Original Article

Effect of Helper Lipids on Stability and Transfection Activity of Lyophilized Lipoplex Formulations of Antisense and DOTAP Nanoliposomes

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Abstract

Survivin, an inhibitor of apoptosis protein is highly expressed in most cancers and considered as an attractive target for cancer antisense therapy. To vectorize antisense molecules, cationic nanoliposomes are generally used; however, their complexes are too instable, during shelf-life and upon exposure to blood components and extracellular matrix, to be used in-vivo. The present study aimed to develop fresh and lyophilized formulations of antisense/DOTAP nanoliposomes with different helper lipids and compare their shelf-life and biologic stabilities and their transfection activities in tumor cell lines. DOTAP nanoliposomes in combination with different helper lipids were prepared in HEPES buffer (20 mM, pH=7.4) by thin-layer hydration followed by thermobarrel extrusion and PTFE membrane filter sterilization. Nanoliposomes were characterized regarding their particle size distribution, final lipid recovery and physical stability. Following antisense loading by direct addition through electrostatic attraction, the degree of complexation was determined by ethidium bromide displacement assay. To stabilize the formulations, they were freeze-dried with 10% sucrose. The potential of the lyophilized and fresh formulations of FITC-labeled antisense to transfect different cell lines (SK-BR-3, MCF-7) was studied by flow cytometry. Fresh nanoliposomes of different formulations had a size in range of 50-100 nm. The degree of complex formation with antisense was determined almost 70-80% (N/P ~ 2) which decreased as incubated with either PBS or complete medium and heparin sulfate. Their average sizes significantly changed after preservation for few days at 4 °C. Lyophilization process compromised the particle size distribution and antisense loading efficiency of different formulations except for DOTAP/DOPE (1:1 mole ratio) which did not change significantly. Both fresh and lyophilized formulations exhibited the highest transfection activity at comparable levels especially in SK-BR-3 cells. As a conclusion, lyophilization process could promote stability and preserve transfection activity of the antisense complexes of DOTAP/DOPE (1:1) nanoliposomes.

Keywords: DOTAP nanoliposome; Lyophilization; Stability; Survivin antisense; Transfection activity.
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1. Introduction

Survivin, a member of inhibitors of apoptosis protein (IAP) family, is expressed in embryonic tissues and in a wide range of cancer tissues, but is not detectable in differentiated normal adult cells for any organ [1]. There are different approaches to inhibit survivin such as small molecule antagonists, dominant negative mutants, immunotherapy and antisense therapy [2, 3]. Antisense oligonucleotides inhibit survivin expression at mRNA level based on ribosomal translation arrest or RNase H activation. Several viral and non-viral vectors are used to deliver antisense

