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Development and characterization of cationic nanoemulsions as non-viral vectors for plasmid DNA delivery

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ABSTRACT: Gene therapy is a promising approach to treat several diseases including cancer. Unfortunately, free nucleic acids are negatively charged and not stable in blood. Cationic drug delivery systems like cationic nanoemulsions (CNEs) are useful to improve the stability and delivery of nucleic acids like plasmid DNA (pDNA) by increasing the interaction between nucleic acids and negatively charged cell membrane. This study is focused on the development and characterization of two CNEs (CNE 1 and CNE 2) for the delivery of pDNA. The CNEs were prepared with microfluidization by investigating the homogenization duration of 1-10 minutes and the droplet size (DS), polydispersity index (PDI), zeta potential (ZP), complexation with pDNA and cytotoxicity on mouse fibroblast cells L929 were characterized. With increasing the microfluidization duration from 1 to 10 minutes, the DS and PDI decreased and the ZP increased for CNE 1, while for CNE 2 the ZP decreased too. Increasing the microfluidization duration does not lead to beneficial results, thus 1 minute seems to be sufficient for obtaining CNEs with appropriate properties. Both CNEs were able to form a complex in a ratio of 1 µl plasmid and 2 µl CNE. The cytotoxicity studies for CNE 1 and its complex revealed that with increasing the applied dose the viability of the L929 cells decreased to 70%, while for CNE 2 and its complex the viability was reduced to 10%. Based on these results it can be stated that CNE 1 is more appropriate than CNE 2 for pDNA delivery.

KEYWORDS: Cationic nanoemulsion; non-viral vector; pDNA; complexation; cytotoxicity; microfluidization.

1. INTRODUCTION

Gene therapy using nucleic acids is a promising strategy for several diseases, especially cancer. Free nucleic acids are negatively charged, large and hydrophilic, thus the cell uptake of free nucleic acids is poor and they are biologically not stable due to degradation by nucleases in the blood. Thus, the delivery of nucleic acids can be improved with help of drug delivery systems like viral or non-viral vectors. In addition, drug delivery systems are able to protect nucleic acids from degradation. The size and surface charge of drug delivery systems are controlling the physical and chemical stability of drugs in biological fluids, interactions with cell membrane and its intracellular behavior. If a drug delivery system is not used, there is no interaction between nucleic acid and negatively charged cell membrane and the entrance of genetic material into the cell is limited. Inverting the negative charge of nucleic acids to positive charge by cationic delivery systems results in an enhanced binding on the cell surface and enhanced entrance of genetic material into the cell by endocytosis [1, 2].

Non-viral vectors have attracted increasing attention compared to viral vectors, due to their advantages like an ease of manufacturing, low immune response, cost-effectiveness and safety. Thus, non-viral vectors can replace viruses as alternative for delivery of nucleic acids [3-7]. Lipid based nanoparticulate carriers like liposomes, solid lipid nanoparticles and emulsions, among others, are attractive platforms for gene delivery [8]. Since more than two decades, cationic nanoemulsions (CNEs) were proposed as delivery system for nucleic acids [9-11]. CNEs are dispersed systems composed of oil phase and water phase (Figure 1), which are normally not miscible, thus the system is thermodynamically not stable.

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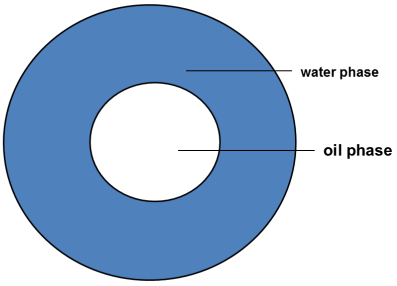

