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A comparative study of cationic liposomes for gene delivery

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ABSTRACT: In the field of gene delivery, non-viral vectors have become more attractive carriers for nucleic acids since they can overcome the significant drawbacks of viral systems such as safety, immunogenicity, and oncogenicity. Among non-viral vectors, cationic liposome-mediated gene delivery gives promising results for gene therapy approaches. This study aimed to develop cationic liposomes and examine the effectiveness in terms of gene delivery. For this purpose, cholesterol, lecithin, and cationic lipid containing liposomes have been developed by film hydration method. Two different cationic lipids, DDAB and EQ, and their different mole ratios were investigated. Characterization studies showed that obtained liposomes have appropriate physiochemical characteristics (~100 nm, homogenous in size and positive zeta potential) for gene delivery. Gel retardation assay revealed that they have DNA binding and protection ability against nucleases. According to the cytotoxicity evaluation performed on L929 cell line, EQ containing liposomes shows significantly less toxicity comparing DDAB containing liposomes ($p<0.05$). Furthermore, *in vitro* transfection study revealed that increasing the EQ mole ratio has increased transfection ability. The stability results showed that the optimal liposome which contains EQ in a higher mole ratio is stable during 90 days at 4°C. Based on these findings, we propose that the developed optimal liposome system in this study could be considered as a suitable nucleic acid delivery vehicle base for gene therapy.

KEYWORDS: Liposome; gene delivery; transfection; cytotoxicity; DDAB; esterquate.

1. INTRODUCTION

In the last decade, gene therapy has become a valuable treatment method for various types of diseases. In general, gene delivery systems are classified such as viral and non-viral vectors [1]. Since the beginning of gene therapy applications, viral vectors have been the first choice due to their high levels of transfection properties. However, concerns about their high immunogenic and oncogenic effects limit their use [2]. On the other hand, non-viral vectors are safer, could be produced cheaper, and have higher nucleic acid loading capacity [3]. The main disadvantage of non-viral systems is that they show lower transfection efficacy than viral vectors [4]. Even so, studies on the production of efficient non-viral gene delivery systems are gaining interest consistently. Especially, after the Covid-19 pandemic, the use of non-viral delivery systems in DNA vaccine has been a frequently applied approach. Based on the promising results obtained by BioNTech-Pfizer and Moderna's mRNA-based Covid-19 vaccine studies, the focus on the design of efficient non-viral vectors has increased [5]. Furthermore, non-viral gene delivery systems are also investigated in terms of their brain targeted delivery for diseases that still have no effective treatment such as brain cancer and Alzheimer's disease [6,7].

Among non-viral delivery systems, liposomes are extensively studied for their ease of manufacture, biocompatibility, and suitable sizes [8]. Briefly, liposomes are vesicles of phospholipid molecules that are self-assembled and consist of either one or more bilayers of phospholipids. By the addition of cationic lipids in the formulation development process, it is possible to obtain cationic charged liposomes [9]. Cationic liposomes are advantageous due to their binding ability to plasmid DNA (pDNA), small interfering RNAs, and mRNAs by electrostatic interactions and facilitating effects on their cellular uptake while protecting them against enzymatic degradation. However, while cationic liposomes provide these advantages, they also lead to major

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limitations such as toxicity, aggregation, and inactivation in serum through surface binding proteins [10]. Therefore, the selection of the appropriate cationic lipid and formulation components is important in developing an efficient cationic liposome for gene transfer.

In this study, we aimed to develop novel cationic liposome systems and compare their efficiency for gene delivery. For this purpose, two different cationic lipid, dimethyldioctadecylammonium bromide (DDAB) and esterquate (EQ), and their different ratios were investigated. The developed liposomes were evaluated in terms of physiochemical properties, DNA binding, and protection ability against nucleases, cytotoxicity, transfection efficiency. The stability of optimal liposome was also investigated. The comprehension obtained from this research may result in the development of better cationic liposomes for gene delivery purposes.

2. RESULTS AND DISCUSSION

2.1. Formation and Characterization of liposomes

In both pre-clinical and clinical trials for the treatment of various diseases, liposomes have been extensively studied for gene delivery. Especially, cationic liposomes were investigated in terms of their complex ability with negatively charged nucleic acids and facilitate nucleic acid uptake through the negatively charged cell membrane [11]. In this study, cationic liposomes that contain cholesterol, lecithin, and DDAB /EQ were fabricated by a film-hydration method with different mol ratios (Table 1).