molecules to intracellular targets. The non-viral vectors such as cationic polymers, liposomes, polymeric micelles, and dendrimers are generally considered to be safer, easier for large scale production, and less immunogenic [4, 5]. Among non-viral vectors, cationic nanoliposomes are mainly used for transfection. They function through packing genetic materials, protecting them from nuclease degradation, and induction of cellular internalization and intracellular trafficking [5, 6]. Nanoliposomes simply embark DNA through electrostatic interaction and form cationic lipid-DNA complexes
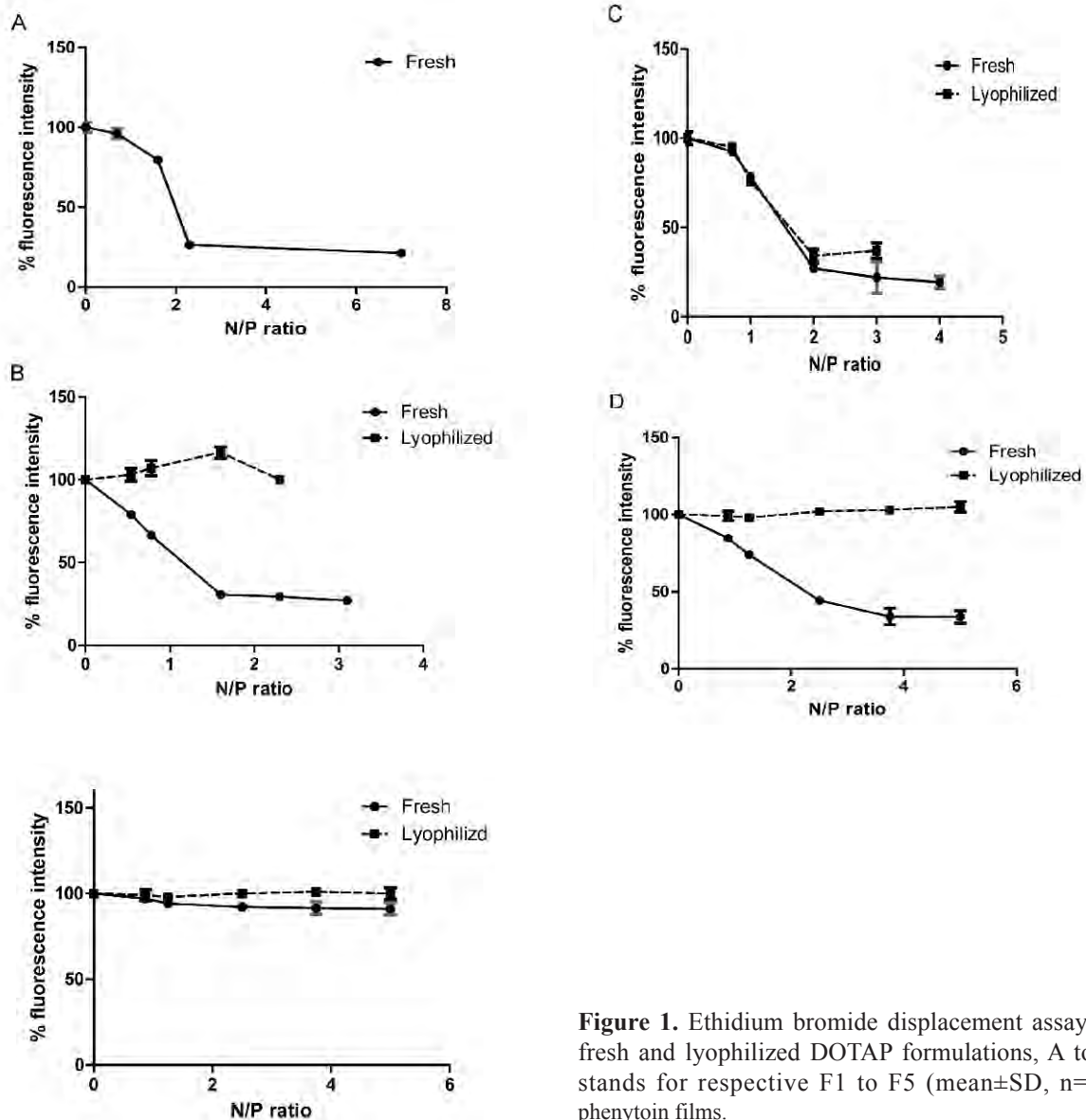

Figure 1. Ethidium bromide displacement assay of fresh and lyophilized DOTAP formulations, A to E stands for respective F1 to F5 (mean±SD, n=3); phenytoin films.

Table 1. Lipid compositions and preparation temperatures of DOTAP liposomal formulations.

Formulation	Composition	Molar ratio	Temperature (°C)
F1	DOTAP	100	25
F2	DOTAP/Chol	50:50	50
F3	DOTAP/DOPE	50:50	22
F4	DOTAP/DOPC	50:50	20
F5	DOTAP/DPPG	50:50	40

(lipoplexes), which are mainly defined by their lipid composition and N/P charge ratio between the cationic lipid ammonium and DNA phosphates, medium ionic strength and mixing style [7].