Figure 1. Schematic illustration of an oil/water nanoemulsion

As the inner phase of the emulsions for gene delivery different oils like medium-chain triglycerides, soybean oil, and squalene can be used [11-13]. The oil phase is stabilized by a cationic lipid like 1,2-dioleoyl-3-trimethyl ammonium propane (DOTAP), 3-b[N-(N0,N0-dimethylaminoethane)-carbamoyl] cholesterol (DC Chol), didodecyldimethylammonium bromide (DDAB) or a mixture with phospholipids like 1,2-dioleoyl-3-phosphatidylethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) and/or non-ionic surfactants (Tween 80, Poloxamer etc.). [14] Using a cationic lipid or agent in the formulation is necessary for the formation of a complex with nucleic acids through electrostatic interactions. According to the optimization of complexes for improving the stability of nucleic acids and transfection efficiency, extensive research was performed. In vitro studies have shown that complexes improved the intracellular delivery of nucleic acids and protected them from nuclease degradation. The *in vivo* transfection efficiency after intravenous [15], nasal [13, 16], and pulmonary administration [17] of CNEs was improved. The first cationic emulsion used for delivering a plasmid through a portal vein injection in mice was composed of castor oil and DC-Chol [9]. Its application resulted in a greater amount of gene product in the liver than traditional cationic liposomes *in vivo*. One of the main advantages of NEs is their versatile possible application as oral, parenteral, intranasal and its dilution with water. Some examples of NEs available in the market are Diazepam® Lipuro, Disoprivan®, Etomidat® Lipuro, Intralipid®, Lipofundin®, Propofol 1%/2% Fresenius and Stesolid®.

In this study, phytosphingosine (PS) was investigated for the first time, to the best of our knowledge, as a complexation agent for plasmid desoxyribonucleic acid (pDNA). Another novelty of this study is the use of the cationic lipid DDAB in CNEs by investigating its complex formation capacity with pDNA.

For manufacturing CNEs, the cationic compound is crucial for the positive charge. DDAB is a well-known substance, often used to create positive charge in formulations like nanobubbles [18] and solid lipid nanoparticles [19] for studies, but there is no report in the literature about its use in CNEs.

In previous studies, PS was used as an excipient for preparing CNEs [20]. PS is part of ceramides and thus of the skin and is generally used as a cationic agent for enhancing the dermal permeation and penetration [21].

This study is focused on the development and characterization of two CNEs as non-viral vectors for plasmid DNA delivery. For that purpose first blank CNEs were prepared by microfluidization and characterized in terms of particle properties like droplet size (DS), size distribution (polydispersity index, PDI) and zeta potential (ZP), formation of complexes with pDNA and cytotoxicity using mouse fibroblast L929 cells.

2. RESULTS AND DISCUSSION

2.1. Solubility of phytosphingosine and DDAB in Oils

The solubility results (Table 1) revealed that after storage of 24 hours at 25°C no precipitation of the cationic agent was observed for the combinations Peceol and PS, Lauroglycol 90 and DDAB and octyldodecanol and PS. Due to the fact that an increased temperature of 100 °C to solve PS is probably not beneficial, the combination Peceol + PS and Lauroglycol 90 + DDAB were used for the preparation of the CNEs. Thus, two different CNEs, CNE 1 and CNE 2 (Table 3) were prepared and investigated.

Table 1. Solubility study results of PS and DDAB in several oils.

Oil Compound	Cationic Agent (0.5%, w/w)	25 °C	50 °C	100 °C	After Storage of 24 h at 25°C
Peceol	PS	-	-	+	n.p.
	DDAB	-	+	n.i.	p.
Lauroglycol FCC	PS	-	-	+	p.
	DDAB	-	+	n.i.	p.
Lauroglycol 90	PS	-	+	+	p.
	DDAB	+	+	n.i.	n.p.
Plurol oleique	PS	v	v	+	v
	DDAB	v	v	n.i.	v
Octyldodecanol	PS	-	-	+	n.p.
	DDAB	-	+	n.i.	p

-=not soluble; +=soluble; n.i.= not investigated; p= precipitation; n.p.= no precipitation; v= high viscosity

2.2. Development and particle characterization of cationic nanoemulsions

For preparing CNEs by microfluidization there are two important groups of parameter existing, formulation parameter, like the type and concentration of the constituents and the preparation parameter, like the microfluidization duration, temperature and pressure.