Table 1. Compositions of developed liposome formulations.

Formulation code	Cholesterol	Lecithin	DDAB	EQ	Mol ratio
Lipo-1D	+	+	+	-	1:1:1
Lipo-2D	+	+	+	-	1:1:2
Lipo-1E	+	+	-	+	1:1:1
Lipo-2E	+	+	-	+	1:1:2

Dynamic light scattering (DLS) measurements were performed to determine the particle size, polidispersity index (PDI), and zeta potential of liposomes and their complexes via pcDNA3-EGFP. According to the results given in Table 2, the obtained liposome's particle size range is between 78.13 and 100.20 nm. The PDI values of liposomes were measured lower than 0.3. This PDI value is attributed to the uniformity in the droplet size distribution of formulations [12,13]. After binding of pDNA, the sizes and the PDI values of formulations have increased except for the formulation Lipo-2E:pDNA. The particle sizes of the complexes were determined between 107.30 to 173.90 nm. In the literature, many articles suggested that the delivery systems with particle sizes around 150 nm are promising systems for gene delivery [9,14]. The PDI value of Lipo-2E:pDNA formulation was measured lowest among others as 0.180 which shows that the pDNAs and Lipo-2E formulation forms monodisperse complexes. The particle size visualized in the scanning electron microscopy (SEM) images and particle sizes found smilar to the data obtained by DLS method. This result revealed that Lipo-2E formulation has a particle size around 100 nm with spherical shape and a uniform distribution (Figure 1).

Table 2. Particle size, PDI, and zeta potential results for prepared liposome formulations. The results were indicated as mean ± standard deviation (SD) (n=3).

Formulation	Particle size (nm ± SD)	PDI (± SD)	Zeta Potential (mV ± SD)
Lipo-1D	91.30 ± 1.19	0.281 ± 0.007	64.6 ± 1.72
Lipo-2D	78.13 ± 1.00	0.298 ± 0.008	66.7 ± 2.73
Lipo-1E	96.43 ± 0.71	0.239 ± 0.011	39.3 ± 0.20
Lipo-2E	100.20 ± 0.96	0.215 ± 0.008	48.0 ± 0.78
Lipo-1D:pDNA	134.10 ± 1.53	0.325 ± 0.036	35.9 ± 0.32
Lipo-2D:pDNA	107.30 ± 1.96	0.356 ± 0.018	31.2 ± 2.36
Lipo-1E:pDNA	173.90 ± 1.62	0.298 ± 0.007	37.6 ± 0.89
Lipo-2E:pDNA	127.30 ± 1.26	0.180 ± 0.010	39.3 ± 1.81

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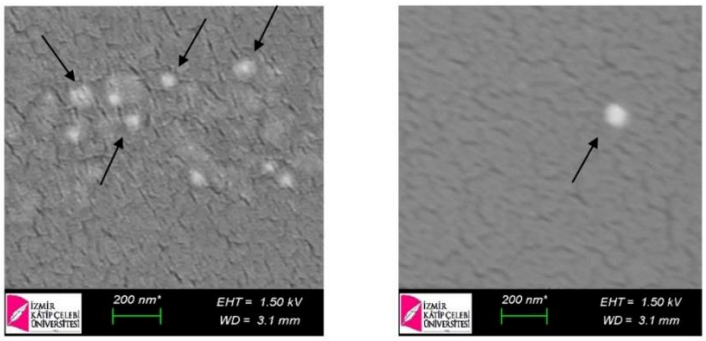

Figure 1. Scanning electron micrograph of Lipo-2E.

The zeta potential measurements indicate that all prepared liposomes have a positive surface charge. This positive charge is due to the cationic lipids used in the formulation stage such as EQ and DDAB [6,15]. The zeta potential of liposomes prepared with DDAB was measured higher than formulations prepared with EQ. By increasing the mole ratio of cationic lipids, the zeta potentials increased from 64.6 to 66.7 for EQ and from 37.3 to 48.0 for DDAB. This positive charge enables electrostatic interactions to bind pDNAs to the outer surface of liposomes [16]. Following the complex formation with pDNA, the zeta potential of complexes has decreased as expected due to the negative charge of nucleic acids [17]. It can be drawn that the physicochemical characteristics of the developed liposome:pDNA complexes are sufficient (particle size ~150 nm, and positively charged) to obtain a suitable long circulating delivery system and to achieve high transfection [18].

