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Preparation and Evaluation of TSP-1 Loaded Pegylated Cationic Liposomes for Inhibiting Angiogenesis

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Abstract

In this study, novel techniques have been proposed for the development of liposome formulations that will transfer the pDNA with a thrombospondin gene, which has been shown to prevent tumor growth by inhibiting angiogenesis, to an active, safe and resistant cell. Our aim was to develop liposome formulations that can directly affect angiogenic tumor cells without harming vasculogenesis exhibiting cells. F5 and F9 coded PEGylated liposomes were selected for investigation of their physicochemical and physicopharmaceutical properties. Positively charged lipoplexes size were around 150 nm and they encapsulated the plasmid at a rate of 35 %. Furthermore, , F5 coded PEGylated liposomes investigated the applicability for gene transfection efficiency at MCF-7 (breast cancer epithelial) cells. Accordingly, the , F5 coded formulation can be further used as a gene delivery system in the in vivo studies to obtain preliminary findings on anti-cancer treatment by the anti-angiogenesis approach

Keywords: Liposome, pDNA, Thrombospondin, Gene therapy, Pegylation

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Introduction

Liposomes are vesicular structures that can be used to deliver a molecular cargo such as DNA for therapeutic benefit. Liposome-derived carriers have advantages imparted by lipid vesicles are; 1. Their diverse range of morphologies and compositions, 2. Abilities to envelope and protect many types of therapeutic biomacromolecules, 3. Lack of immunogenic response, and 4. Their differential release characteristics [1, 2].

Some cells can display uncontrolled growth through division beyond normal limits. This uncontrolled growth may cause tumors. Studies have already generated strong evidence indicating that angiogenesis plays a key role in the growth of solid tumors [3]. Thrombospondin-1 (TSP-1) has a potent effect on tumor growth by inhibiting angiogenesis. The anti-angiogenic effect of TSP-1 is mediated through inhibition of vascular endothelial cell adhesion and migration [3,4]. It was reported that various extracellular stimuli alter TSP-1 gene expression at the level of transcription [4].

In our study, we prepared cationic liposomes as a molecular cargo for TSP-1 encoding plasmid. Cationic liposomes are formed from either a single cationic amphiphile or commonly from a combination of a cationic and a neutral lipid with positive zeta potential. Positively charged cationic liposomes interact electro-statically with negatively charged nucleic acid to form lipoplexes which are capable of entering a cell [5, 6]. Different functional groups, such as polyethylene glycol (PEG) can easily modify to cationic lipids [7, 8]. The aim of this study was to examine the use of PEGylated cationic liposomes as a candidate to use in anti-angiogenic gene carrier systems.

Materials and Methods

Chemicals

N-(N', N'-dimethylaminoethane)-carbamoyl]-cholesterol (Dc-Chol), 1, 2-Dioleoyl-sn-Glycerol-3-Phosphoethanolamine, (DOPE), and 1, 2-di-(9z-octadecenoyl)-3-trimethylammonium-propane, (DOTAP) were purchased from Avanti Polar Lipids. Polyethylene glycol (PEG) with a molecular weight of 2.000 Da was purchased from Merck and first purified with hexane from tetrahydrofuran and activated according to the method described by Petersen et.al. [9]. pBluescriptR (clone ID: 5266513) and pcDNATM3.1/V5-His-TOPO with its kit were purchased from ATCC

and Invitrogen, respectively. TSP-1 forward and reverse primers were ordered from Alpha-DNA. Dialysis bags (MWCO:12,000) and anti-thrombospondin mouse antibody were purchased from Sigma.

Anti-V5-FITC and restriction enzymes were ordered from Invitrogen and Fermentas, respectively. Bidistilled water was used to prepare analytical solutions and buffer.

Construction of TSP-1 into pCDNA3.1 plasmids

The human TSP-1 (from plasmid pBluescriptR) was inserted into the pCR3.1 plasmid as described by our previous work [10]. The integrity of the plasmid was analyzed by 0.8% agarose gel electrophoresis (Thermo Scientific, Turkey) at 60 V, 150 mA.