Several lines of evidence have demonstrated nanoliposomes and their complexes (lipoplexes) are not stable in liquid suspension during long-term storage or may demonstrate DNA displacement following exposure to proteins [8]. Some strategies are introduced to improve their stabilities that include the use of high pH diluents and conjugation of polyethylene glycol to the surface of lipoplexes. Such strategies although may be effective, but limited by the cost of production and diminished cellular uptake. Lyophilization is proposed as another method for long-term storage and possibly enhancement of lipoplex stability and protection against extracellular medium. Lyoprotectants such as disaccharides are generally supplemented in freezing medium. They promote the particle stability during lyophilization process as explained by the particle isolation and vitrification theories. They refer to the formation of a glassy sugar matrix in which diffusion is inhibited [9].

In the present study, we prepared different nanosized dioleoyltrimethyl-ammoni-umpropane (DOTAP) liposomes differ regarding their helper lipids at constant mole ratio (1:1). Helper lipids may help the stability of lipoplex in blood stream and releasing DNA cargo into cytoplasm [8]. Lipoplexes formed from survivin antisense were lyophilized. Their loading efficiency, stability in heparin sulfate modeling extracellular matrix and transfection activity in breast

tumor cells were compared with the respective fresh formulations.

2. Materials and methods
2.1. Materials

Dioleoyltrimethylammoniumpropane (DOTAP), dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylglycerol (DPPG), cholesterol (chol) and fluorescence-labeled lipid rhodamine- dioleoylphosphatidy lethanolamine (Rh-DOPE) were purchased from Avanti Polar Lipids (Albaster, AL, USA). Intercalating dye ethidium bromide (EtBr) was from Sigma (St. Louis, Missouri, USA). Survivin phosphorothioate antisense (ASOSURV1, CCC AGC CTT CCA GCT CCT TG) was obtained from Metabion (Germany). Metafectene reagent was received as a gift from Biontex (Germany). Breast cancer cell lines SK-BR-3 and MCF-7 were obtained from Pasteur Institute (Tehran, Iran).

2.2. Preparation of cationic nanoliposomes (transfection agents)

DOTAP and helper lipid solutions in chloroform were mixed according to Table 1 and dried under vacuum for 4 h by rotary evaporator (Heidolph, Germany) resulting in deposition of thin lipid film on the round bottomed flask wall. The dried lipid films were hydrated overnight with 20 mM HEPES (pH=7.4) to the final lipid concentration of 5 mM. The resulted liposomes were then down-sized and homogenized using thermobarrel extruder (Northern lipids, Vancouver, Canada) 10 times above their critical temperatures through double-stacked polycarbonate filters of 100 nm. Nanoliposomes were sterilized

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Table 2. Mean particle sizes and SPAN indices of different DOTAP formulations freshly prepared, stored in refrigerator, following reconstitution of the lyophilized liposomes and lipoplexes at N/P=2.

Formulation	Average volume diameter (nm), SPAN index			
	Fresh	After 6 months	Lyophilized liposome	Lyophilized lipoplex
F1	50.0, 0.38	104.5, 0.65	ND*	ND*
F2	56.5, 0.69	105.0, 0.95	289.0, 47	293.3
F3	64.6, 0.65	105.0, 0.97	122.5, 0.46	166.0
F4	70.0, 0.69	130.5, 1.07	182.7, 0.44	365.0
F5	163.0, 0.63	261.5, 1.21	567.0, 0.59	649.0

*ND=not determined

with PTFE syringe membrane filter of 0.22 μm (Millipore, USA).

To determine the lipid recovery after extrusion and membrane filtration, the lipid membrane was labeled with Rh-DOPE (1% lipid mole ratio); the filters were soaked in methanol overnight at 4 followed by 30 sec bath sonication. The fluorescence intensities of the samples were measured at excitation and emission wave lengths of 550 and 600 nm (TECAN, Austria). Lipid recovery was determined according to the equation 1:

$$\% \text{ Lipid recovery} = (I_{Liposome} \times 10 - I_{Filter}) / (I_{Liposome} \times 10 - I_0) \times 100$$
Equation 1

Where $I_{Liposome}$, I_{Filter} and I_0 are the fluorescence intensities of 10X diluted liposomes, adsorbed lipids on the filters and methanolic blank. The liposome concentrations were corrected according to the final lipid recovery percentages.

