DEVELOPMENT OF SOLID LIPID NANOPARTICLES AS GENE DELIVERY SYSTEM

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ABSTRACT

As a natural result of the progresses made in the recombinant DNA technology, the gene treatment has been brought forward for the improvement of genetic disorders. The purpose of the gene therapy is the treatment of the infection and genetic disorders with the introduction of new genetic materials among cells taken from the body. The objective of our study is to develop a new gene carrier system that can bind the DNA through electrostatic interaction by cationic solid lipid nanoparticulate (SLN) system with the help of a cationic lipid. In preparation of the SLNs, two different lipids including mono-, di- and triglyceride mixtures (Gelucire 50/13 Pastilles) and glycerol distearate (Precirol ATO 5) were selected as lipid matrixes. The particle sizes of the prepared cationic solid lipid particles were found to be between 50.55 ± 0.12 and 168.2 ± 0.35 nm. Their zeta potentials which constitute of an important characteristic for the stability were determined to be between -50.2 ± 0.16 and +37.1 ± 0.63 mV. Increasing the ratio of cationic lipids in SLN have been observed to increase the zeta potentials and the toxic properties on HEK 293 cells of SLNs. The DNA binding abilities of SLNs were found to be varied.

Key words: Solid lipid nanoparticle (SLN), gene delivery system, cationic carrier system.

Kati Lipit Nanopartiküllerin Gen Taşıyıcı Sistem Olarak Geliştirilmesi

Rekombinant DNA teknolojisindeki ilerlemelerin doğal sonucu olarak, kalıtsal hastalıkların iyileştirilmesi için gen tedavisi gündeme gelmiştir. Gen tedavisinde amaç, vücuttan alınan hücreler içinde yeni genetik materyalin tanıtılmasıyla, enfeksiyon ve genetik hastalıkların tedavisidir. Çalışmamızın amacı, katı lipit nanopartiküler (KLN) sisteme, katyonik bir lipit yardımıyla katyonik özellik kazandırılarak, DNA'ya elektrostatik etkileşme ile bağlanabilecek yeni bir gen taşıyıcı sistem geliştirmektir. SLN'lerin hazırlanmasında lipit matris olarak mono-, di- ve trigliserid karışımı (Gelucire 50/13 Pastilles) ve gliserol distearat (Precirol ATO 5) olmak üzere farklı iki lipit seçilmiştir. Hazırlanan katyonik katı nanopartikülleri, partikül boyutu 50.55 nm ve 168.2 ± 0.35 nm aralığında bulunmuştur. Kararlılıkları için önemli özellik olan zeta potansiyelleri ise -50.2 ± 0.1 ile +37.1 ± 0.63 mV aralığında belirlenmiştir. Katyonik lipidin oranının arttırılması ile SLN'lerin zeta potansiyelinde ve 293 HEK hücrelerinde toksik özelliğinin arttığı gözlenmiştir. SLN'lerin DNA bağlama yetilerinin farklılık gösterdiği belirlenmiştir.

Anahtar Kelimeler: Katı lipit nanoparltikül (KLN), gen taşıyıcı sistem, katyonik taşıyıcı sistem

INTRODUCTION

The studies on gene treatment are promising in the treatment and prevention of the genetic and infectious diseases as well as the cancer because of the recent developments in the field of the molecular biology (1). In the process to date, the conventional use of drugs in the treatment of cancer and viral diseases are now being replaced with the gene therapy. This type of treatment aims to deliver the corrected genes to the patients or to correct the patient's genes. The gene therapy is defined as transferring the genetic material to the specific cells to create a therapeutic impact (2). The replacement of the damaged genes, putting back the lost genes or the disease treatments by means of silencing the gene definitions have steered many studies in recent years with the gene therapy that declared as the future revolution of the modern medicine (3). The applications of gene therapy are either divided as somatic and germline gene therapy in terms of the target issue, or as ex vivo and in vivo gene therapy in terms of its administration to the patient (4).

For the ex vivo gene therapy, the cells are taken out from the affected tissue, and genetically straight genes are transferred to these cells within a laboratory conditions. After the generation of these cells, the treated cells are picked up from other cells, and applicated to the patient via vaccination or transplantation methods. Using the patient's own cells, no immunological impacts are seen following the vaccination or transplantation (4).

For the in vivo gene therapy, it is tried to be performed through the direct transfer of the healing gene to the patient's cells in the body, without removing the sick cells, with the help of vectors (delivery systems) (5).