In previous studies, the formulation parameter like the type and concentration of the used compounds and process parameter like preparation temperature, pressure and duration during the development of NEs were investigated [22]. It could be shown that investigating the PS concentration (0.3-0.7%) revealed that only the NE formulations containing 0.5 and 0.6% were stable after storage at 25 °C and 40 °C. Regarding the ZPs after storage have shown that only for the NE containing 20% Eutanol G® as the oil phase the ZP remained unchanged, whereas decreased ZPs were observed for the NEs containing 15% and 10% oil phase, respectively. According to the influence of the different concentrations of the two stabilizers Lipoid E 80 in the oil phase and Tween 80 in the aqueous phase on the physical stability, the NEs prepared with 2% Lipoid E80 and Tween 80 were most stable. All other NEs showed either an increase in the particle size or non-constant ZPs. Furthermore, it was shown that for developing NEs the preparation parameter were more effective than the formulation parameter [22, 23]. Based on these findings, the NE containing 0.6% PS, 20% oil phase and 2% Lipoid E80 and Tween 80, respectively, prepared with 25 °C and 700 or 50 °C and 500 bar were determined as appropriate parameter [22].

Here, some modifications were made according to these parameters like novel oil compounds like Peceol and Lauroglycol 90, and novel cationic agents for complexation of pDNA like DDAB and PS or an intermediate pressure of 600 bar. The oil compounds (Peceol and Lauroglycol 90) and the cationic agents (DDAB and PS) are crucial for preparing CNEs with appropriate properties like a small DS of < 300 nm, a narrow size distribution with a PDI of <0.3 and a high ZP of >+30 mV. All particle properties like DS, PDI and ZP are essential for the purpose of this study, but the ZP is the most crucial property due to formation of complexes with pDNA. The effects of the used compounds and of the microfluidization duration from 1-10 minutes on particle properties (DS, PDI and ZP) were studied to optimize the preparation of the CNEs. The CNEs were prepared with microfluidization method. Due to the fact that the here used microfluidizer (Microfluidizer ML100L) does not allow to vary the preparation temperature, a temperature of 25 °C was used for preparing the CNEs. Previous studies have shown that the combination of 25 °C and 700 or 50 °C and 500 bar were appropriate for preparing NEs and that the microfluidization duration or number of homogenization cycles was more crucial than the homogenization pressure [22-24].

Thus, the two NEs were prepared using 0.5% PS, 20% oil phase and 2% lecithin and Tween 80, at 25 °C and a pressure of 600 bar, by investigating the microfluidization duration of 1, 2, 3, 4, 5, 6, 8 and 10 minutes. An intermediate pressure between 500 and 700 bar, namely 600 bar, was chosen for the preparation of the CNEs, because the CNEs will be blank, without a drug, and thus less pressure input into the NE system could be sufficient.

With increasing the microfluidization duration from 1 to 10 minutes, the DS of NE 1 (Figure 2a) decreased, from 104 nm (1 min) to 93 nm (10 min), just like the PDI slightly decreased from 0.11 (1 min) to 0.10 (10 min) (Figure 2a), and the ZP increased from +44.8 mV (1 min) to +46.8 mV (10 min) (Figure 2b) for CNE 1. The DS of CNE 2 decreased from 194 nm (1 min) to 159 nm (10 min) (Figure 3a), the PDI decreased from 0.17 (1 min) to 0.11 (10 min) (Figure 3a), and the ZP decreased from +48.7 mV (1 min) to +43.6 mV (10 min) (Figure 3b). Increasing the microfluidization duration does not lead to beneficial results, thus 1 minute seems to be sufficient for obtaining CNEs with appropriate properties like a small DS and PDI and a high ZP.