2.3. Lipoplex formation

Lipoplexes were formed by direct addition of equal volumes of cationic nanoliposomes to ASOSURV1 at various N/P mole ratios and let them stand for 15 min at room temperature prior to application. Formation of lipoplexes was studied by ethidium bromide exclusion assay and native 20% acrylamide gel electrophoresis (Biorad, USA).

2.4. Lyophilization process

To overcome liposome problems during drying and rehydration, lyoprotectants such as carbohydrates, e.g. sucrose, lactose and glucose were compared in a preliminary study [10, 11]. The preferred lyoprotectant (sucrose 10% with higher glass transition temperature) was admixed with 2 ml of the nanoliposomes or their ASOSURV1 lipoplexes individually in PTFE-stoppered glass vials (Supelco, USA). The mixtures were kept in polystyrene cryobox and gradually cooled down to -70 °C overnight. The frozen vials were thoroughly wrapped with cotton fibers and placed in
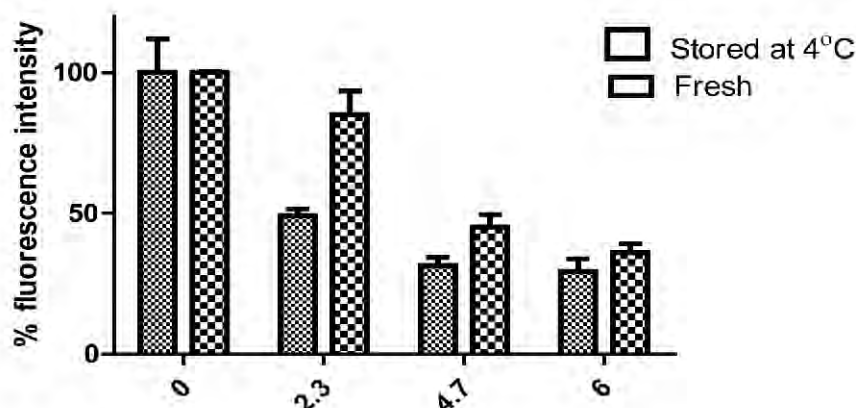

Figure 2. DOTAP lipoplex stability after 1 week storage at 4 °C as determined by ethidium bromide displacement assay (mean±SD, n=3).

Table 3. Extracellular stability of fresh and lyophilized lipoplexes modeled by heparin sulfate interaction on ethidium bromide displacement assay

Formulation	Fresh		Lyophilized	
	alone	**+ heparin sulfate**	**alone**	**+ heparin sulfate**
F1	41.9±0.5	49.7±2.3	22.7±1.3	72.6±2.1
F2	30.68±0.7	106.0±3.9	28.0±2.5	91.2±3.7
F3	27.12±1.7	85.7±1.7	38.3±0.6	57.7±1.2
F4	44.36±2.0	98.7± 1.0	30.0±0.8	84.5±2.4
F5	92.2±0.3	104.2±2.4	ND*	ND*

Above header spanning: **% Excluded EtBr**

mean±SD, n=3; *ND=not determined

containers belong to the freeze-drier apparatus (Zirbus, Germany). They were thermally sealed from the environment following vacuum formation. Dehydration step lasted for 2 days at condenser temperature of-50 °C until a dried sponge cake formed. The vials were filled with nitrogen gas to prevent lipid oxidation and stoppered. During rehydration step just prior to application, the dried mass was reconstituted in aqueous medium, either distilled water or antisense solution, for respective lipoplex or nanoliposome formulations.

2.5. Particle size measurement

The average volume-weighted diameter and polydispersity SPAN indices of the fresh and lyophilized formulations were determined by dynamic light scattering method (Shimadzu SALD2101, Japan) SPAN indices were calculated from Equation 2:

$$SPAN = (D_{90\%}-D_{10\%}) / D_{50\%} \quad \text{Equation 2}$$

Physical stability of the particles during shelf life was studied with respect to the changes of the average volume diameters using repeated measure analysis of variance statistical method.