There needs to be a suitable genetic carrier for the transfer of the gene. For this purpose, viral and nonviral vectors were used for gene delivery (6). It has been demonstrated that the viral vectors have a much higher transfection impact in many cells compared to the non-viral vectors. However, as the viral vectors have a mutation, a recombination or an oncogenic impact and high costs, the studies on the non-viral vectors have been increased (7).

The non-viral vectors are such systems that can carry a gene material that has a low immune response, ease of synthesis, and an unlimited size (8). The mostly studied non-viral vectors are liposomes and nanoparticulate systems. These are polymeric, lipidic, and peptide-protein structures allowing for simple packaging of genetic material, majority of which have cationic characteristics, and enable the transfection of the genetic material into the cell (9).

In our study, the solid lipid nanoparticles (SLN) were selected as non-viral vectors. At the preparation stages, the cationic lipid (octadecylamine) added into the matrix lipid brought a

positive characteristics to the negative charge SLNs (10, 11). It is known that the positive charge SLNs and pDNA electrostatically interact with each other and create a complex (12). The SLNs are offered as an alternative drug delivery systems to the liposomes, polymeric nanoparticles, and emulsions (13). SLNs are similar to the emulsions and liposomes, and consist of such materials that can be physiological well-tolerated (14).

The objective of this study is to prepare the nano-size DNA binding solid lipid nanoparticulate systems so as to be used in gene treatment, and to determine their characteristics and toxic effects.

METHODS

Materials

As solid lipids, three lipids with different melting points (50 °C and 56 °C) including mono-, di- and triglyceride mixture (Gelucire 50/13 Pastilles) and glycerol distearate (Precirol ATO 5) were supplied from Gattefosse. As the surfactants, polyoxyethylene-20-sorbitan monooleate (Tween 80, Fluca) and Sorbitan Trioleate (Span 85, Sigma) were used. As cationic agent, the stearylamine (octadecylamine, Fluka) was chosen. The Plasmid DNA (MB113, Pharm. Dev) was supplied by Cambio Ltd. and the gWiz™ GFP plasmid used for transfection was obtained from Aldevron. To determine the cytotoxicities and transfection activity of the formulations, there was studied on Human Embryonic Kidney 293 (293 HEK) cells (ATCC).

Preparation of SLNs

In preparation of the cationic SLNs, the simple technique of generating oil-in-water emulsion was used. The lipids used in the preparation of SLNs each containing cationic lipid, were melted at about 10 °C above the melting point of each lipid and added to the hot aqueous surfactant solutions that were heated to same degree as lipids. The molten lipids were added in the hot surfactant solutions and the mixture was sonicated for 2 min at 20 % power using a sonicator (Sonics, USA). The codes and contents of the formulations are provided in the **Table 1**.

Table1. The codes and contents of the SLNs

Codes	**G**	**GI**	**GII**	**GIII**	**GIV**	**P**	**PI**	**PII**	**PIII**	**PIV**
	Gelucire 50/13					**Precirol ATO 5**				
Solid Lipid **(% w/w)**	4	4	4	4	4	4	4	4	4	4
Cationic Lipid **(% w/w)**	-	0.25	0.30	0.35	0.50	-	0.25	0.30	0.35	0.50
Tween 80® (% w/w)	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8

Span 85® (% w/w)	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Heating Degrees	60 ± 1°C					66± 1°C				

Characterization of SLNs

Particle size and zeta potential

Mean diameter of the bulk population and the particle distribution via the PI and zeta potentials of SLNs analyzed by a Zetasizer NanoZS (Malvern Instruments, UK). Distilled water with a conductivity value of 50 µS/cm was adjusted using sodium chloride (0.1 N) at pH 7.4 and used in zeta potential analyses. Electrostatic mobility was converted to zeta potential using the Helmholtz Smoluchowski equation. For this purpose, 50 µL of the SLNs were dispersed in 1 mL of this distilled water and particle size, PI, and zeta potentials were determinated.

Gel Retardation

Gel retardation studies were used to determine DNA binding ratio of SLNs. Different ratios of pDNA/SLN (µg/µL) complexes were prepared and loaded on an agarose gel and visualized by 1.5 % ethidium bromide staining for 2 h at 25 V. Images were obtained using Kodak Image Station 440 CF (USA).

