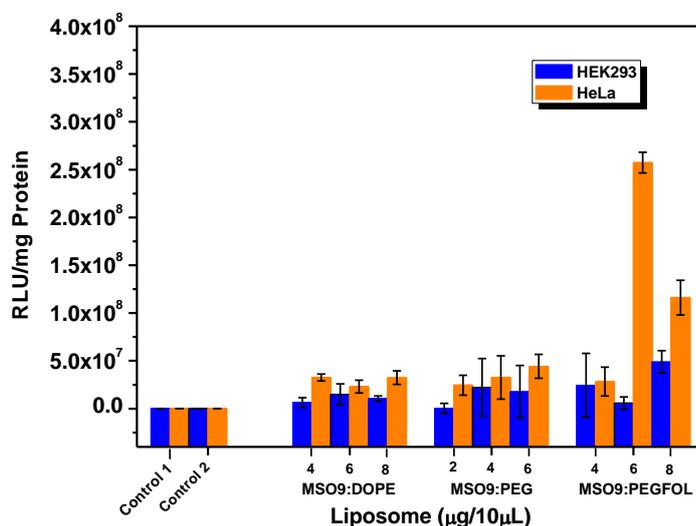


Fig.3: Transfection studies of liposome-plasmid DNA complexes in HEK293 and HeLa cells in vitro. Incubation mixtures (10µL) contained 0.5µg of plasmid DNA. Varying amounts of liposome from slightly below to slightly above end point ratios were assayed. Luciferase activity is expressed as RLU/mg soluble protein. Control 1: untreated cells; Control 2: plasmid DNA alone. Data are presented as means \pm SD (n= 3).

In Fig.3, the folate:liposome:DNA complexes showed a two-fold increase in transfection activity compared to plain (without PEG or FA) liposomes and pegylated liposomes. This suggests that FA presence in liposome;DNA complexes facilitates the uptake of the FA-liposome:pDNA into the HeLa cells via receptor mediation. Low transfection levels were achieved for HEK293 cells (receptor negative cells). Significant transfection levels for HeLa cells for FR-targeted liposomes were seen at their 3:1 (+/-) ratio or (6 µg/0.5 µg). These findings also support the notion that the lipoplexes with the sizes range from 100-200 nm are suitable to traverse the cell membrane to reach the nucleus. Lipoplexes achieved high transfection levels falls in this range.

4. Conclusions

FR-targeted liposomes, synthesized using F-PEG-DSPE has been shown previously to effectively target FR-expressing tumor cells. It is further shown in this study that FR-targeted lipoplexes had poor cytotoxicity and this indicates that FR-targeted liposomes are potentially useful for delivery of therapeutic agents. In addition, FR- targeted lipoplexes showed poor cytotoxicity, high transfection levels, and can be specifically

taken up by FR over expressing cells. In summary, the cationic liposome (MSO9) containing FA ligand had good physical chemical and FR targeting properties. The results obtained from this study suggest that, FR-targeted liposomes may constitute a better candidate for future clinical development of gene/drug in vivo.

5. Acknowledgements

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6. References

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