2.2. Gel retardation assay result

To examine the nucleic acid binding affinity of a cationic liposome, gel retardation assay was conducted with increasing Liposome:pDNA ratios (0.5:1, 1:1, 2:1, 3:1, 4:1 (v/v)). As can be seen in Figure 2, all liposomes retarded pDNA migration in agarose gel and showed that Lipo-1D, Lipo-2D, Lipo-1E, and Lipo-2E have the ability to complex with pDNA. This result can be attributed to the neutralizing effect of positively charged liposomes on the negatively charged phosphate groups in the DNA backbone [19]. Hence, the movement of DNA from the well has been inhibited.

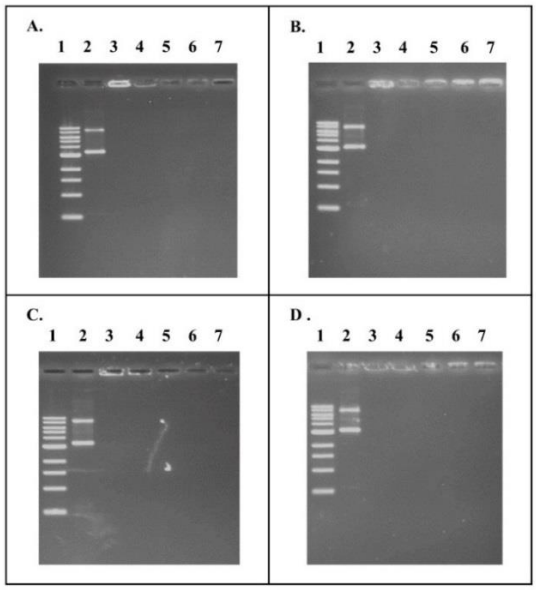

Figure 2. Agarose gel electrophoresis results of complexes containing constant amount of pDNA and increasing amount of Lipo-1D (A), Lipo-2D (B), Lipo-1E (C), and Lipo-2E (D). (1: 1kb plus DNA ladder, 2: Free pcDNA3-EGFP, 3-7: Liposome:pDNA complexes for the ratio 0.5:1, 1:1, 2:1, 3:1, 4:1 (v/v), respectively).

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2.3. Protection ability against DNase I

To develop an efficient gene delivery system, it is required to protect the integrity of nucleic acids during the circulation and transportation to the target side [6]. For this purpose, pDNA should be protected against enzymatic degradation by the use of appropriate delivery systems [20,21]. According to the DNase I protection study result presented in Figure 3, naked pDNA was fully digested within 30 min (lane 3). Lanes 4-8 show the protection ability of liposomes formulations with increasing complex ratio (0.5:1, 1:1, 2:1, 3:1, 4:1 (v/v)). For the formulations Lipo-1D, Lipo-1E, and Lipo-2E most of the DNA was degraded for the ratio 0.5:1 (v/v). However, for the same formulations when the complex ratio reached 1:1 (v/v), the protection ability of formulations increased, and the pDNA is prevented from degradation. For the formulation Lipo-2D, pDNA was protected from degradation starting from the ratio 0.5:1 (v/v). The higher zeta potential of the formulation compared to other formulations may have led to this result [9]. On the basis from these findings, Lipo:pDNA vectors with the ratio of 1:1 (v/v) were selected as the optimal ratio and used for the further studies.

