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Long-term Stability of Cationic Phytosphingosine Nanoemulsions as Delivery Systems for plasmid DNA

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Abstract

Gene delivery systems like cationic nanoemulsions are in general not ready to use for any time. Thus, delivery systems for genetic materials like nucleic acids have to be prepared freshly before application. This study is focused on the preparation and characterization of cationic nanoemulsions using phytosphingosine for plasmid DNA delivery. Repurposing of cationic agents guided us to phytosphingosine, previously used for enhanced interaction with negatively charged surfaces. It was reported that phytosphingosine may act anti-apoptotic, but without using it in an appropriate delivery system like nanoemulsions. This gap attracted our interest in preparing and characterizing long-term stable cationic nanoemulsions and their cytotoxic effects on MDA-MB-231 and MCF-7 breast cancer cells using phytosphingosine. The cationic nanoemulsions 1, 2, and 3 were prepared and characterized in terms of droplet size, polydispersity index, zeta potential, long-term stability after storage at 25 and 40 °C, complexation with pDNA, release and cytotoxicity on MDA-MB-231 and MCF-7 cells. The CNEs showed appropriate properties like a small droplet size (<200 nm), a narrow size distribution and a high zeta potential (>+30 mV). Unfortunately, each cationic nanoemulsion showed some disadvantages. Cationic nanoemulsion 1 decreased the viability of cancer cells to only 25 %. Phase separation was observed for cationic nanoemulsion 2 after storage of six months at 40 °C. And cationic nanoemulsion 3 was not able to form a complex with pDNA.
To sum up, cationic nanoemulsion 1 is more appropriate than the other cationic nanoemulsions for delivering pDNA.

Keywords: cationic nanoemulsion, stability, pDNA, complexation, phytosphingosine, cytotoxicity.

1. Introduction

Large and hydrophilic free nucleic acids are negatively charged, which disables their cellular uptake. Furthermore, they are biologically not stable and degradation by nucleases in the blood is occurring. Drug delivery systems like viral or non-viral vectors are useful tools for improved delivery of nucleic acids and they can protect nucleic acids from degradation. Loading anionic nucleic acids onto cationic delivery systems resulted in an enhanced binding on the cell surface and enhanced entrance of genetic material into the cell by endocytosis [1, 2]. Easy manufacturing, low immune response, cost-effectiveness, and safety are some of the advantages of non-viral vectors, representing alternative drug delivery systems to viral delivery of nucleic acids [3-7].

Cationic nanoemulsions (CNEs) were proposed for more than three decades as delivery system for nucleic acids [8-10]. CNEs are dispersed systems composed of an oil phase and an aqueous phase. Both phases are normally not miscible, thus the dispersed system is thermodynamically not stable. The inner oil phase of oil-in-water (O/W) emulsions can be stabilized by non-ionic surfactants (Tween 80, Poloxamer etc.) and additionally a cationic lipid like didodecyl-dimethylammonium bromide (DDAB) can be used [11]. A cationic lipid or agent in the formulation is required due to the formation of a complex with anionic nucleic acids through electrostatic interactions. The first cationic emulsion for the delivery of a plasmid through a portal vein injection in mice was composed of castor oil and 3-b[N-(N0,N0-dimethylaminoethane)-carbamoyl] choles-terol [8]. After its application, higher amounts of the gene product in the liver were observed, compared to traditional cationic liposomes. Oral, parenteral, and intranasal application and dilution with water are some of the advantages of NEs. Diazepam® Lipuro, Disoprivan®, Etomidat® Lipuro, Intralipid®, Lipofundin®, Propofol 1%/2% Fresenius and Stesolid® are some examples of NEs available in the market.