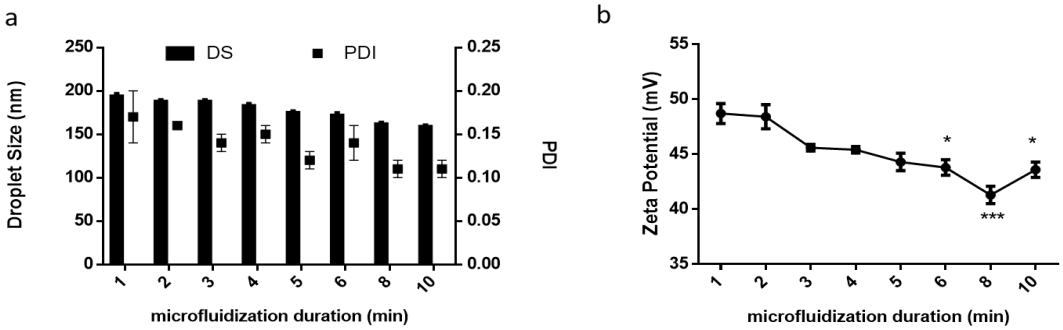

Figure 2. The droplet size and polydispersity index (a) and zeta potential (b) results of CNE 1 with increasing microfluidization duration. * p<0,05 , ***p<0,001.

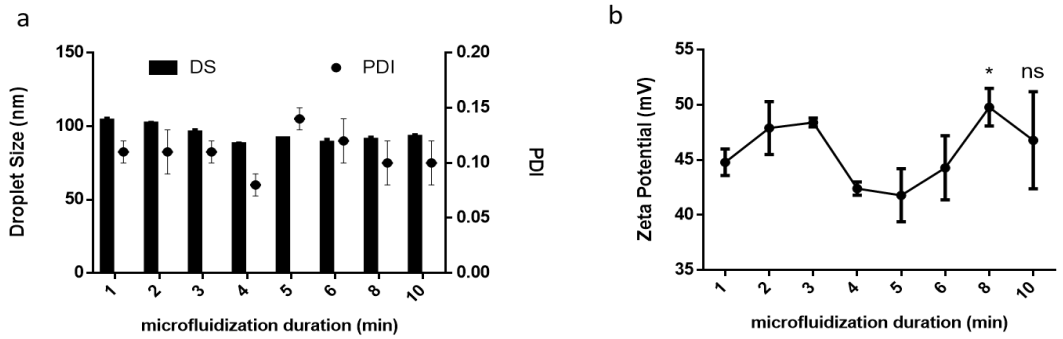

Figure 3. The droplet size and polydispersity index (a) and zeta potential (b) results of CNE 2 with increasing microfluidization duration. * p<0,05.

Due to the fact that the ZP is the most crucial property for the formation of complexes with nucleic acids like pDNA, a microfluidization duration of 1 min was considered as appropriate for CNE 1 having the highest ZP of +44.8 mV. For CNE 2, longer microfluidization durations than 1 min are not necessary, because increased durations resulted in some minor changes without any significant differences of DS, PDI and ZP. Thus, the CNEs prepared with a microfluidization duration of 1 min were used for further investigations.

Comparing the DS, PDI and ZP of the two CNEs showed that CNE 1 had a smaller DS (114 nm), a lower PDI (0.11) and a lower ZP (+44.8 mV), compared to CNE 2 with a DS of 194 nm, a PDI of 0.17 and a ZP of +48.7 mV. Both formulations CNE 1 and CNE 2 prepared with a microfluidization duration of 1 min were investigated according to their complex formation ability with pDNA and cytotoxicity on mouse fibroblast cells L929.

2.3. Gel Retardation assay of cationic nanoemulsion-pDNA complexes

According to the CNEs-pDNA complexation results, the complex of 1 µl plasmid and 2 µl CNE 1 (Figure 4a) and CNE 2 (Figure 4b), respectively, (1:10 diluted) were appropriate for further cytotoxicity studies. Following the preparation of blank CNEs, the complex formation of these CNEs with pDNA was investigated. Then, the properties of blank CNEs and CNE:pDNA complexes were characterized.