Cytotoxicity of SLNs

Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method was used for the quantitative determination of cell cytotoxicity. 293 HEK cell line was used in the method. Briefly, 2×10^4 cells were seeded within a 96-well plate (Greiner, Sigma-Aldrich). Cells were incubated at 37°C for 24 hours in a humidified atmosphere containing 5% CO_2. After 24 hours of incubation, supernatant of each well was replaced with different concentrations of formulations, and the plates were incubated at 37°C for another 4 hours. Then the medium was withdrawn, fresh medium was added to cells, and the plates were re-incubated for 48 hours. At the end of the incubation time, supernatant of each well was replaced with 20 µL MTT dye (Sigma-Aldrich) (diluted with PBS for 5 mg/mL concentration) solution and the plates were incubated in the same previous conditions for 4 hours. 200 µL spectrophotometric DMSO was then added to each well to dissolve formazan crystals. After 30 min of incubation, absorbance of the plate was measured at 570 nm using a spectrometric microplate reader (Perkin Elmer Victor X5, England).

Transfection of SLNs

To determine the transfection efficacy of lipidic particles, gWiz™ GFP plasmid that encodes the gene for the Green Fluorescent Protein (GFP) was used as pDNA. 293 HEK cell lines were seeded with antibiotic-free medium in a 6-well plate and incubated at 37°C for 24 h with 5 % CO2 until the cell intensity elevated to 60-70 %. gWiz™ GFP plasmid/PP (2 µg) 25 µL SLNs were incubated at room temperature for 30 min to maintain adsorption of negatively charged pDNA on positively charged particles. Than these complexes were diluted with 1 mL antibiotic/FBS-free medium and added drop-wise to the cells. After 48 h incubation, 20 different areas were selected among the all wells for counting both the transfected and non-transfected total cells (10). Transfection efficiency (TE) was calculated using following Equation (1).

$$TE\ (\%) = \frac{\text{Number of transfected cell}}{\text{Number of transfected cell} + \text{Number of nontransfected cell}} \times 100 \qquad \text{(Eq.1)}$$

RESULTS

Particle size and zeta potential

Mean particle size, particle sizes distributions and zeta potentials of the SLNs are given in **Table 2**.

Table 2. Mean particle sizes and particle size distributions of the SLNs.

	G	GI	GII	GIII	GIV
PS (nm) ± SE	50.55 ± 0.12	87.11 ± 0.34	61.22 ± 0.19	52.56 ± 0.21	51.66 ± 0.34
PI ± SE	0.670 ± 0.134	0.585 ± 0.231	0.492 ± 0.213	0.476 ± 0.019	0.568 ± 0.126
ZP (mV) ± SE	-26.3 ± 0.12	15.9 ± 0.03	19.5 ± 0.10	20.2 ± 0.14	20.2 ± 0.27
	P	**PI**	**PII**	**PIII**	**PIV**
PS (nm) ± SE	110.8 ± 0.11	149.5 ± 0.09	133.4 ± 0.32	153.4 ± 0.46	168.2 ± 0.35
PI ± SE	0.409 ± 0.263	0.250 ± 0.248	0.272 ± 0.546	0.297 ± 0.265	0.398 ± 0.154
ZP (mV) ± SE	-50.2 ± 0.16	10.4 ± 0.51	27.1 ± 0.27	30.5 ± 0.26	37.1 ± 0.63

Experiments were carried out in triplicates. PS; Particle size, PI; Polydispersity index, ZP; Zeta potentials and SE; Standard error.

Gel Retardation

Codes of the SLNs and ratios of pDNA/SLN (µg/µL) complexes are given in **Table 3-5.** Complete binding of pDNA with SLNs was determined by the absence of free DNA bonds on the gel images in **Figure 1-3**.

Table 3. Ratios of SLN:pDNA (µL/µg) and application area of GI and PI coded SLNs/pDNA complexes on the gel.

pDNA	GI				PI			
-	I	II	III	IV	I	II	III	IV
2µg	1:1	1:2.5	1:4	1:5	1:1	1:2.5	1:4	1:5
	Ratios of SLN:pDNA (µL/µg)							

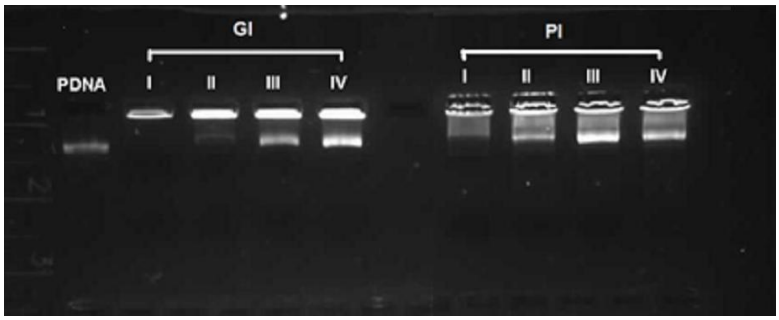

Figure 1. Gel image of GI and PI coded SLNs/pDNA complexes.