Phytosphingosine (PS) is an important point of this study and was investigated as a complexation agent for plasmid desoxyribonucleic acid (pDNA) in CNEs with appropriate properties. PS is one representative of sphingoid bases, which are constituents of ceramides in the stratum corneum. PS is known to have antibacterial activity against epidermal and mucosal bacterial infections [5-Schuh] and antimicrobial and antiinflammatory activity and efficacy on acne vulgaris [12]. In addition, PS induced apoptosis-like cell death in *Neurospora crassa*, caspase-independent apoptosis in *Aspergillus nidulans* and is a potent inducer of apoptosis [13-16]. Furthermore, PS induced mitochondria-involved apoptosis and cell-cycle arrest in Jurkat cells, reduced cytochrome *c* release independently of caspase activation[17]**.** Moreover, PS dephosphorylated Akt to inhibit pro-growth signal, protein phosphatase inhibitor attenuated PS-induced Akt dephosphorylation and overexpression of mitochondria-localized anti-apoptotic protein Bcl-2 prevented PS apoptotic stimuli [17]. It was reported that PS caused a strong induction of caspase-8 activity and caspase-independent Bax translocation to the mitochondrial in Jurkat (human T-cell lymphoma) and NCI-H460 (human non-small cell lung cancer) cells [18]**.** Besides, PS induced activation of caspase 9 and 3, cytochrome *c* release from mitochondria and mitochondrial translocation of Bax from the cytosol without changes in the protein levels of Bcl-2, Bcl-xL, and Bax [19]**.** These studies have in common that PS was not investigated in a delivery system like NE, which is a serious lack.

For manufacturing CNEs, the cationic compound is crucial for the positive charge and gene delivery systems using cationic agents are expected to be non-toxic, but potent to deliver the cargo to the target to a high extent. Within the last decades, several cationic agents have been studied for the complexation and delivery of nucleic acids. This study aims to point out PS and PS-Hydrochloride (PSHCl), which has a higher aqueous solubility than PS, as cationic agents in cationic nanoemulsions (CNEs) for delivery of pDNA. In that context, the effects of PS and PSHCl on particle properties, complexation and cytotoxicity among others were investigated.

The challenge of using PS is to find an appropriate solvent. The low aqueous solubility of PS is disabling its use in simple aqueous formulations. Due to this PS was used in previous studies in NEs for dermal [20, 21] and oral [22] application. This is the first study about using PS as a cationic agent in CNEs with appropriate properties for delivering pDNA. A previous study about the preparation a CNE using PS and Peceol was not appropriate due to phase separation after storage of 1 month at 40 °C [23].

This study is focused on the preparation and characterization of three CNEs as non-viral vectors for plasmid DNA delivery and one blank NE (OD-NE) as a control for the cytotoxicity studies. For that purpose, the CNEs were prepared by microfluidization and characterized in terms of particle properties like droplet size (DS), size distribution (polydispersity index, PDI), zeta potential (ZP) and cytotoxicity using human embryonic kidney (HEK 293) and the two breast cancer cell lines, MDA-MB-231 and MCF-7. Formation of complexes with pDNA and SDS release studies were only performed with the CNEs.

2. Material and Methods

2.1 Materials

Phytosphingosine (PS; 2S-amino-1, 3S, 4R-octadecanetriol) and PS-Hydrochloride (PSHCl) were gifts from Evonik (Essen, Germany). Polysorbate 80 (Tween 80, Merck, Germany) and lecithin from soybean 90% (Applichem, Darmstadt, Germany) were chosen as surfactants. Octyldodecanol (OD; Eutanol® G, Caesar and Lorenz GmbH, Hilden, Germany) as an oil compound was purchased.

The plasmid pEGFP-C1 from Invitrogen, California, USA was used as a model. The pDNA was amplified in *Escherichia coli DH5α* strain. Maxiprep plasmid DNA purification kit from Invitrogen (USA) was performed to purify plasmid. After obtaining pDNA, restriction enzyme digestion and visualized by agarose gel electrophoresis was performed to check plasmid unity. Moreover, the purity and the concentration of the plasmid were 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.

2.2 Methods

2.2.1 Preparation of Cationic Nanoemulsions

The CNEs were prepared with microfluidization method by using Microfluidizer ML100L.

The CNEs were prepared using 0.5% PS (CNE 1 and 3) and PSHCl (CNE 2), respectively, 20% OD (CNE 1 and 2) and 5% OD (CNE 3), 2% lecithin and Tween 80, at 25 °C, 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, due to the fact that the CNEs will be blank, without a drug, and thus less pressure input into the NE system could be sufficient for obtaining CNEs with appropriate long-term properties.

The composition of the CNEs is given in Table 1. The CNEs were prepared by microfluidization as described in the literature [22, 24].