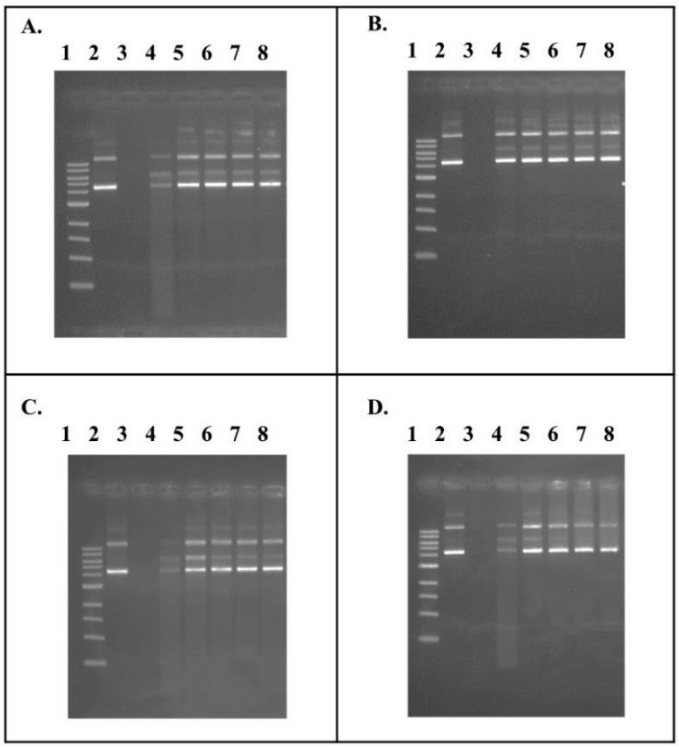

Figure 3. DNase I protection ability of formulations Lipo-1D (A), Lipo-2D (B), Lipo-1E (C), and Lipo-2E (D). (1: 1kb plus DNA ladder, 2: Free pcDNA3-EGFP, 3: Free pcDNA3-EGFP + Dnase I, 4-8: Liposome:pDNA complexes for the ratio 0.5:1, 1:1, 2:1, 3:1, 4:1 (v/v), respectively).

2.4. Cytotoxicity evaluation

Cytotoxicity evaluation of liposome formulations was evaluated on mouse fibroblast (L929) cells. Cell viability of the cells exposed to the liposome formulations was presented in Figure 4. All formulations showed increasing cytotoxicity with the increasing formulation concentration. For the formulations Lipo-1E and Lipo-2E; no significant cytotoxicity was determined at concentrations between 3-15 µL/well ($p<0.05$). For these formulations, cell viability was detected more than 80% even for the highest concentration. On the other hand, cell viability decreased dramatically by the application of Lipo-1D and Lipo-2D on the L929 cells. Lipo-1D showed 57% viability for the dose 1.5 µL/ well and 19% viability for the dose 3 µL/well (significantly higher than Lipo-1E, $p<0.005$). Moreover, Lipo-2D showed 12.8% viability for the dose 1.5 µL/ well and less than 10% viability for the higher doses (significantly higher than Lipo-2E, $p<0.005$). This may be caused by the destructive effect of DDAB on the cell membrane [22]. Previously, it was reported that the cationic head group within liposomes could lead cytotoxicity [9]. This toxic effect occurs due to the binding effect of serum proteins to the cationic charged liposomes. In many studies it has been shown that decreasing the positively charge

results in reduction of cationic liposomes toxicity [23]. Our results support the same fact. Therefore, Lipo-1E and Lipo-2E were selected for the further use in transfection studies due to their non-toxic properties.

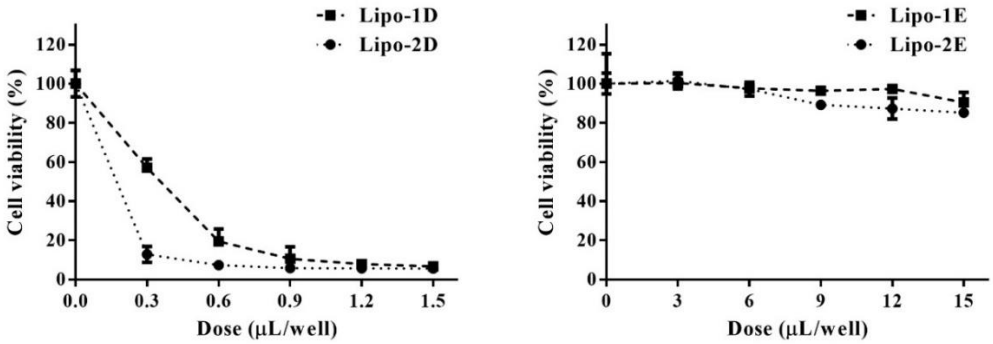

Figure 4. Cell viability of L929 cells exposed to Lipo-1D, Lipo-2D, Lipo-1E, and Lipo-2E formulations. (Data are shown as mean ± SD, n=4).