Table 4. Ratios of SLN:pDNA (µL/µg) and application area of GII,GIII and GIV coded SLNs/pDNA complexes on the gel.

pDNA	GII				GIII				GIV			
-	I	II	III	IV	I	II	III	IV	I	II	III	IV
2µg	1:1	1:2.5	1:4	1:5	1:1	1:2.5	1:4	1:5	1:1	1:2.5	1:4	1:5
	Ratios of SLN:pDNA (µL/µg)											

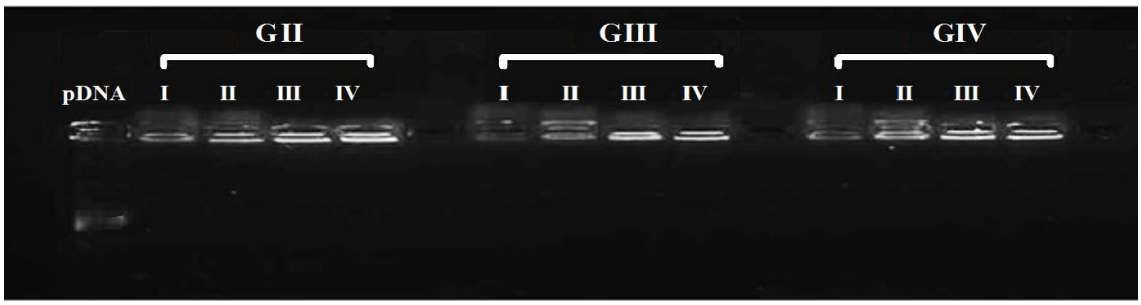

Figure 2. Gel image of GII,GIII and GIV coded SLNs/pDNA complexes.

Table 5. Ratios of SLN:pDNA (µL/µg) and application area of PII, PIII and PIV coded SLNs/pDNA complexes on the gel.

pDNA	PII				PIII				PIV			
-	I	II	III	IV	I	II	III	IV	I	II	III	IV
2µg	1:1	1:2.5	1:4	1:5	1:1	1:2.5	1:4	1:5	1:1	1:2.5	1:4	1:5
	Ratios of SLN:pDNA (µL/µg)											

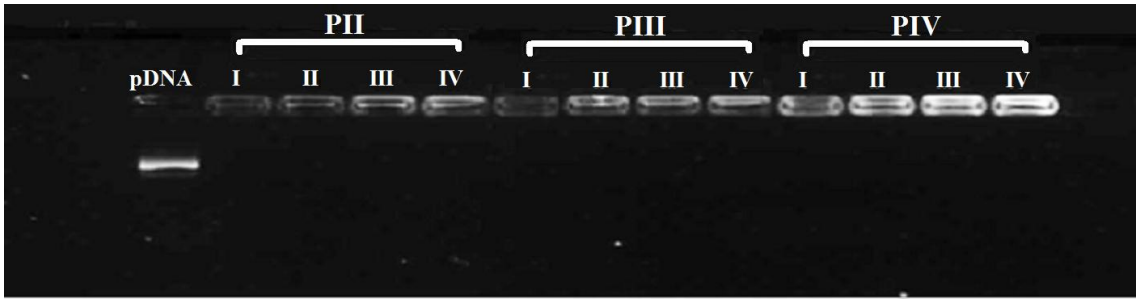

Figure 3. Gel image of PII, PIII and PIV coded SLNs/pDNA complexes.

Cytotoxicity of SLNs

Cell culture studies were performed using G, GI, P and PI coded dispersions for the determination of cytotoxic effects. Toxic effects of different amount SLNs (without pDNA) on 293 HEK cell line after 48 hours were evaluated in comparison. Cell viability/concentration curves of SLNs are given in **Figures 4 and 5**.