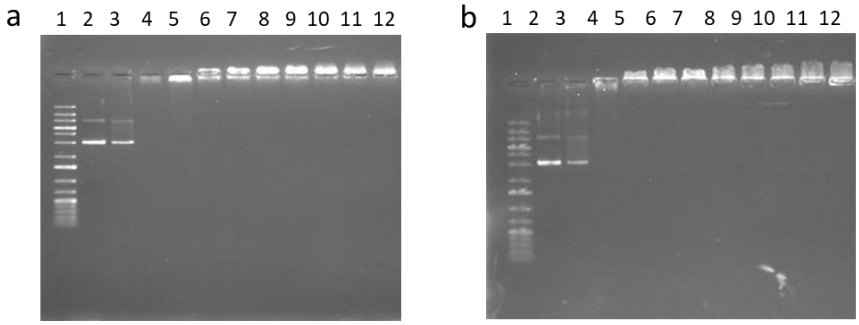

Figure 4. Gel electrophoresis image of the complexation assay between the constant amount of pEGFP-C1 with the increasing amount of CNE 1 (a) or CNE 2 (b). (1:1Kb plus DNA ladder, 2: Naked DNA as positive control, 3,4,5,6,7,8,9,10,11,12: pDNA:CNE complexes for the ratio of 1:0.5, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9 (v/v), respectively ; CNEs were used as 1:10, diluted).

2.3.1. Particle characterization of cationic nanoemulsion-pDNA complex formulations

For CNE 1, the DS only slightly changed from 194 nm to 208 nm after complexation with pDNA, the PDI slightly decreased from 0.17 to 0.15 after complexation with pDNA, but the ZP significantly decreased from +48.7 mV to +5.7 mV after complexation with pDNA (Table 2). The formation of a CNE 2-pDNA complex resulted in a slight increase of the DS from 104 nm to 137 nm, an increase of the PDI from 0.11 to 0.26, and a significant decrease of the ZP from +44.8 mV to +14.1 mV. Comparing both CNE-pDNA complex formulations revealed that the DS of CNE 1-pDNA is higher (208 nm) than of CNE 2-pDNA (137 nm), the size distribution of CNE 1-pDNA is narrow (PDI of 0.15) compared to CNE 2-pDNA (PDI of 0.26) and the ZPs are below +30 mV, +5.7 mV for CNE 1-pDNA and +14.1 mV for CNE 2-pDNA. Based on these particle characterization results, it could be stated that both CNEs are appropriate for the formation of a complex with pDNA. However, the cytotoxicity on L929 mouse fibroblast cells is essential.

Table 2. The droplet size, size distribution and zeta potential results of the CNEs and their pDNA complex results.

Formulation	DS [nm ± SD]	PDI [± SD]	ZP [mV± SD]
CNE 1	194 ± 2.9	0.17 ± 0.03	+48.7 ± 0.9
CNE 1-pDNA	208 ± 2.2	0.15 ± 0.04	+5.7 ± 0.6
CNE 2	104 ± 1.8	0.11 ± 0.01	+44.8 ± 1.2
CNE 2-pDNA	137 ± 5.3	0.26 ± 0.01	+14.1 ± 0.9

2.4. Cytotoxicity

The cytotoxicity study results of the CNE 1 and its complex with pDNA (Figure 5a) revealed that with increasing the applied dose of CNE 1 and CNE 1-pDNA complex from 2 to 10 µL, the viability of the L929 mouse fibroblast cells decreased from 90% (CNE 1) and 80% (CNE 1-pDNA), respectively, to finally 70% at 10 µL. For cytotoxicity studies using normal cells, obtaining high viabilities is preferred. On the contrary, CNE 2 and its complex with pDNA (Figure 5b) reduced the viability of the L929 mouse fibroblast cells to a viability of less than 10%, which is not preferred for normal cells, but requested for cancer cells.

Based on these results it can be stated that CNE 1 is probably more appropriate than CNE 2, because of higher viability results of normal cells.

3. CONCLUSION

Here, the developed CNEs revealed suitable properties like a small DS of approximately 200 nm (CNE 1) and smaller (CNE 2), a narrow size distribution, expressed as PDI <0.3, and a high zeta potentail of >+30 mV for complexation and delivery of pDNA. Although all these properties are important for gene delivery, the ZP is very crucial for a successful gene delivery because of forming complex with nucleic acids like pDNA. Both CNEs revealed appropriate particle properties and formed a complex with pDNA without any significant changes of these properties, except for ZP, which was expected due to negatively charged pDNA.

To sum up, PS is able to form a complex with pDNA in form of CNE, representing an alternative to the well-known cationic agents like DOTAP, DOPE etc.