2.5. *In vitro* transfection studies

Liposomes have been utilized as carriers for many active molecules either genes or proteins for therapeutic and even diagnostic purposes [24]. To develop a successful liposome-based gene delivery system, the evaluation of the transfection is one of the most crucial step. Transfection ability of developed Lipo-1E and Lipo-2E formulations were investigated on human embryonic kidney (HEK293) cell line under a fluorescence microscope. Fluorescence from GFP expression in the cells is shown with the green color (Figure 5C, 5D). According to the transfection results, Lipo-1E and Lipo-2E formulations have transfection ability. For Lipo-2E formulation, GFP expression was observed in more cells comparing Lipo-1E formulation in 48 h. This increase may be due to the difference of the zeta potential. The zeta potential of Lipo-2E:pDNA complex is slightly higher comparing Lipo-1E:pDNA complex (39.3 and 37.6 mV, respectively) (Table 2). Moreover, as mentioned previously, both Lipo-1E and Lipo-2E formulations are able to protect the pDNA against nucleases. However, pDNA transforms from the supercoiled state to the open circular and linear form due to enzymatic degradation for the formulations Lipo-1E. The degradation level is reduced for the formulation Lipo-2E depending on the increasing amount of liposome. It has been stated in the literature that the conversion of supercoiled pDNA to the open circular form results in a decrease in the efficiency of gene transfer [26]. Hereby, this phenomenon may have caused an increase in transfection for Lipo-2E.

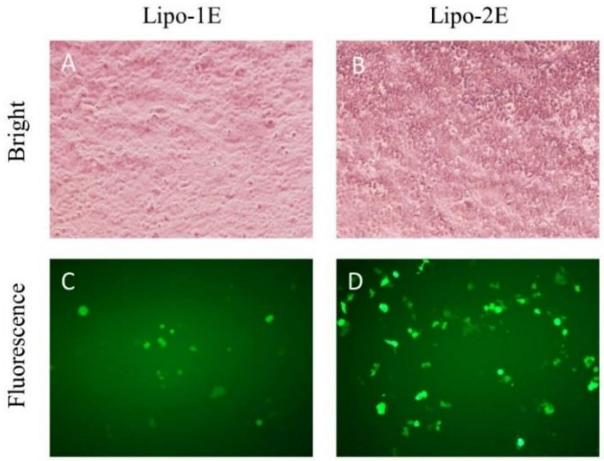

Figure 5. Light and fluorescent images of HEK293 cells following transfection by Lipo-1E:pDNA and Lipo-2E:pDNA complexes. (A and B in the bright field; C and D under fluorescence microscope).

2.6. The physicochemical stability of liposome formulation

Since Lipo-2E showed higher transfection efficiency, physicochemical stability monitoring was performed specifically for this formulation. No turbidity or aggregation was determined during the 3 months period. According to the DLS measurement results given in Figure 6, no significant difference was observed on the formulation Lipo-2E stored at 4 °C during this period ($p > 0.05$). As a result, it can be said that Lipo-2E is a suitable gene delivery system for longterm storage.

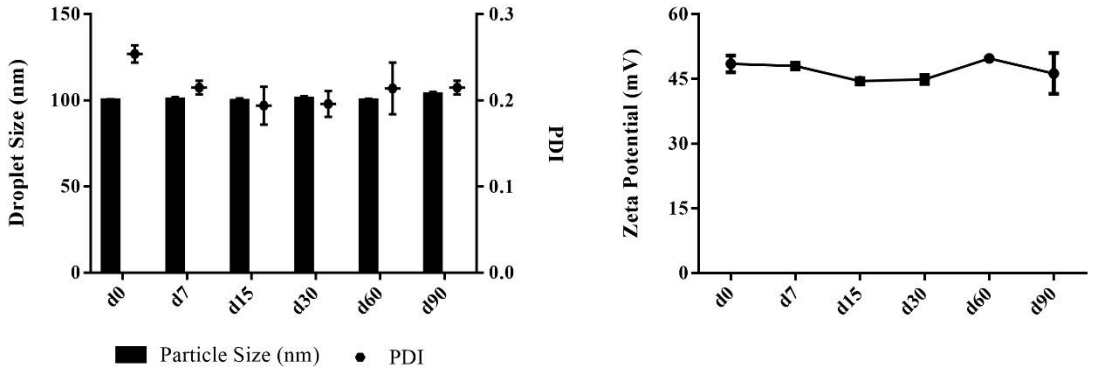

Figure 6. Stability study results of Lipo-2E over 90 days. (d represents days. The results are shown as mean ± SD, n=3).

