Preparation and characterization of non-viral gene delivery systems with pEGFP-C1 Plasmid DNA

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In recent years, non-viral delivery systems for plasmid DNA have become particularly important. They can overcome the disadvantages of viral systems such as insertional mutagenesis and unpredicted immunogenicity. Some additional advantages of non-viral gene delivery systems are; good stability, low cost, targetability, delivery of a high amount of genetic materials. The aim of the study was to develop novel non-viral nanosystems suitable for gene delivery. Two formulations were developed for this purpose: water-in-oil microemulsion (ME) and solid lipid nanoparticles (SLN). The microemulsion was composed of Peceol, Tween 80, Plurol oleique, ethanol and water. The SLN was consisting of Precirol, Esterquat-1 (EQ1), Tween 80, Lecithin, ethanol and water. Characterization studies were carried out by measuring particle size, zeta potential, viscosity and pH. TEM imaging was performed on SLN formulations. Protection against DNase I degradation was examined. Cytotoxicity and transfection efficacy of selected formulations were tested on L929 mouse fibroblast cells. Particle sizes of complexes were below 100 nm and with high positive zeta potential. TEM images revealed that SLNs are spherical. The SLN:DNA complexes have low toxicity and good transfection ability. All results showed that the developed SLN formulations can be considered as suitable non-viral gene delivery systems.

Keywords: Microemulsion. Solid lipid nanoparticle. DNA delivery. Transfection.

INTRODUCTION

Gene therapy using plasmid DNA molecules for specific diseases gained great importance in last decades. The main purpose of gene therapy is replacing diseased genes with new healthy ones. Selection of a proper gene delivery system is a very important issue when considering gene therapy because naked plasmid DNA (pDNA) is rapidly degraded following administration to the human body (Park *et al.,* 2015). There are several methods developed for gene delivery. Generally, these methods can be divided into two main groups; 1) methods applying viral delivery systems, 2) methods applying non-viral delivery systems. Viral gene delivery systems have been the main choice during early gene therapy trials (Jomary *et al.,* 1994; Edelstein *et al.,* 2004). However, there were important drawbacks concerning the safety of these systems. For example, unpredicted immunogenicity and insertional mutagenesis are very dangerous adverse reactions that can develop after the treatment (Hackett *et al.,* 2013; Solinís *et al.,* 2014). Because of these complications, researchers developed non-viral gene delivery systems. Nowadays in gene delivery trials non-viral delivery systems are preferred over viruses because of their safety (Fornaguera *et al.,* 2015). In addition, these systems possess other advantages such as good stability, targetability to specific sites, ability to deliver a high amount of materials, high therapeutic efficacy and low cost. However, non-viral vectors possess some disadvantages too. One of these disadvantages is that non-viral systems exhibit lower delivery efficacy than viral vectors (Mellott *et al.,* 2013). Also, another important issue is their low transfection efficiency. Challenges in preserving the DNA intact during transportation in the biological fluids, low rate of endosomal escape and poor nuclear entry are known to contribute to the lower transfection efficiency of non-viral gene delivery systems (Glinka, 2012).

In recent years, lipid nano-carriers such as liposomes, MEs and SLNs gained attention as therapeutic

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Braz. J. Pharm. Sci. 2018;54(1):e00265

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gene delivery systems. SLNs have a particle size distribution varying from approximately 50 nm to 1000 nm (Carrillo *et al.,* 2013). Cationic SLNs are especially promising systems for gene delivery purposes (Severino *et al.,* 2015). They are good alternatives to conventional formulations such as nanoemulsions and liposomes for gene delivery because of their good physical stability and biocompatibility (Jin, Kim, 2014). It is known that positively charged nanoparticles are most likely to be taken by cells via electrostatic interaction with negatively charged cell membranes (Jin, Kim, 2014; Remaut *et al.,* 2014). They are easily and cost effectively produced in large scale, and easy to generate. To transfect cells, these systems need a positive surface charge which can be provided by adding cationic lipids such as etylpyridinium chloride, cetyltrimethylammonium bromide (CTAB), dimethyldioctadecylammonium (DDAB), N-[1-(2,3-dioleoyloxy)-propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP) and esterquat-1 which are some of the most widely used cationic lipids for obtaining cationic SLNs (Severino *et al.,* 2015). The advantages of cationic SLNs are their good biocompatibility and low toxicity. These properties make them suitable for application by various routes (nasal, oral, ocular, topical, intravenous etc.) (Fàbregas *et al.,* 2014). Another important advantage of SLNs is the ability to be produced from hot microemulsion intermediates.

MEs are thermodynamically stable colloidal systems that form spontaneously under certain ratios of ingredients. MEs contain at least three main components; oil, emulsifier and water. MEs are easy to produce because of the self-assembling ability of the components. The most important property of MEs is to provide protection of encapsulated compounds (Chatzidaki *et al.,* 2015). Water droplets formed in water-in-oil (W/O) microemulsions are good candidates for confining DNA. Because of DNA's high water solubility, it can be easily incorporated into the inner water phase of a W/O microemulsion (Swami *et al.,* 2008). The high viscosity of W/O MEs increases the stability and makes them suitable for topical application (Kajbafvala *et al.,* 2016; Callender *et al.,* 2017). Chitosan is a widely used excipient in pharmaceutical formulations. It is regarded as non-toxic and biocompatible material (Shaji, Jain, Lodha*,* 2010) and is widely used as a DNA condensing cationic polymer.