Thus, it can be concluded that CNE 1 containing PS as a novel cationic complexation agent for nucleic acids is more appropriate for delivery of pDNA, compared to CNE 2, revealing low viability on L929 cells.

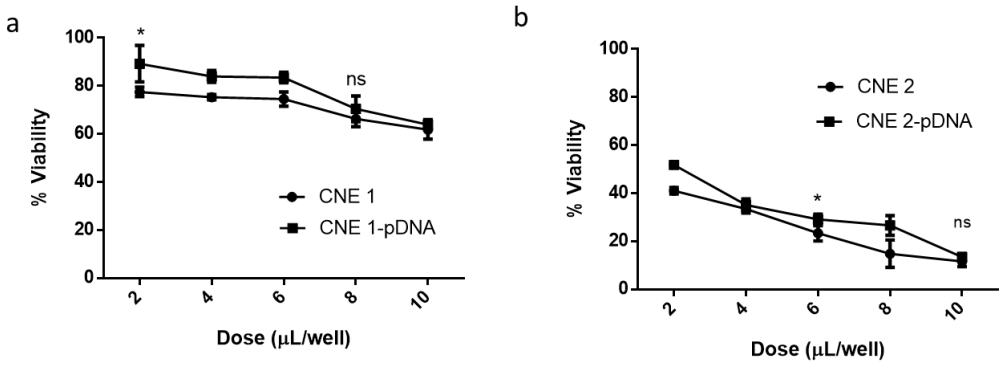

Figure 5. Cytotoxicity study results of CNE 1 and its complex with pDNA (a) and CNE 2 and its complex with pDNA (b) on mouse fibroblast cells . * p<0,05

4. MATERIALS AND METHODS

4.1. Materials

Phytosphingosine (PS; 2S-amino-1, 3S, 4R-octadecanetriol) from Evonik (Essen, Germany) and the oil compounds Lauroglycol 90, Lauroglycol FCC, Peceol and Plurol Oleique (Gattefosse, France) were gifts. Polysorbate 80 (Tween 80, Merck, Germany) and lecithin from soybean 30% (Applichem, Darmstadt, Germany) were chosen as surfactants. Octyldodecanol (Eutanol® G, Caesar and Lorenz GmbH, Hilden, Germany) as oil compound, benzalkonium chloride (Signa, St. Louis, USA) as preservative and didodecyldimethylammonium bromide (DDAB; TCI, Tokio, Japan) were purchased.

The plasmid pEGFP-C1 from Invitrogen, California, USA was used as a model. The pDNA was amplified in *Escherichia coli DH5a* strain. Maxiprep plasmid DNA purification kit from Invitrogen (USA) was performed to purify plasmid. After obtaining pDNA, restriction enzyme digestion and visualizion by agarose gel electrophoresis was performed to check plasmid unity. Moreover, the purity and the concentration of the plasmid was mesured at 260/280 nm wavelengths by UV/Vis spectrophotometer. The concentration of pDNA was adjusted to 100 µg/mL and stored at -20°C until use.

4.2. Methods

4.2.1. Solubility screening of phytosphingosine and DDAB

Before preparing the CNEs, the solubility of PS and DDAB were investigated by solving a final concentration of 0.5% (w/w) of them in the oil components Lauroglycol 90, Lauroglycol FCC, Peceol, Plurol Oleique and octyldodecanol, at 25 °C, 50 °C and 100 °C, respectively. These solutions were stored for 24 hours and observed for precipitation.

4.2.2. Preparation of cationic nanoemulsions

The composition of the CNEs are given in Table 3. The CNEs were prepared by microfluidization as described in the literature [23, 24].

Briefly, the oil phase was prepared by adding the cationic agent PS to Peceol at approximately 50 °C and adding another cationic agent DDAB to Lauroglycol 90 at 25 °C and stirred with a magnetic stirrer until complete solvation was obtained. After that, the surfactant lecithin was added and stirred until complete solvation was achieved. The water phase was obtained by solving the surfactant Tween 80 and the preservative benzalkonium chloride in water at 25 °C.