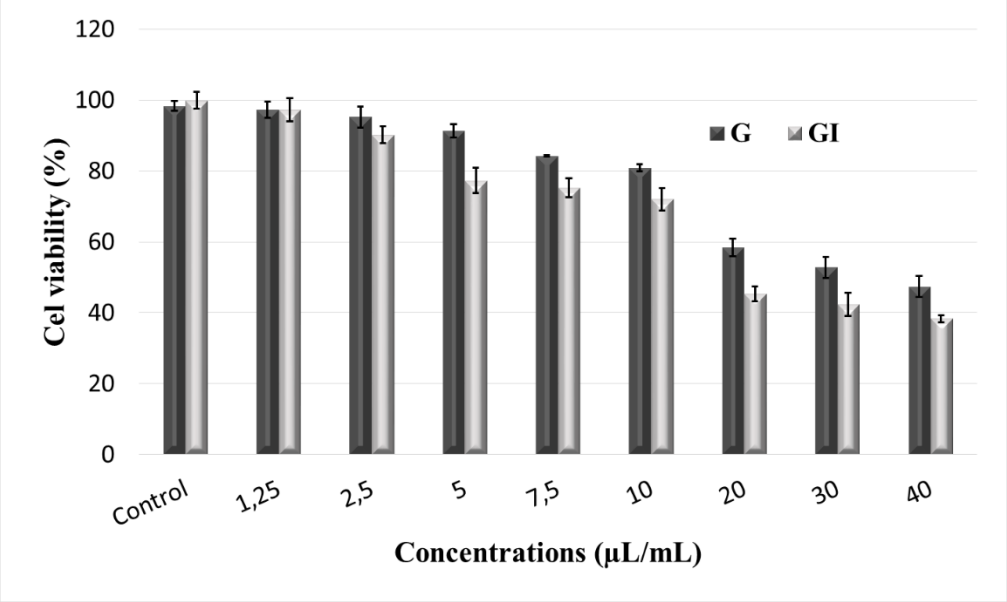

Figure 4. Viability of 293 HEK cells after being treated with G and GI coded SLNs. Each condition was tested in eight replicates for 48 hours.

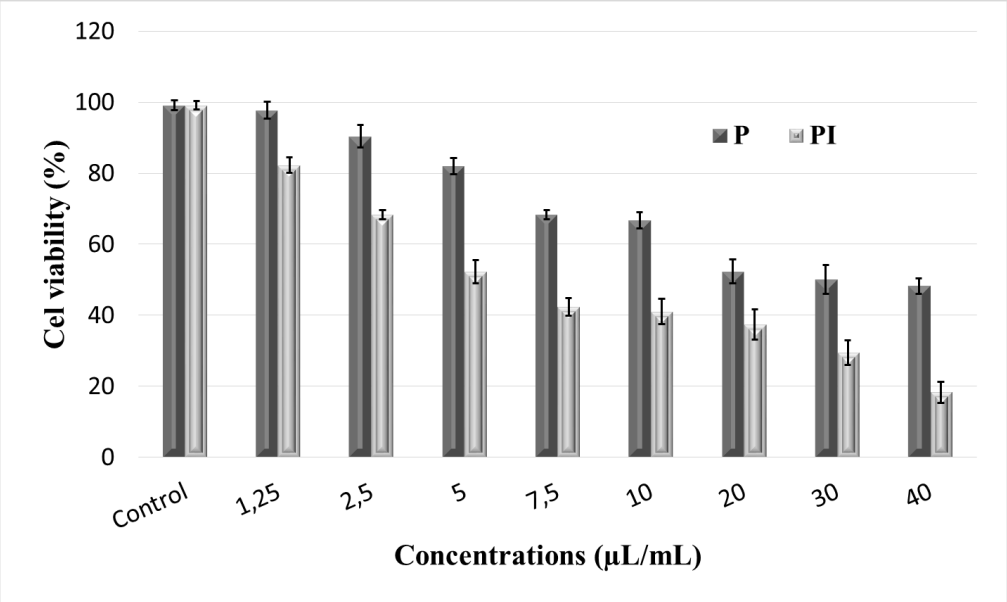

Figure 5. Viability of 293 HEK cells after being treated with P and PI coded SLNs. Each condition was tested in eight replicates for 48 hours.

Cellular uptake

Uptake of the lipidic particles in 293 HEK cells was determined using gWiz™ GFP plasmid adsorbed formulation and investigated under fluorescence microscope (Figure 6). According to

the transfection results obtained on 293 HEK cells by counting the cells at the end of 48 hours, transfection ratios determined using Eq. 1 were 48 % for PI and 63 % GI respectively.