The first aim of this study was to develop and characterize a non-toxic solid lipid nanoparticle system employing hot o/w microemulsion dilution technique. The second aim was to prepare and characterize a w/o microemulsion formulation for plasmid DNA delivery.

MATERIALS AND METHODS

Materials

Trizma base, Ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), Didecyldimethylammonium bromide (DDAB), Cetyl trimethylammonium bromide (CTAB), Tween 80, Chitosan (medium molecular weight), Phenol, 1-propanol and Span 80 were purchased from Sigma Aldrich GmbH (Steinheim, Germany). Soybean Lecithin (90% phosphatidylcholine) and Phosphate-buffered saline (PBS) tablets (pH: 7.4) were purchased from AppliChem GmbH (Darmstadt, Germany). XTT cell proliferation kit, Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, L-glutamine and penicillin-streptomycin were purchased from Biological Industries (Beit-HaEmek, Israel). Precirol ATO-5, Labrafac PG, Labrafac Lipophile, Plurol Oleique CC, Lauroglycol FCC, Lauroglycol 90, Capryol PGMC, Labrafil M and Peceol were donated by Gattefossé (Cedex, France). DNase I (Enzyme I) was purchased from Biolabs (New England, USA). Ethanol was purchased from Merck KgaA (Darmstadt, Germany). Glacial acetic acid was purchased from Atabay Kimya (İstanbul, Turkey). Esterquat-1 (N,N-di-(β-stearoylethyl)-N,N-dimethyl ammonium chloride) was purchased from Gerbu Biotechnik GmbH (Heidelberg, Germany). Agarose was purchased from Conda Laboratories (Madrid, Spain). Ethidium bromide was purchased from Fluka Chemie GmbH (Steinheim, Germany). pEGFP-C1 plasmid DNA and maxiprep plasmid DNA purification kit were purchased from Invitrogen (Carlsbad, CA, USA). DNA molecular weight standards (100bp and 1000bp) and DNase I (Enzyme II) were purchased from Fermentas, ThermoFischer Scientific (Waltham, Massachusetts, USA).

Plasmid DNA

pEGFP-C1 from Invitrogen (Carlsbad, CA, USA), which encodes green fluorescence protein (GFP) under CMV promoter, was used as the model plasmid in this study. pEGFP-C1 plasmid DNA was amplified in *Escherichia coli* JM109 strain and purified with Invitrogen maxiprep plasmid DNA purification kit (Carlsbad, CA, USA) according to manufacturer's instructions.

The integrity of the plasmid was assessed by restriction enzyme digestion, followed by agarose gel electrophoresis.

The purity and the concentration of the plasmid were measured with nanoVette UV/Vis spectrophotometer

by measuring the absorbance of samples at 260/280 nm wavelengths. After isolation and purification, the plasmid DNA was diluted to 100 µg/mL and stored at -20 °C until use.

Preparation of formulations

Preparation of SLN formulations

SLNs were produced by using hot microemulsification method. Microemulsions were obtained with Precirol ATO-5 as the solid lipid, Tween 80 and Soybean lecithin as hydrophilic and lipophilic surfactants, respectively, and ethanol as the cosurfactant (Cos). Tween 80 and lecithin were used at a ratio of 2:1 (wt/wt) and represented the surfactant (S) phase, and the S/Cos ratio was 1:2 (wt/wt). For obtaining cationic SLNs either Esterquat-1 or CTAB were included in the composition of formulations.

Briefly, Precirol (100 mg), S/Cos (1440 mg), cationic lipid (56 mg) and water (2400 mg) were weighed and mixed with magnetic stirrer at 1500 rpm. The mixture was heated at 10 °C above the melting point of Precirol ATO-5 and a transparent hot microemulsion was obtained. Subsequently the microemulsion was rapidly transferred into 20 ml cold water and SLN dispersion was formed (Kotmakçı *et al.*, 2016).

Preparation of W/O ME formulation

Following materials were selected to construct pseudo-ternary phase diagram; Peceol (HLB: 1) as the oil phase, Plurol oleique (HLB: 6) and Tween 80 (HLB: 15) as surfactants and Ethanol as co-surfactant. The purpose of generating phase diagram was to find optimum concentration ranges for formulation. Oil, S and Cos mixture which consist of predetermined amounts of the ingredients was titrated by adding 10 µL portions of water each time, and the appearance of the system was visually observed. The point where the appearance of the mixture turns to blurry was noted for each sample and used for the construction of the phase diagram. Titration was performed with constant stirring rate (300 rpm) at room temperature. Optimal ME existence areas were determined with phase diagrams drawn by means of a software developed at Ege University Faculty of Pharmacy (Ege, Karasulu, Güneri, 2004).

Chitosan was used for providing cationic charge to MEs. Chitosan solution was prepared by dissolving chitosan with 1 % (v/v) glacial acetic acid (final chitosan concentration 0.3 % (wt/wt)). In order to obtain cationic ME, stable transparent w/o systems were selected from the phase diagrams and chitosan solution was added to these systems as the water phase.

Preparation of Cationic SLN:DNA complexes

To obtain the optimum complexation ratio, the following cationic SLN-to-plasmid DNA ratios were tested; 1:1, 2:1, 3:1, 4:1, 5:1, 7:1, 10:1 (v/v). Complexation was achieved at room temperature under stirring with vortex (300 rpm) for 30 minutes. Concentration of DNA working solution was 100 µg/mL.