Afterwards, a pre-emulsion was obtained by using the high speed stirrer Silverson L5M with 10000 rpm for 5 min and subjected afterwards to the Microfluidizer ML100L. The CNEs were prepared at 25 °C with 600 bar and different microfluidization durations (1, 2, 3, 4, 5, 6, 8 and 10 min) by microfluidization to obtain CNEs.

Table 3. Composition of the cationic nanoemulsions.

Substance	CNE1	CNE2
Oil phase	**% (w/w)**	**% (w/w)**
Peceol	20	-
Lauroglycol 90	-	20
PS	0.5	-
DDAB	-	0.5
Lecithin	2	2
Water phase		
Tween 80	2	2
Benzalkonium chloride	0.1	0.1
Water	ad 100	ad 100

After preparing the CNEs, their complex formation ability with pDNA was evaluated by the use of different relations of CNEs and pDNA, showed with gel retardation assay. The cytotoxicity studies of the CNEs were performed using mouse fibroblasts L929 cell line.

4.2.3. Particle Characterization of Cationic Nanoemulsions

Droplet Size, Size Distribution and Zeta Potential

The DS, PDI and ZP of the CNEs were measured directly after the preparation. Mean DS and PDI were determined by Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) per photon correlation spectroscopy with dynamic light scattering (DLS). The ZP, representing the surface charge of the NEs was determined by measuring the electrophoretic mobility. 20 µl of the sample was added to 20 ml purified water and measured. The Helmholtze Smoluchowski equation was applied for the calculation of the ZP **[25]**. The CNE with appropriate properties like a small DS, a narrow size distribution and especially a high ZP of >30 mV was investigated according to formation of a complex with pEGFP-C1 in ratios between pEGFP-C1:CNE of 1:0-1:3.

Preparation of Cationic Nanoemulsion-pDNA Complexes

To determine the saturation of pDNA binding, different volume ratio of CNEs to pDNA solution were examined. The augmenting amount of CNEs (0.5 - 9 µl, 1:10 diluted) were added into the constant amount of pDNA solution (1 µl of 100 ng/ml plasmid stock solution) and shaken on a bench-top shaker for 30 min to complete the binding of pDNA onto the CNEs *via* electrostatic interaction.

The resultant CNE:pDNA complexes were characterized by gel retardation assay [19]. The agarose gel electrophoresis (1% agarose/1xTAE, w/v) was carried out for 40 min under the voltage of 100 V and imaged *via* gel documentation system (Vilber Lourmat, France). The CNE:pDNA complexes were freshly prepared before each use for further studies.

4.2.4. Cytotoxicity

The *in vitro* cytotoxicity of CNEs were investigated on mouse fibroblast cell line L929 (ATCC® CCL-1) Cells were plated in 96-well plates at a density of $1x10^4$ cells per well. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS was used as culture medium. Cells were incubated for 24 h at 37°C in a humidified 5% CO_2 atmosphere. Then, the medium was removed, the cells were washed and treated with CNE1, CNE1:pDNA complex, CNE2 and CNE2:pDNA complex. Samples were applied at doses 2, 4, 6, 8, 10 µL/well (with respect to the volume of CNE formulations for the complexed forms). Following the incubation for 24 h, the medium was removed and the cells washed twice with PBS (pH=7.4). Then, 100 µL DMEM and 50 µL activated XTT reagent was added to each well and the cells were incubated for 2 h as recommended by the manufacturer. The absorbance of the orange-colored metabolite of XTT reagent was measured at wavelengths of 475 nm and 660 nm by Varioskan Multiplate Reader (Themo Scientific, USA). Specific Absorbance (SA) values were calculated using the following equation.

$$SA = A_{475}(test) - A_{475}(blank) - A_{660}(test) \quad [Eq. 1]$$

Cell viability was calculated based on the specific absorbance of CNE treated cells compared to the absorbance of the control group consisting of untreated L929 cells. Experiments were carried out at least in triplicate.

4.2.5. Statistical analysis

GraphPad Prism v6.01 software was used for statistical analysis of all data. $P < 0.05$ was considered to be statistically significant. Results are expressed as means ± SD. For analysis between groups, a two-way ANOVA was performed followed by multiple comparison to compare differences between groups. Measurements and experiments were carried out at least in triplicate.

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