3. CONCLUSION

To sum up, in this study cationic liposomes that contain DDAB or EQ as a cationic lipid were successfully prepared. The effect of cationic lipid and their mole ratios was examined in terms of gene delivery purpose. The characterization studies revealed that obtained liposomes have an appropriate particle size, PDI, and zeta potential. Gel electrophoresis studies proved DNA binding and protection ability against serum nucleases. According to cytotoxicity results, EQ containing liposomes found to be more appropriate compared to DDAB containing liposomes, revealing low viability on L929 cells. Furthermore, transfection studies showed that developed liposomes are capable of carrying genetic materials into the cells efficiently. Consequently, it can be inferred that the cationic liposomes prepared herein are a promising carrier for the transfer of genetic material and can be used to treat a wide variety of genetic diseases as a non-viral delivery system.

4. MATERIALS AND METHODS

Green fluorescence protein (GFP) encoding plasmid (pcDNA3-EGFP) was kindly gifted by Doug Golenbock (Addgene plasmid # 13031). pcDNA3-EGFP was amplified in the DH5α strain of *E. coli* and plasmid was extracted with Invitrogen's plasmid DNA purification kit (Carlsbad, USA) following the manufacturer's instructions. 1 kb plus DNA ladder was purchased from ABM (Vancouver, Canada). Cholesterol and DDAB were purchased from Sigma-Aldrich Chemical Company (St.Louis, USA). Esterquat was provided from Gerbu Biotechnik (Gaiberg, Germany). Lecithin was obtained from Applichem GmbH (Darmstadt, Germany). L929 and HEK293 were purchased from the American Type Culture Collection (Virginia, USA). All other chemicals used in this study were of analytical grade. Ultrapure nuclease-free water (UPH2O) was used in all stages needed.

4.1. Preparation of liposomes

Totally, four formulations were developed; formulations 1 and 2 were prepared with DDAB and formulations 3 and 4 with EQ as cationic lipids. Both liposomes contain cholesterol and lecithin and were prepared using the method of film-hidration [2,8]. Briefly, neutral and cationic lipids were dissolved in 5 mL chloroform. The solution was then evaporated for 30 min at 45°C. Under N_2 flow, the residual solvent was

evaporated. The formed thin film layer was rehydrated in ultrapure H_2O. The suspension was then sonicated for 15 min by prob type sonicator (Bandelin sonoplus, HD2070, Berlin, Germany). The final concentration of neutral lipids was 1.5 mg/mL for all formulations.

4.2. Characterization studies

The developed liposome formulations were characterized in terms of surface charge, particle size, and polydispersity index via DLS method (NanoZS, Malvern, UK). DLS measurements were reported as averaged intensity weighted distribution for particle size measurements. The refractive index of the cholesterol (1.525) as a main ingredient of the lipid matrix was used for DLS calculations [26]. For morphological examinations of the formulation Lipo-2E, Carl ZEISS Sigma 300 VP SEM (ZEISS Group, Germany) was employed. The samples were coated under a high vacuum with a conductive layer of gold using Quorum Q150R ES (Quorum Technologies, UK) prior to analysis. Then, SEM analysis was performed by an InLens secondary electron detector operating at 2.00 kV in The Central Research Laboratory of Izmir Katip Celebi University, Turkey.