Confinement of DNA in ME formulation

To confine the DNA in the ME formulation, first Chitosan:DNA complex was prepared. Chitosan solution (0.003 mg/mL) and DNA solution (100 µg/mL) were mixed at 1:1 (v/v) ratio and stirred with vortex (300 rpm) for 30 minutes at room temperature. Subsequently, the Chitosan:DNA complex dispersion was used as the water phase for the microemulsion.

Characterization studies

Particle size distribution

Particle size distribution of cationic SLNs was determined by dynamic light scattering using Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Low volume disposable polystyrene cuvettes were used for size measurements. Formulations were diluted 10 fold with ultrapure water and the measurements were performed at 25 °C. Particle size was measured in triplicate and mean zeta average diameter (±SD) was reported.

Zeta potential measurement

Zeta potential value of cationic SLNs was measured with Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Standard zeta cuvettes were used and the working temperature was set to 25 °C during zeta potential measurements. Values were measured in triplicate and mean value (± SD) was presented.

pH measurement

pH value of formulations was measured with SevenGo Duo SG 23 pH meter (Mettler Toledo Ohio, USA) at room temperature.

Refractive index and Viscosity measurements

Refractive index was measured with Krüss DR301-95 refractometer (Hamburg, Germany), the viscosity was measured with Brookfield SV-10 vibrating viscometer (Brookfield Engineering Labs, Inc., Middleboro, MA). Measurements were carried out at room temperature.

TEM (Transmission Electron Microscopy) analysis

The morphology of formulations was investigated with FEI Tecnai G² high contrast transmission electron microscope (FEI Co., USA). Approximately 10 µL samples were placed on carbon coated 200 mesh copper grids and allowed to dry overnight at room temperature. Observations were carried out at 120 kV.

SDS-induced DNA release study

The release of DNA from the SLNs was assessed with agarose gel electrophoresis. SDS was added immediately after complex formation. Following 5 minutes incubation at 25°C, samples were loaded on 1% agarose gel for electrophoresis. Two SLN:DNA ratios were tested - 1:1 and 1:2 (v/v). Optimal SDS amount was determined by applying the following SDS percentages to gel; 0.5, 1, 1.5, 2, 2.5.

DNase I protection study

Two different commercial DNase I enzymes were used for this study (Fermentas, ThermoFischer Scientific (Waltham, Massachusetts, USA) and Biolabs, (New England, USA)). SLN:DNA ratios were 1:1 and 1:2 (v/v). 0.4 IU DNase I was applied per each 1 µg DNA.

After complex formation DNase I was added and incubated for 30 minutes at 37°C in a heating water bath. Subsequently, SDS (%1) was added to release the DNA for analysis and incubated as described above. Samples were loaded to %1 agarose gel. Electrophoresis was carried out for 1 hour at 100 volts.

Cell culture

All cell culture studies were carried out on L929 mouse fibroblast cell line, as it is a commonly used cell line. Formulations were freshly prepared and SLN:DNA complexes were obtained immediately before the application.

Cytotoxicity study

Cytotoxicity of formulations was estimated by measuring cell viability with XTT cell proliferation assay. For this purpose, cells were inoculated in 96 well plates at a density of 6×10^3 cells/well. Plates were incubated for 24 h at 37 °C in a humidified atmosphere (%5 CO_2) to allow the cell to adhere to the wells. After 24 hours, cells were treated with SLNs and SLN:DNA complexes at the following doses; 3, 5, 7, 10, 15, 20 µL/well. Each dose was applied to the wells in a final volume of 100 µL. After another 24 hours of incubation, cells were washed with PBS and fresh medium was added. 50 µL of XTT solution was added to each well. After 150 minutes of incubation, absorbance was measured at 450-630 nm using Varioskan Flash microplate reader (ThermoFisher Scientific, USA). Untreated cells were used as control group.

Transfection ability

Doses with low toxicity (3 µL and 5 µL) were used in transfection studies. Transfection ability was evaluated under a fluorescence microscope (Olympus, Japan). In order to evaluate the transfection ability considering vitality, cells were stained with DAPI (4',6-diamidino-2-phenylindole) for 1-5 min after performing the transfection.

Stability

SLNs in suspension form were evaluated for change in particle size, zeta potential and PDI with Nicomp 380 ZLS (Particle Sizing Systems, USA) for two months. ME formulation was observed visually for clarity and phase separation for two years. Formulations were stored at room temperature.

Statistical analysis

All results are presented as mean ±SD of at least three measurements. Statistical analysis was performed using Student's t-test and analysis of variance (ANOVA). A "p" value of less than 0.05 was considered significant.

RESULTS

ME formulation

As shown in Figure 1, pseudo ternary phase diagram was constructed by titration procedure with water. Optimal S:Cos ratio was 1:1 (wt/wt). The selected microemulsion formulation is presented as a black point on the microemulsion area and consists of 28 % Peceol, 21 % Plurol Oleique, 7 % Tween 80, 28 % ethanol, and 16 % water.