Preparation of liposomes

We prepared liposomes with the most widely used method, named as lipid hydration method. This method involves drying a solution of lipids with rotary evaporator so that a thin film is formed at the bottom of 100 mL round bottom flask and then hydrating the film by adding aqueous buffer and vortexing the dispersion for some time. Briefly, a known amount of lipids (Table 1) were dissolved in chloroform. Lipid solution was dried at rotary evaporator for 30 minutes at 30°C to form the lipid film. The conditions of evaporation were 150 rpm and 200 bar pressure. If the evaporation doesn't finish, extra 15 minutes could be applied. 3 mL Bidistilled water containing 50 µg/mL pDNA was added to the dry film and rotates for hydration in 10 min. If necessary 2 mL Bidistilled water with 10% activated PEG (w/v) was added and agitated for more 10 minutes at 45°C. Liposomes are filtrated once from 0, 45 µm filter and three times from double 0, 22 µm filters under nitrogen.

Table 1: Prepared liposome contents and their properties

Formulation	Type of lipids	Ratio of lipids	Particle Size (nm±SD)	Zeta Potential (mV)
F1	DOTAP/DOPE	50 / 50 / 0	385 ± 49	51.12
F2	(DOTAP/DOPE)/PEG	45.5 / 45.5 / 9	212 ± 53	48.96
F3	DOTAP/DC-Chol	50 / 50 / 0	440 ± 54	38.17
F4	DOTAP/DOPE	67 / 33 / 0	390 ± 39	48.50
F5	(DOTAP/DOPE)/PEG	57 / 33 / 10	149 ± 18	34.58
F6	DOTAP/DC-Chol	67 / 33 / 0	175 ± 24	50.25
F7	(DOTAP/DC-Chol)/PEG	57 / 33 / 10	225 ± 97	39.12
F8	DC-Chol/DOPE	67 / 33 / 0	150 ± 54	43.58
F9	(DC-Chol/DOPE)/PEG	57 / 33 / 10	135 ± 24	36.56
F10	DC-Chol/DOPE	50 / 50 / 0	170 ± 40	39.47
F11	(DC-Chol/DOPE)/PEG	45.5 / 45.5 / 9	119 ± 92	36.79
F12	DC-Chol/DOPE	67 / 33 / 0	126 ± 35	34.69

Liposomes characterization

Particle size and zeta potential measurements were performed using an aqueous dip cell in the automatic mode by Zetasizer Nano ZS (Malvern Instruments, UK). Encapsulation efficiency of the process was determined by ultracentrifugation (at 300.000 g up to 5 hours at 25°C) rpm of the disintegrated lipoplexes. The number of free plasmids

was determined in a clear supernatant by spectrophotometry at 260 nm. The amount of pDNA encapsulated in the liposomes was calculated by measuring the difference between the total amount of pDNA added in the liposome formulation preparation and the amount of non entrapped pDNA remaining in the aqueous supernatant. The pDNA loading capacity was calculated by using equations [11] as indicated below (Eq1). The morphology of the lipoplexes was visualized by transmission electron microscopy (TEM, Leo 906E). The stability of pCR3.1-TSP1 in formulations against DNase-I was evaluated by agarose gel electrophoresis. Naked 1 mg pCR3.1-TSP1 and liposome formulations (equivalent to 1 mg of pDNA) were incubated with a final concentration of 4 or 40 µg/ml DNase I for 15 min, at 37°C. The reaction was stopped by adding 100 µl of DNase-I inhibitor (5mM iodoacetic acid). After this treatment, applied liposomes were subjected to high temperature for rapid liposome degradation. Then, the integrity of the plasmid was analyzed at 0.8% agarose gel electrophoresis and stained with ethidium bromide.