2.6. Ethidium bromide displacement assay

EtBr, a DNA-intercalating dye, was used to examine the association of DNA with the cationic liposomes [12, 13]. ASOSURV1 20 µM was incubated with 1 µg/ml EtBr, and the maximum fluorescence intensity was recorded

at emission wavelength of 595 nm (excited at 510 nm) by fluorescent microplate reader (TECAN, Austria). Various amounts of fresh and freeze-dried nanoliposomes were added to the solutions in polystyrene microtubes and the changes in the fluorescence intensity were measured. The degree of DNA-cationic liposome complex formation was calculated from Equation 3:

$$\text{Degree of complex formation} = (I_1-I_2) / (I_1-I_0) \times 100$$
$$\text{Equation 3}$$

Where I1, I2 and I0 are the fluorescence intensities of liposome free DNA solution, lipoplex sample and EtBr blank solution.

Degrees of complex formation were calculated during shelf life at 4 . Besides, heparin sulfate 0.9% (w/v) was added to the certain tubes and incubated for 15 min to model lipoplex exposure with extracellular matrix and the resulting DNA unpacking. The fluorescence intensity of EtBr was measured and compared before and after heparin sulfate addition by paired t-test.

2.7. Cell transfection and flow cytometry

Cells were transfected according to the previously reported method [14]. Breast tumor cells (SKBR-3 and MCF-7) were seeded in 6-well plates at 100000 cells/ml of 10% FBS supplemented RPMI 1640 medium. They were incubated for 48-72 h at 37 °C/ 5% CO2. After medium aspiration, cells were rinsed twice with sterile normal saline or PBS. Fresh or lyophilized lipoplexes of 700

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Table 4. Transfected cell percentages for different DOTAP formulations compared to Metafectene in MCF-7 and SK-BR-3 cells

	% Gated cells containing FITC-labeled antisense oligonucleotides	
Formulation	MCF-7	SK-BR-3
Free	24.89±0.13	26.39±0.3
F1	67.98±0.16	82.22±0.4
F2	60.57±0.1	39.33±0.35
F3	80.87±0.6	88.39±0.8
F4	73.33±0.18	87.23±0.32
F5	43.22±0.13	30.6±0.63
Metafectene®	74.38±0.84	98.47±0.15

mean±SD, n=3

nM FITC-labeled ASOSURV1 in culture medium were added to the wells and incubated for 4 h. Cells were detached from the wells by adding 400 µl trypsin-EDTA 0.25% and neutralized by 4 ml PBS containing 10% bovine serum albumin followed by centrifugation for 5 min at 1000 rpm. The supernatant was discarded and the cell pellets were re-suspended in 500 µL of 20 µM monensin solution in PBS to neutralize endosomal pH which interferes with FITC fluorescence intensity. Cells were kept on ice until the cell associated fluorescence measured by FACScalibur flow cytometer (Becton Dickinson, USA). Cell Quest software (Becton Dickinson, USA) was used for data acquisition through FL1 (530 nm) and analysis for 10,000 gated events. Cell-associated fluorescence intensities and percentages of the transfected cells were expressed as the average of these measurements. The results were compared to those of Metafectene®, considered as a reference transfection agent, by analysis of variance and Bonferroni follow up test

2.8. Statistics

All statistical tests were performed using Graphpad software version 5 at 0.05 significance level.

3. Results and discussion

3.1. Particle size measurement

Literature data about the effect of liposome and lipoplex size on the transfection activity is controversial. In the present study, the sizes of different formulations (freshly prepared, after 6 month storage in refrigerator, following reconstitution of 6 month old lyophilized liposomes or lipoplexes) are summarized in Table 2. Sizes of the freshly prepared formulations ranged from 50-70 nm except for F5 (DOTAP: DPPG) which was extremely higher due to neutralization of DOTAP charge by DPPG and probable particle aggregations. The average sizes were comparable with the previously reported results of around 100 nm for DOTAP/DOPE, DOTAP/cholesterol and the PEGylated nanoliposomes [9]. They are totally favorable for passive targeting and escaping from reticuloendothelial system. It was shown that reduction of liposome size facilitates absorption of nanoliposomes trough physiologic barriers and the possibility of passive targeting to the tumor site due to enhanced permeability and retention effect [15].