DNA-SLN complexation

Figure 2 shows the agarose gel photograph of samples tested for complexation at different SLN_{EQ1}:DNA ratios.
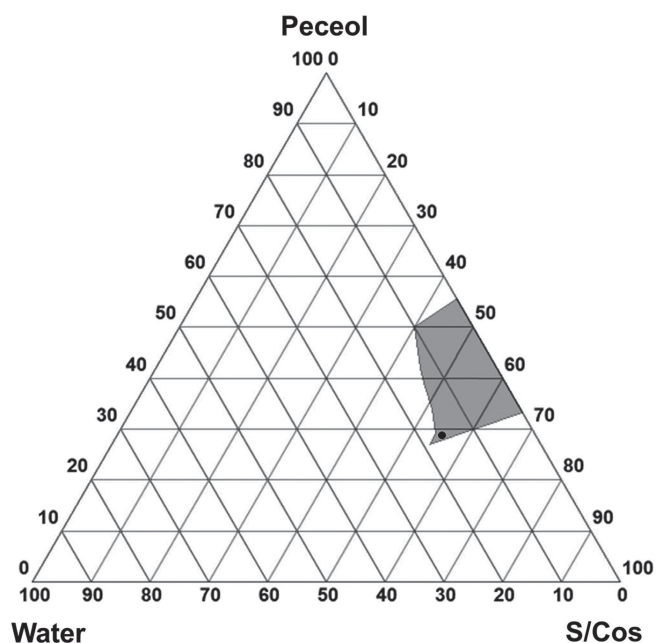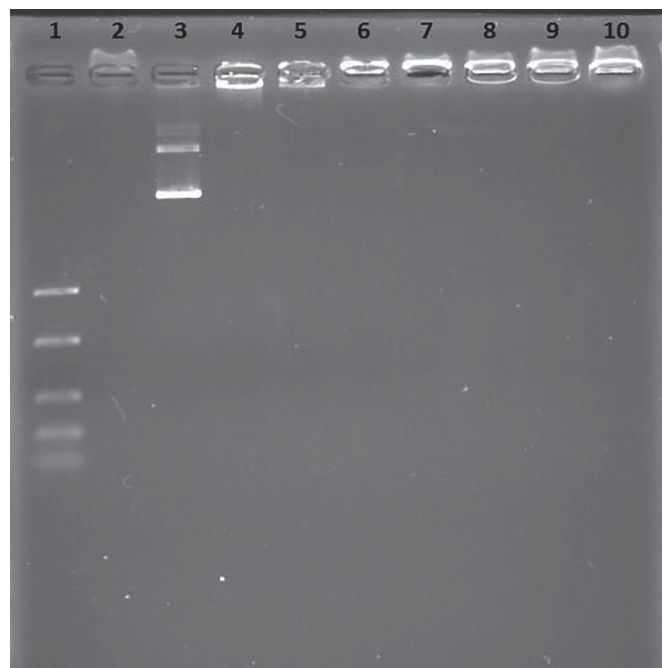
FIGURE 1 - Pseudo ternary phase diagram obtained with peceol, plurol oleique, T80 and ethanol. S/Cos ratio: (1:1). Grey area represents W/O microemulsion formation area. Black dot represents the selected W/O microemulsion

FIGURE 2 - Agarose gel photograph showing SLN_{EQ1}:DNA complexation. Lane 1: GeneRuler 1 kb DNA ladder; Lane 2: SLN; Lane 3: naked DNA as control; Lane 4: SLN:DNA 1:1; Lane 5: SLN:DNA 2:1; Lane 6: SLN:DNA 3:1; Lane 7: SLN:DNA 4:1; Lane 8: SLN:DNA 5:1; Lane 9: SLN:DNA 7:1; Lane 10: SLN:DNA 10:1.

Characterization of obtained microemulsion, SLNs and SLN:DNA complexes

pH values of SLN_{EQ1} and ME formulations were determined as 3.9 and 3.4, respectively. Viscosity and refractive index values of ME formulation were obtained as 36 cP and 1.42, respectively. Table I shows the particle size, PDI and zeta potential values of SLN formulations and SLN:DNA complexes. Figure 3 shows the intensity-size distribution of SLN:DNA formulations.

SDS induced DNA release and protection against DNase I degradation

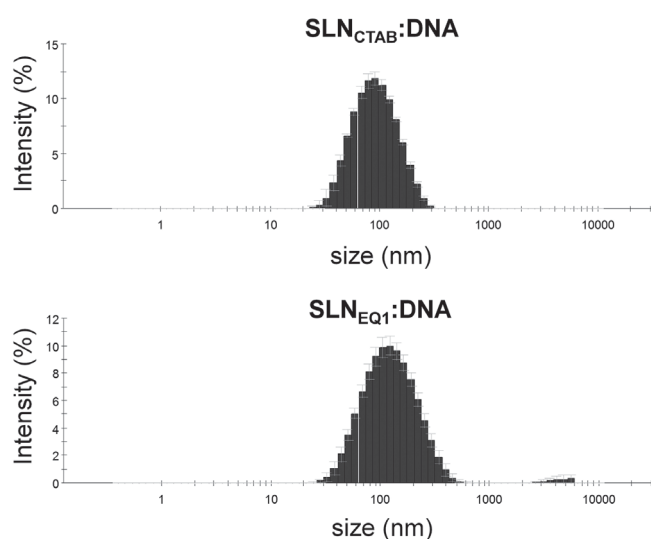For SDS induced DNA release study, SLN:DNA complexes were prepared in two ratios; 1:1 and 2:1 (v/v).

In this preliminary study, it was seen that increasing SLN ratio from 1:1 to 2:1 has no significant effect on DNA release. It was also seen that 1% SDS will be appropriate for further studies. We aimed to compare cationic SLNs prepared with different cationic lipids in terms of complexation and SDS induced DNA release. According to the (Figure 4) lane 6 and lane 7, both formulations released DNA properly in the presence of 1% SDS.