EE (%) = [(a-b) x d / c – (a-b)]/a x 100 [Eq1]

A: total amount of pDNA added to formulation, b: free amount of pDNA, c: precipitate amount of formulation weight, d: total amount of liposome ingredients.

The release of pCR3.1-TSP1 from formulations was determined with dialysis bags (MWCO: 12,000) in 20 mL sterile phosphate-buffered saline (pH 7.4) and samples were stored at 37°C under shaking conditions. 400 µl of sample was taken at the time intervals and the same amount of the fresh medium was added. After serial sampling at specified time intervals, the amount of released pCR3.1-TSP1 was determined spectrophotometrically at 260nm. The release data were plotted as a function of time and evaluated by kinetic equations.

Human breast epithelial cells (MCF-7) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids with 1% penicillin-streptomycin at 37°C in a humidified 5% carbon dioxide incubator. Cells were seeded at a density of 0.5 x 106 cells (Nikon, Eclipse TS100) per well in 24-well culture plates and were transfected with 50µL of liposome formulations. Fifty microliters of lipids and 20 µL/ well-naked DNA was used as a negative control. After 3 hours incubation, media was refreshed with fresh complete media, and cultured for another 24 hours. After all, incubations were done; cells were washed with PBS and fixed with 50 µL of methanol to the 24-well culture plates. Anti-V5- FITC with the 1:500 dilutions were added and the pCR3.1-TSP1 transfections were observed with a fluorescent microscope. Incubated cells were harvested by using an SDS-based lysis buffer (62.5 mm Tris-HCl (pH: 6.8); 2% (w/v) SDS; 10% (v/v) glycerol; 0.1% (w/v) bromphenol blue, 50 mm DTT). Samples were subjected to 8% with 15% discontinuous SDS-PAGE gels. Proteins were then electrophoretically transferred to PVDF membranes and incubated with mouse antibodies against TSP-1 (1:400) overnight at ambient conditions. Protein bands were visualized by using a horseradish peroxidase-linked anti-mouse secondary antibody (1:1000). Protein band density was quantified by using a densitometry computer programme.

Result and Discussion

We obtained optimum conditions with F5 and F9 (Table1). Their particle size was 149.34 ± 18 µm and 135.56 ± 24 µm respectively. The morphological appearance of liposomes (Figure 1) was visualized by using TEM. Zeta potential of liposomes was between 36-51 mV and increased encapsulation efficiency slightly decreases the zeta potential. Encapsulation efficiency of liposomes was calculated as 35.12 ± 1.7 % and 32.78 ± 1.3%, respectively. The low encapsulation can be explained by the ruptures at vesicular structures while pressure applied to form small vesicles.