It was reported that lyophilization inherently induced particle aggregation for DOTAP/cholesterol, DOTAP/DOPE and PEGylated formulations if no excipient was added, but its effect was inhibited by sucrose, trehalose, etc. Sucrose was determined as efficacious as inulin for PEGylated liposomes while average sizes increased for dextran and trehalose [16-18]. It was reported that stabilization effect of sucrose in DC-Chol/DOPE prepared by dehydration-rehydration was attributed to direct interaction with DNA rather than by vitrification to a high Tg solids [19].

The sizes were significantly increased

beyond 100 nm after 6 month at 4 °C and lyophilization. Among the lyophilized formulations, F3 was more preserved and still suitable for lipoplex formation. The size changes may happen as small unilamellar nano-vesicles are transformed mainly into larger multilamellar structure during lyophilization. The sizes of lyophilized lipoplexes were even more than nanoliposomes, but less than 200 nm for F3

and were considered acceptable for application.

3.2. Displacement of EtBr intercalation

Displacement of bound EtBr from DNA is usually employed to measure the intercalation of cationic liposome and DNA [10]. Figure 1 shows that the fluorescence intensity decreased as N/P ratio increased until reaching a plateau. Maximum DNA/DOTAP complex
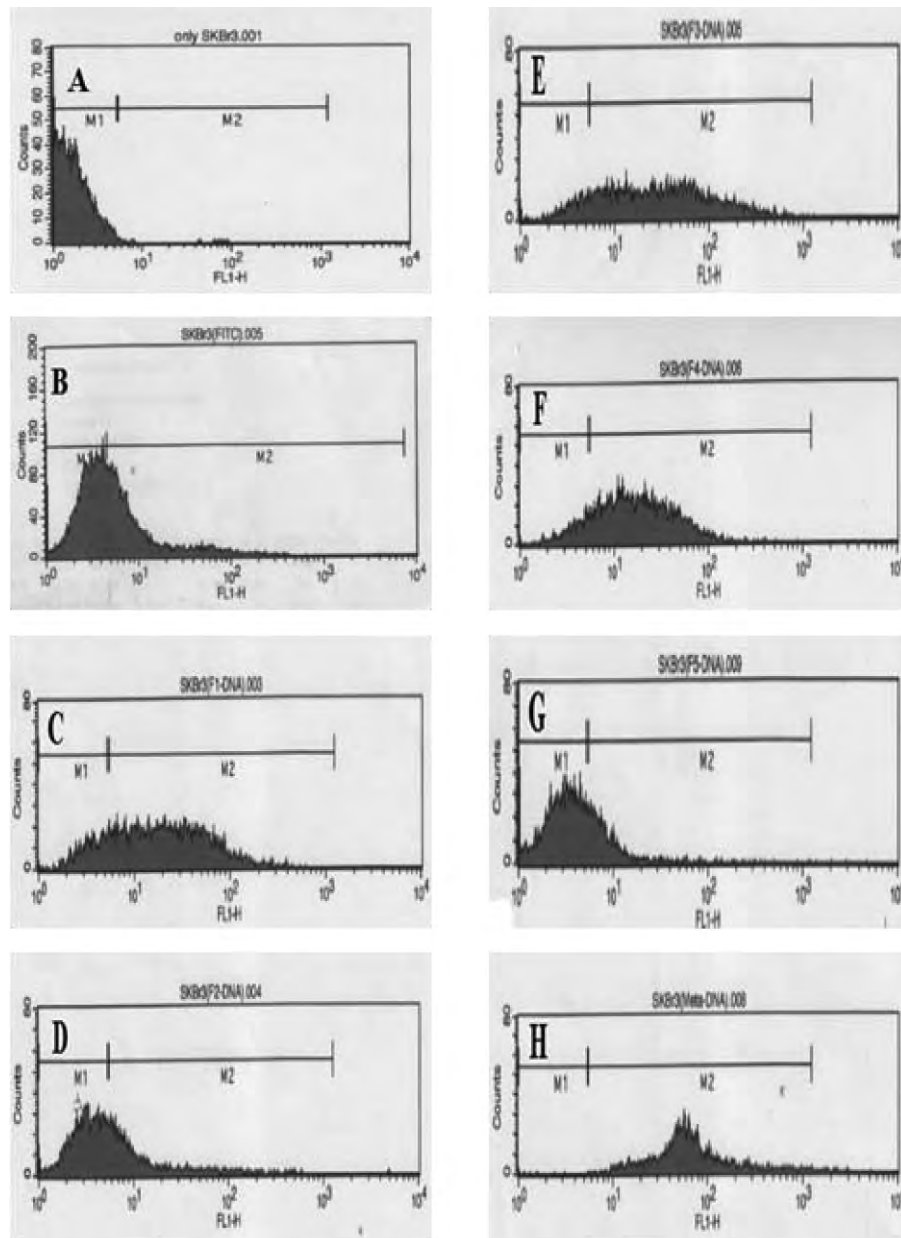