4.3. Preparation of liposome:pDNA complexes

Liposome:pDNA complexes were formed by electrostatic interactions between cationic charged liposomes to anionically charged pDNA. To determine the optimum complex formation ratio, increasing volume of liposomes to constant volume pDNA were examined (0.5:1, 1:1, 2:1, 3:1, 4:1, v/v). For this purpose, the specified proportions of liposomes were added into the pDNA solution (100 ng) and allowed to incubate at 25^{o}C for 30 min on a shaker to complete the complex formation. In order to load the complexes into the wells on the gel, glycerol (2%) was added to each sample. Agarose gel electrophoresis (1% agarose in 1xTAE, w/v)) was performed for 50 min at a constant voltage of 100 V. Following the electrophoresis, the gel was stained in 0.5 µg/ml solution of ethidium bromide. The pDNA migration pattern was then visualized under a UV transilluminator (GEN-BOX, imagER CFx ER Biyotek, Turkey). Naked pDNA was used as a control.

4.3. DNase I protection study

In vitro DNase I protection analysis was performed to determine the protection capability of liposome:pDNA complexes against serum nucleases.. For this purpose, the complexes were prepared as explained above. Then, 0.4 IU DNase I (New England Biolabs, USA) was added per each 1 µg DNA and incubated at 37^{o}C for 30 minutes in an incubator. Following the incubation, SDS (1%) was added to release the DNA for analysis and incubated for a further 5 min at room temperature. pDNA degradation was observed by agarose gel electrophoresis. The integrity of pDNA was compared with the naked pDNA exposed to DNase I during the same period.

4.4. Cytotoxicity evaluation of Liposomes

The cytotoxicity studies were performed on L929-mouse fibroblast cell line [27]. L929 is one of the suggested cell line to investigate the biocompatibility and cytotoxicity of the compounds and delivery systems in the literature [14,28,29]. For this purpose, the cells were cultured in complete media containing Dulbecco's Modified Eagle's Medium (DMEM, low glucose) with 10% fetal bovine serum (FBS) and penicillin–streptomycin (100 UI/ml penicillin, 100 µg/ml streptomycin) to avoid bacterial contamination. At the beginning of the cytotoxicity experiment, the L929 cells were seeded into 96 well plate at the concentration 1x10^{4} cells/well and incubated overnight in a 5% CO_2 atmosphere incubator at 37 °C. Then, the medium was replaced with fresh DMEM that contained liposomes at increasing concentrations (3, 6, 9, 12, 15 µL/well for Lipo-1D and Lipo-2D; 0.3, 0.6, 0.9, 1.2 and 1.5 µL/well for Lipo-1E and Lipo-2E) and incubated for a further 24 h. The percentage of viable cells was evaluated according to Alamar Blue cell viability assay kit at the end of the incubation period (Thermo Fisher Scientific, USA) by using CLARIOstar Plus multimode plate reader (BMG Labtech, Germany). Viability of cells was calculated by normalizing the fluorescence of untreated cells. Experiments were performed at least in quadruplicate.

4.5. Transfection study

The transfection ability of formulations with low cytotoxicity was evaluated by transfecting pcDNA3-EGFP to HEK293 cell line. In order to perform a transfection study, the HEK293 cells were plated in 12 well plates as 5x10^{4} cells/well and incubated until the cells reached 70% confluency. Hence, the medium was removed and cells were washed twice with PBS (pH 7.4). Following 500 µL of fresh growth medium addition, the liposome: pDNA complexes (equivalent to 2.5 µg of pDNA) was added onto the cells and incubated at 37 °C for 4 h in a 5% CO_2 atmosphere. At the end of that period, the medium containing the liposome:pDNA

complexes were aspirated and fresh growth medium was added. The cells were incubated for a further 48 h to allow GFP gene expression. Transfection ability was then visualized by fluorescence microscope (Olympus, Japan).

4.6. Stability

The stability of Lipo-2E formulation was followed based on particle size, PDI, and zeta potential data during 3 months. The measurements were performed on days 7, 15, 30, 60, and 90 by DLS method (Nano ZS Zeta sizer, Malvern Instruments Ltd., UK). The formulation was stored at 4 °C during this period.

4.7. Statistical analysis

GraphPad Prism 6.0 (GraphPad Software, Inc., USA) was used for statistical analysis. Data are expressed as means ± SEM. A non-paired t-test and one-way ANOVA followed by multiple comparison tests were used to evaluate the statistical analysis between the groups. The difference was considered statistically significant when the "p" value was calculated less than 0.05.

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