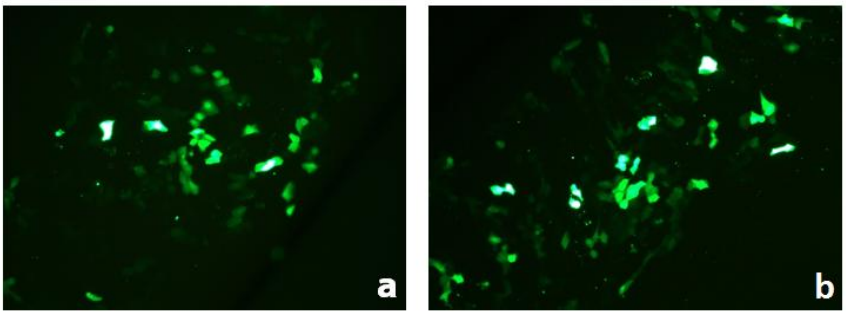

Figure 6. Fluorescent images demonstrating of gWiz™ GFP plasmid delivery

to the 293 HEK cells with PI (a) and GI (b).

DISCUSSION

The particle size of DNA-delivery system complexes is one of the critical parameters in the preparation of the lipid complexes and liposomes (15). In the studies carried out, it has been suggested that the size is really important for the cells and tissues to up take the particles (16). The particle sizes of the SLNs were found to be between 50.55 nm and 168.2 ± 0.35 nm. It was observed that the particle sizes of the formulations prepared using Gelucire were much less than those of the PI SLNs prepared with Precirol ATO5. With the increased amount of the cationic lipid in the formulations, it was also observed that the particle sizes of the SLNs also increased. This results show that the increased lipid amount in the formulations results in the enlarged particle size (17).

The zeta potential is, in practice, a carefully considered measure in the assessment of the suspensions (18). To ensure the stability of prepared formulations, the particles needs to have a certain zeta potential in order to be able to create electrostatic interaction between pDNA and particles, and to bind the complex created as a result of such interaction to the cell membrane (19, 20).

In a study carried out, it has been determined that the SLNs have a negative zeta potential without the addition of cationic lipids (Table 2). It was also observed that the zeta potential of the particles increased depending on the increased cationic lipid amount.

In the formulations prepared using Precirol as solid lipid, it was determined that the zeta potential has a higher increase rate. This result made it easier to electrostatically bind the formulations to the pDNA.

It was observed on the gel images achieved that all the formulations containing 0.30%, 0.35%, and 0.50% cationic lipids can be completely bound to various ratios of pDNA (Figure 2 and 3).

As the formulations containing 0.25% cationic lipid have low zeta potential values, it was determined that the pDNA binding rates of the particles decreased depending on the increased pDNA amount. When these formulations were compared among themselves, it was determined that the PI coded SLN have a low rate of pDNA binding compared to the GI coded SLNs (Figure 1).

In toxicity studies, it was determined that the SLNs not containing any cationic lipids when the applied above the concentration of 20 μL/mL had toxic characteristics. It was observed that the G formulation is much toxic compared to the P formulations (Figure 4 and 5).

It was observed that the cytotoxicity of the SLNs, which have positive characteristics by adding cationic lipids increased (Table 2). It is thought that the cytotoxicity increased because of the toxic characteristics (8, 21) of cationic type lipids which contain amine group. It was found that the PI coded formulation shows a quite higher cytotoxicity compared to the GI coded formulation. It was found that GI coded SLN lowers down the cell vitality under 50% at a concentration of 20-40 μL/mL, while PI coded formulation lowers it down under 50% at a concentration of 7.5-40 μL/mL.

Considering the cytotoxicity results of the SLNs containing 0.25% cationic lipid, it was not deemed necessary to carry out cytotoxicity studies on the SLNs containing 0.30%, 0.35%, and 0.50% cationic lipids. It was found out in the results obtained that the cytotoxicity ratio would increase with the increased cationic lipid amount.

CONCLUSIONS

Considering all the data as obtained, it was observed the particle sizes, zeta potentials, and toxic effects of the SLNs prepared with the increased cationic lipid amount increased. It is thought that although the increased zeta potential as observed is superior in pDNA binding ability, the formulations (GI and PI) containing the lowest rate of (0.25%) cationic lipid may be appropriate gene carrier systems because of the toxic effect of cationic lipids. It is also thought that the genetic materials with different characteristics can be adsorbed onto these systems, and they can be used after having been optimized for different therapeutic purposes such as DNA vaccinations, treatment of cancer and genetic diseases, and antisense technology.

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