Figure 3. Flow cytometric histograms of the cell associated fluorescence intensities of A: untreated cells, and cells treated with B: free antisense oligonucleotide, C to G: respective lipoplexes of F1 to F5, and H: Metafectene.

formation was observed at minimum N/P ratios of 2.3, 1.6, 2 and 2.5 for F1 to F4, respectively. F5 formulation (DOTAP/DPPG) did not show any significant reduction in fluorescence intensity after antisense addition, because DOTAP is not still cationic when combined with DPPG (an anionic phospholipid).

Freeze drying process compromised DNA binding activity of cationic liposomes (F2 and F4) presumably because uni- to multi-lamellar vesicle transition, which generally happens in lyophilization process, may reduce available active surface for DNA binding. On the contrary, F3 exhibited a completely different behavior and DNA binding capacity was preserved at the same N/P ratio. This may be attributed to DOPE unique property of inducing reverse hexagonal phase during dehydration and transformation into lamellar structure when rehydrated.

3.3. Lipoplex stability

Lipoplex stability during shelf life and in extracellular medium is a prerequisite for successful antisense delivery. Figure 2 shows that N/P ratio shifted to higher value if the lipoplex stored in refrigerator for 1 week. This implies destabilization and weakening of the association bounds between DNA and cationic liposomes. Besides, this unstable nature of lipoplexes necessitates their preparation immediately before administration.

The stability of lipoplexes in extracellular medium is usually modeled by incubating lipoplexes with serum and heparin sulfate. Inclusion of helper lipids in DOTAP liposome formulation made them more susceptible to heparin sulfate addition. This caused finally a complete DNA exposure to EtBr and raised the fluorescence intensities of DNA intercalated EtBr to almost 100% (Table 3). Although this effect related to helper lipids seems undesirable in extracellular medium, it guarantees DNA unpacking inside the cells.

Freeze-drying process was helpful to induce heparin sulfate resistance depending on the lipid composition. F3 (DOTAP/DOPE) was more resistant to heparin sulfate action. This may imply formation of a different lipoplex structure than spaghetti meatball. Although it was not demonstrated here, it was thought formation of DNA sandwich between lipid bilayers is responsible for stabilization of the lipoplexes in heparin sulfate supplemented medium.

3.4. Flow cytometry

Flow cytometry experiment was performed to show the ability of lipoplexes to transfect the tumor cells. The percentages of transfected cells calculated from the gated events was not only depends on the cell line but also the liposome formulation (Figure 3 and Table 4). It was revealed in both cell lines that lipoplexes increased cellular uptake of FITC-labeled antisense except for F5 that no functional lipoplexes was formed. The percentages were significantly higher in SKBR-3 than MCF-7 cells. The highest transfection percentage was obtained following F3 treatment for 4 h. This calculated to be about 88.39% in SKBR3 cells, which is comparable with the commercially available Metafectene reagent.

4. Conclusion

Lyophilization process may stabilize nanoliposomes or lipoplexes especially if DOTAP combined with DOPE at 1:1 mole ratio. This promotes extracellular as well as shelf life stability without compromising transfection activity.

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