Figure 5 shows the agarose gel photograph of SLN_{EQ1} for DNase I protection study. Lane 2 is the negative control (untreated plasmid). According to lane 2, uncomplexed DNA was fully digested by the DNase I. Lanes 3-10, show the protection of SLN_{EQ1} formulation against two different nuclease enzymes at

TABLE I - Particle size, PDI and Zeta potential values of SLN formulations (n=3)

Formulation	Particle Size (nm) (±SD)	PDI (±SD)	Zeta Potential (mV) (±SD)
SLN_{CTAB}	44.9 (±0.77)	0.394 (±0.011)	44.000 (±0.16)
SLN_{EQ1}	104.7 (±1.25)	0.309 (±0.004)	47.333 (±2.32)
SLN_{CTAB}:DNA	72.9 (±2.00)	0.266 (±0.001)	45.933 (±3.26)
SLN_{EQ1}:DNA	98.9 (±0.28)	0.287 (±0.003)	41.567 (±0.32)

SD: standard deviation, SLN_{CTAB}: SLN formulation prepared with CTAB as the cationic lipid, SLN_{EQ1}: SLN formulation prepared with EQ1 as the cationic lipid.

**FIGURE 3 -** Intensity-size distribution graphs of SLN:DNA complexes.
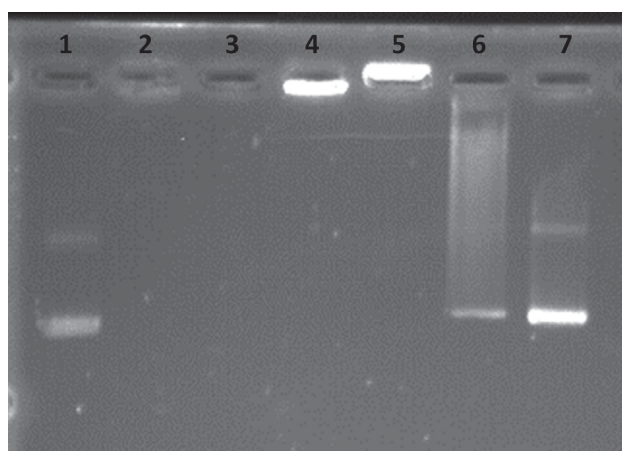
FIGURE 4 - Comparison of SLN_{EQ1} and SLN_{CTAB} formulations for complexation and SDS induced DNA release abilities. Lane 1: naked plasmid DNA as control; Lane 2: SLN_{EQ1}; Lane 3: SLN_{CTAB}; Lane 4: SLN_{EQ1}:DNA (1:1); Lane 5: SLN_{CTAB}:DNA(1:1); Lane 6: SLN_{EQ1}:DNA treated with %1 SDS; Lane 7: SLN_{CTAB}:DNA treated with %1 SDS.

two different SLN_{EQ1}:DNA ratios (1:1 and 1:2 (v/v)). DNase I protection study results of SLN_{CTAB} were not given because the formulation was determined to be cytotoxic on L929 cells.

Cytotoxicity and transfection of L929 cells

Cytotoxicity and transfection abilities of formulations were tested on L929 mouse fibroblast cells. 5, 7, 10, 15, 20 µL/well doses were tested. Cell viability after treatment with the ME, ME:DNA, SLNs and SLN:DNA formulations were presented in Figure 6.
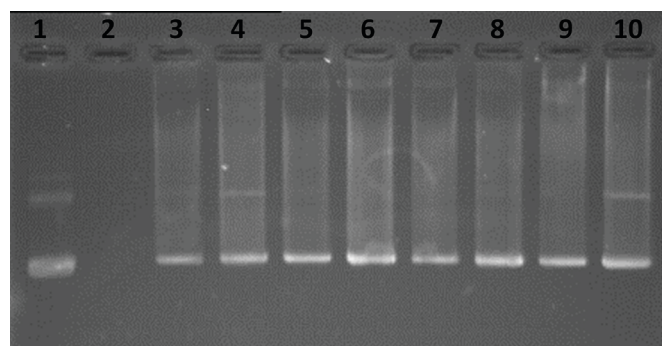
FIGURE 5 - DNase I protection of SLN_{EQ1}:DNA complexes. Lane 1: naked DNA as control; Lane 2: naked DNA+ Enzyme I as positive control; Lane 3: SLN_{EQ1}:DNA 1:1; Lane 4: SLN_{EQ1}:DNA 1:2; Lane 5: SLN_{EQ1}:DNA 1:1; Lane 6: SLN_{EQ1}:DNA 1:2; Lane 7: SLN_{EQ1}:DNA 1:1; Lane 8: SLN_{EQ1}:DNA 1:2; Lane 9: SLN_{EQ1}:DNA 1:1; Lane 10: SLN_{EQ1}:DNA 1:2; Lanes 3,4,7,8 treated with Enzyme I; Lanes 5,6,9,10 treated with Enzyme II.

Figure 7 shows the transfection ability of selected formulations. Blue colored images indicate DAPI staining. DAPI staining shows living cells under an inverted microscope. Green color indicates fluorescence from GFP expressed in the cells.

Stability

ME formulation was observed visually for 2 years. No turbidity or phase separation was determined during this period. The formulation was stored in a dry atmosphere in the dark in sealed glass vials. The storage temperature was 25 °C.

The SLN_{EQ1} formulation in suspension form was observed for 6 weeks for particle size and PDI change. The formulation was stored as described for ME. At the end of the 45th day, stability study was terminated because of turbidity formation. Measurement results are presented in Table II.