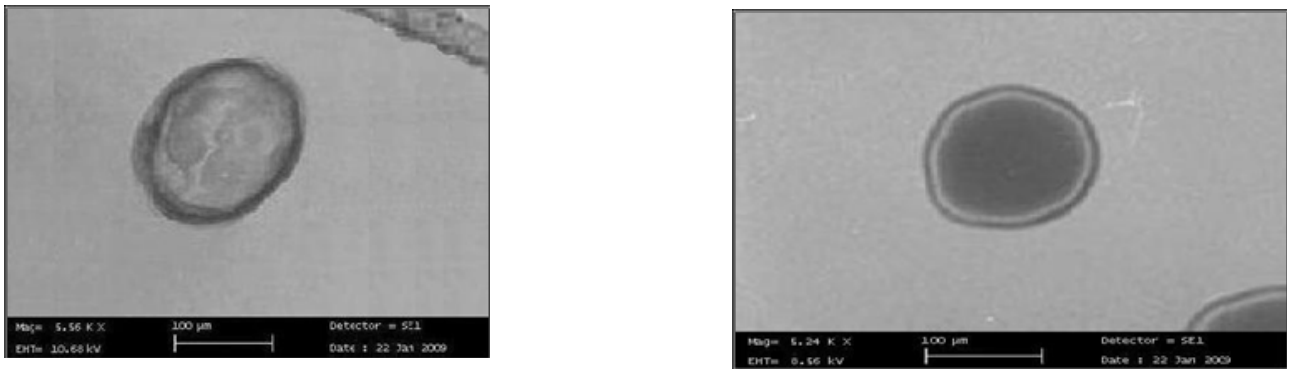

Figure 1: TEM micrograph of F5 and F9, respectively

When encapsulated pDNA in F5 and F9 liposome formulations were compared to naked pDNA, less degradation of pDNA was observed (Figure 2, lane 3-5). Free pDNA was fragmented within 15 min. (lane 3), whereas the pDNA recovered from formulations after same treatment are mostly remained intact (lanes 4-5). This suggests that at the physiological condition, where the nuclease concentration is markedly lower than the tested concentration, this formulation should render a significant protection of the pDNA. Also, these analyses revealed no change in the size or confirmation of the plasmid as compared with the original one. These findings showed us, this formulation process was mild enough not to inflict any damage on the pDNA.

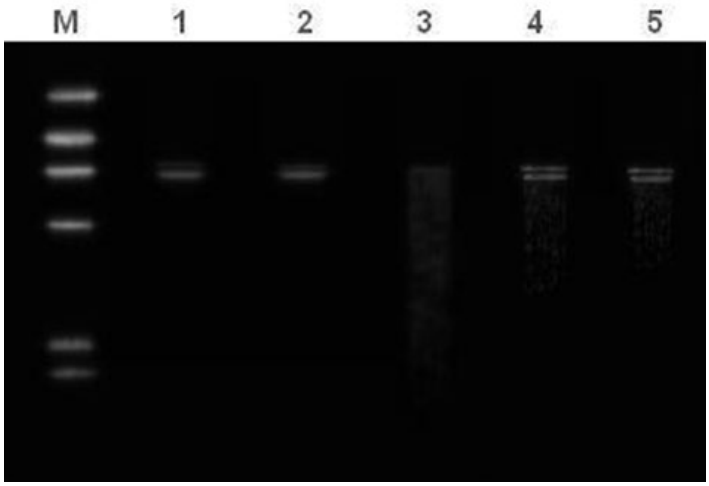

Figure 2: Electrophoretic mobility analysis of naked and liposome entrapped pDNA following DNase I digestion

Lane M: Lambda phage DNA marker, hind III digested,
lane 1: F5 formulation applied high temperature,
lane 2: F9 formulation applied high temperature
lane 3: naked pDNA + DNase I (4 µg/ml),
lane 4: F5 liposomes + DNase I (40 µg/ml) + liposome digestion,
lane 5: F9 liposomes + DNase I (40 µg/ml) + liposome digestion.

In-vitro release studies were carried on serum and blood protein free environment, so that effect of PEGylation couldn't understand in our in-vitro conditions. According to PEGylated liposomes releases studies, active ingredient showed rapid release by following to reach the plateau with a simple first-degree kinetic (r2>0, 96). This shows that time independent controlled release was occurred (Figure 3).