TEM analysis

ME formulation could not be visualized because of its high oil content. SLN_{EQ1} and SLN_{EQ1}:DNA formulations were imaged with transmission electron microscope. TEM micrographs can be seen in Figure 8. The morphology of SLNs appeared to be spherical (Figure 8A). On the other hand, the morphology of SLN_{EQ1}:DNA complexes are observed to be granular (Figure 8B).

DISCUSSION

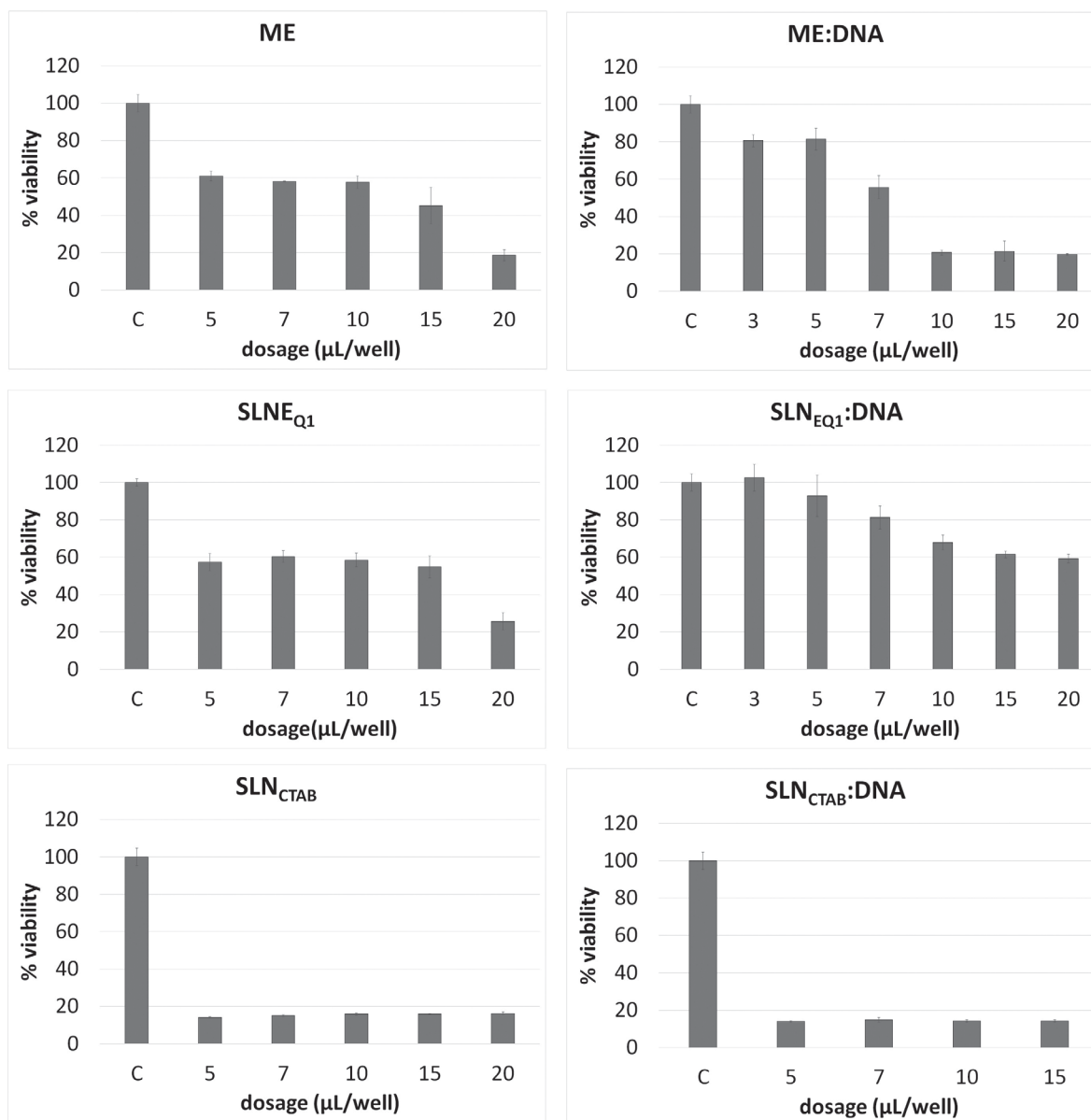Microemulsion formulations have advantages for

FIGURE 6 - Cell viability after treatment with formulations at different doses.

DNA delivery because of their transparent appearance, spontaneous formation and thermodynamical stability. The optical density of formulations can provide indications about the reproducibility of formulations. Monitoring the solubilization is another advantage for transparent formulations (Ulrich, 2002). Besides, cationic materials (in this case chitosan), that facilitate DNA encapsulation in microemulsions, can easily be incorporated into the formulation (Lee *et al.,* 2010). Another advantage of W/O microemulsion is confining and protecting DNA in its inner phase (Baroli *et al.,* 2000). In this study, Plurol oleique was selected as a lipophilic surfactant because of its ability to create transparent ME systems. Employing Plurol oleique, the HLB value of the system was decreased

dramatically. To adjust HLB value which is appropriate for w/o ME system, Tween 80 was used as a hydrophilic surfactant. The location of selected formulation in ME area can be seen in Figure 1. HLB value of ME system was calculated as 5.63. The viscosity of ME was 36 cP, which is an appropriate value for topically administered formulations. It was previously shown that increasing viscosity values renders MEs suitable for topical application.

SLNs are good delivery tools for genetic materials. Factors such as the protection of genetic materials from degradation and efficient delivery potential into cells make SLNs a perfect choice for gene delivery purposes (Torrecilla *et al.,* 2015). In this study, Precirol ATO5 was
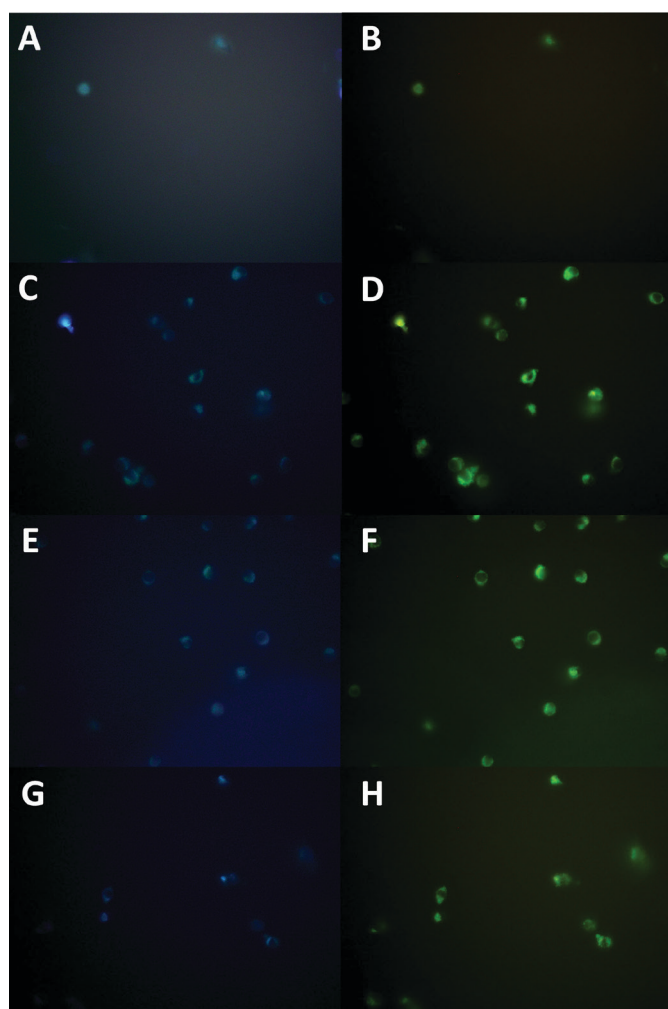
FIGURE 7 - Transfection ability of formulations (A and B: ME:DNA 5 µL, C and D: SLN$_{EQ1}$:DNA 3 µL, E and F: SLN$_{EQ1}$:DNA 5 µL, G and H: Control (naked DNA+L929 cells)). A,C,E,G images represent DAPI staining; B,D,F,H images represent fluorescence visualization.

used as the oil phase for SLN formulation. Ethanol was used as the co-surfactant, because of its ability to easily dissolve lecithin and its non-toxic characteristics. For an efficient transfection, the particle size of formulations should be approximately 100 nm because transfection activity strongly depends on the cellular uptake (Ulrich, 2002). SLN$_{CTAB}$ (44.9 nm) and SLN$_{EQ1}$ (104.7 nm) formulations have a suitable particle size for DNA delivery. PDI values of SLN formulations are 0.3 which is acceptable for monodisperse systems (Gaur *et al.,* 2014). SLN systems with zeta potential greater than ±30 mV can be considered as stable systems (Ulrich, 2002; Carrillo *et al.,* 2013). As shown in Table I the zeta potential values of developed formulations are sufficiently high, so these formulations can be considered stable systems (Carrillo *et al.,* 2013; Fàbregas *et al.,* 2014).

Considering its small particle size, SLN$_{CTAB}$ appeared as a suitable formulation. However, it was highly cytotoxic. On the other hand, the cytotoxicity of SLN$_{EQ1}$ was more acceptable in comparison with that of SLN$_{CTAB}$. As shown in Figure 6, SLN$_{EQ1}$:DNA complex exhibited approximately 80% cell viability at the dose 7 µL/well, which is suitable for cell culture studies. According to previous studies it is generally accepted that a zeta potential greater than +25 mV is appropriate for SLNs forming complexes with DNA (Fàbregas *et al.,* 2014). SLN$_{EQ1}$ showed high positive zeta potential (+47.3 mV), which is essential for the formation of stable complexes with DNA. Determining the optimum SLN:DNA ratio provides insights about maximum DNA amount that can be loaded to a given formulation (Fàbregas *et al.,* 2014). From Figure 2, it can be seen that the ratio of 1:1 (SLN-to-DNA) is optimal. At this complexation ratio, all DNA molecules are entrapped by the complex and show no
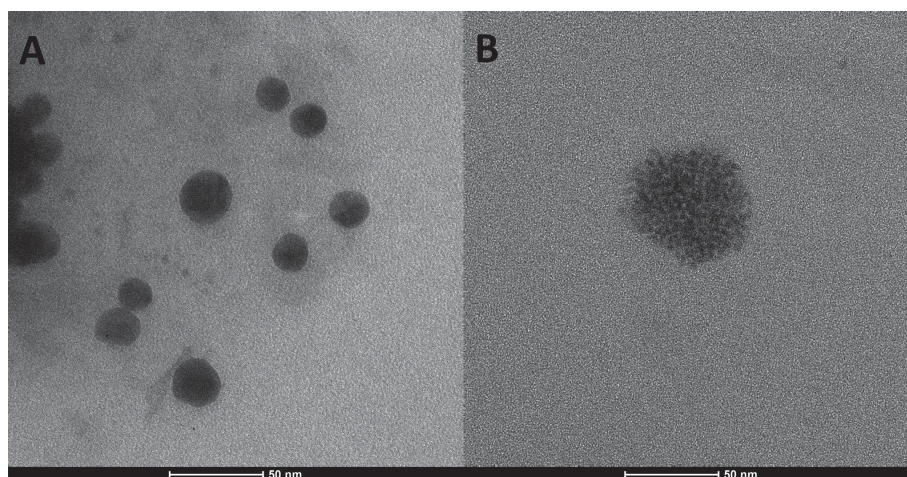
FIGURE 8 - TEM imaging of SLN$_{EQ1}$(A) and SLN$_{EQ1}$:DNA (B).