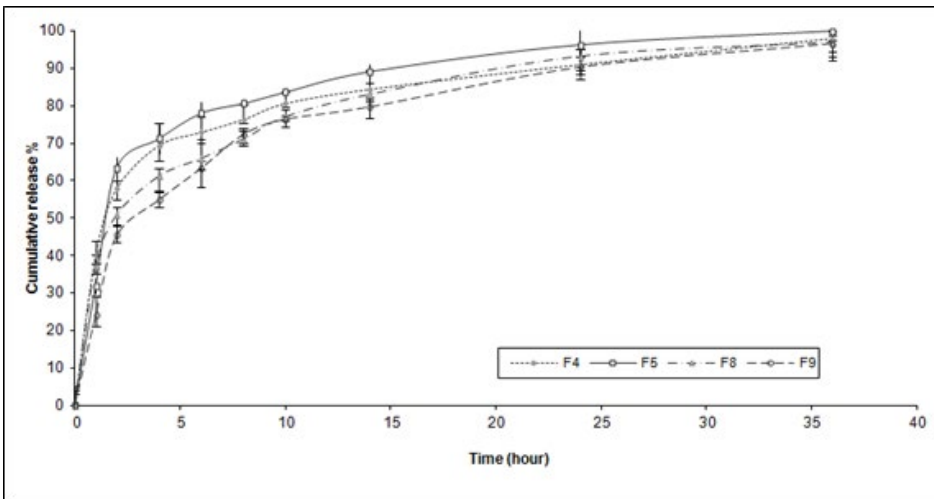

Figure 3: The in-vitro release profile of unPEGylated liposome (F4, F9) and PEGylated liposomes (F5, F9) in PBS medium at 37°C.

In vitro transfection process includes the complex formation between liposomes and DNA, the endocytosis of the complex and the DNA transfer to the nucleus being the moderate size of vesicles (from 0.4 to 1.4µm) more efficient for the gene transfection by endocytosis [12]. When we compared the transfection of cationic carriers, the most transfection was seen in liposome formulation (F5) than nanoparticle structures, which were prepared in our previous work [10]. Probably the lipoplexes can adhere to cells owing to their surface characteristics. Thus, the fusion process between cationic liposomes and cell membrane can play the key role in the transfection facilitating and promoting the cell uptake of large complexes through the endosomal pathway (Figure 4). Therefore, the zeta potential of cationic liposomes can be suggested as one of the factors involved in the process as already emphasized.

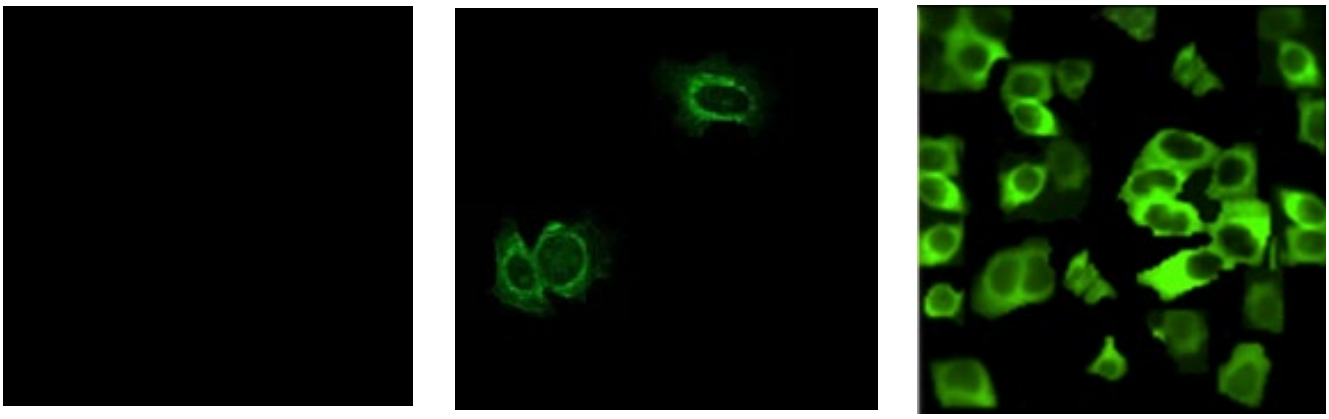

Figure 4: Fluorescent micrograph of a) lipid only, b) naked pDNA and c) F5 liposome formulation (x40)

Conclusion

We successfully prepared PEGylated DNA-lipoplexes with a proper particle size and positive zeta potential that might contribute to the high transfection efficiency and seemed to be more efficient carriers for in vitro gene transfer. Further studies will be carried on PEGylated liposomes to determine their stability and efficacy in animal model transfection process.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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