mobility during electrophoresis. As can be seen from the lanes 6-10 in Figure 2, the complex enters the gel at the opposite side. Considering this fact, it can be suggested that at higher SLN-to-DNA ratios, the complex becomes more positively charged and its movement is observed to be towards the cathode.

As it can be seen in Figure 3 both SLN:DNA complexes have narrow size distributions. Comparison of SLN_{CTAB} and SLN_{EQ1} can be seen in Figure 4. Gel image confirms the zeta potential measurements. According to Figure 4 (lanes 4 and 5), CTAB contributed to production of cationic SLNs with higher positive charge than EQ1. Lanes 6 and 7 indicate that both SLN_{EQ1} and SLN_{CTAB} are able to release DNA in the presence of SDS.

In order to obtain more reliable results, two different commercial enzymes were used for DNase I protection assay. According to lane 2 in Figure 5, naked DNA was completely digested by DNase I enzyme. DNA is in supercoiled form and only small amounts of nick DNA are observed in the wells loaded with the complexes. According to these results, all SLN_{EQ1}:DNA complexes are protecting DNA from enzymatic degradation. SLN_{CTAB}:DNA results were not given because of its poor cytotoxicity results.

Agarose gel electrophoresis studies couldn't be performed on ME formulations due to their high content of oil which makes them impossible to load on agarose gel wells.

The cytotoxicities of all formulations were dose dependent except SLN_{CTAB} formulations (Figure 6). The ME showed approximately 60% viability for the doses 5, 7 and 10 µL/well. The cytotoxicity decreases when DNA complexed with ME. ME:DNA formulation showed approximately 80% viability for the doses 3 and 5 µL/well (significantly higher than ME, p<0.0025). At higher doses, cell viability decreased drastically. Therefore, doses higher than 5 µL/well were accepted as cytotoxic and were not tested for the transfection experiments. SLN_{EQ1}:DNA complexes showed 80% cell viability at the dose 7 µL/well, so 3,5,7 µL/well doses were safely used for transfection studies. Moreover, the viability of cells treated with SLN_{EQ1}:DNA at the dose 5 µL/well was not significantly reduced as compared to the control group (p=0.2867). SLN_{CTAB} showed high toxicity on L929 cells at all tested doses. Therefore, this formulation was not included in any further experiment. DAPI binds to A-T rich regions of DNA and shows living cells in blue color. Merging DAPI-stained and GFP filter micrographs provides information about the transfection ability.

As shown in Figure 7 A and B, a small number of transfected cells were observed with ME:DNA. ME

formulation contains DNA in its inner phase. Because of the fact that the volume of the inner phase is very low (%16), the ME system contains a small amount of DNA (0.04 µg DNA in 5 µL dose). However, SLN 5 µL dose contains 0.25 µg DNA in 1:1 (v/v) complexation rate.

As shown in Table II, the particle size of SLN_{EQ1} decreased slightly during the storage period. However, this decrease is not an abnormal situation exactly. Related literature shows that SLNs' particle sizes could decrease during storage nearly to 30-40% (del Pozo-Rodríguez et al., 2009). After 45 days of storage, formulations' appearance became cloudy. That was a sign that SLNs' integrity was breaking down. Therefore, stability measurements were terminated at this point (Bose, Michniak-Kohn 2013; Dolatabadi et al., 2014). The stability of ME was monitored by visual inspection for 2 years. No change in the appearance was observed during this period.

According to TEM images, the shape of SLN_{EQ1} is spherical and the size of particles is about 50 nm (Figure 8 A). As shown in Figure 8 panel B, pDNA is successfully complexed with SLN_{EQ1} and the morphology of the complex appears wrinkled. TEM images reflects particle size more directly than the DLS measurements (Lee et al., 2013).

TABLE II - Stability of SLN_{EQ1} at room temperature for 1.5 months

Day	Particle Size (nm)	Polidispersity Index
1	87.3	0.238
15	96.8	0.203
30	93.8	0.241
45	77.8	0.251

CONCLUSIONS

In conclusion, two non-viral DNA carrier systems were developed and evaluated for plasmid DNA delivery. Because of its high viscosity, microemulsion system is suitable for topical application. Cytotoxicity of formulations can be adjusted by screening for safe doses as evidenced by cytotoxicity studies. In this study, at lower non-toxic doses SLN_{EQ1}:DNA complexes provided good transfection efficiency with EGFP-encoding plasmid DNA. The SLN formulation described in this study is suitable for investigating novel treatment modalities for various disease models by replacing EGFP-encoding plasmid DNA with therapeutic gene-encoding plasmids.

ACKNOWLEDGEMENT

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