Table 1: Composition of the cationic nanoemulsions.

Compound	CNE 1	CNE 2	CNE 3
oil phase	**% (w/w)**	**% (w/w)**	**% (w/w)**
octyldodecanol	20	20	5
PS	0.5	-	0.5
lecithin	2	2	2
aqueous phase			
PS-HCl	-	0.5	-
Tween 80	2	2	2
bidistilled water	ad 100	ad 100	ad 100

Briefly, for CNE1 and 3 the oil phase was prepared by adding the cationic agent PS to OD at approximately 100 °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 at approximately 50 °C. The aqueous phase was obtained by solving the surfactant Tween 80 and in bidistilled water at 25 °C.

For CNE2, the oil phase was prepared by solving the surfactant lecithin at 50 °C. The aqueous phase was obtained by solving PSHCl and Tween 80 in bidistilled water at 25 °C.

Afterwards, the oil phase was added to the aqueous phase and 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 (Figure 1).

Figure 1: Photograph of the prepared cationic nanoemulsions.

2.2.2 Droplet Size and Zeta Potential Measurements of Particle Characterization of Cationic Nanoemulsions

The DS, PDI and ZP of the CNEs were measured directly after the preparation (d0). The mean DS and PDI were determined by Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) per photon correlation spectroscopy with dynamic light scattering (DLS) and the ZP, representing the surface charge of particles, was determined by measuring the electrophoretic mobility. 20 µl of the sample was added to 20 ml bidistilled 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 of less than 300 nm, a narrow size distribution (PDI <0.3) and especially a high ZP of >+30 mV was investigated according to the aimed formation of a complex with pEGFP-C1 (pDNA).

2.2.3 Complexation Studies of Cationic Nanoemulsion-pDNA

After freshly preparing the CNEs, their complex formation ability with pDNA was evaluated by the use of different relations of CNEs and pDNA,

shown with gel retardation assay. The augmenting amount of CNEs (0.5 - 5 µl, 1:10 diluted) was 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 [26]. The agarose gel electrophoresis (1% agarose/1xTAE, w/v) was carried out for 60 min under the voltage of 80 V and imaged *via* a gel documentation system (Vilber Lourmat, France). The CNE:pDNA complexes were freshly prepared before each use for further studies.

2.2.4 SDS Release Studies of pDNA from Cationic Nanoemulsion-pDNA Complex

SDS release studies of pDNA by agarose gel electrophoresis are necessary to provide that the cargo is released to reach the target. After forming the CNE-pDNA complex, SDS was immediately added and incubated for 10 min at 25 °C. Following the addition of glycerine, the samples were loaded on 1 % agarose gel electrophoresis.

SDS was added immediately after complex formation. Following 10 minutes incubation at 25°C, samples were loaded on 1% agarose gel for electrophoresis. Six CNE:pDNA ratios were tested – 0.5:1, 1:1, 2:1, 3:1, 4:1 and 5:1 (v/v).

The resultant CNE:pDNA complexes were characterized by gel retardation assay [26]. The agarose gel electrophoresis (1% agarose/1xTAE, w/v) was carried out for 60 min under the voltage of 80 V and imaged *via* a gel documentation system (Vilber Lourmat, France). The CNE:pDNA complexes were freshly prepared before each use for further studies.

2.2.5 Cytotoxicity Studies

The cytotoxicity studies of the freshly prepared CNEs were performed using non-cancerous HEK 293 (human embryonic kidney cells) cells and the two breast cancer cells MDA-MB-231 (human breast adenocarcinoma) and MCF-7 (human breast adenocarcinoma). Cells are cultured in DMEM/F12 (1:1) medium which supplemented with 10% fetal bovine serum (FBS) and 0.1% Pen-Strep (10.000 Units/mL penicillin and 10.000 µg/mL Streptomycin) at 37°C and 5% CO_2 with humidified conditions. Cells were inoculated into 96-well plates ($1x10^5$ cells per mL) and then incubated for 24 h at 37°C, 5% CO_2 and humidified conditions. Then, the medium was removed, the cells were washed and treated with 3 different concentrations of CNE 1, CNE1:pDNA complex, CNE 2, CNE2:pDNA complex and CNE 3. In addition, OD-NE, PS, PSHCl and Doxorubicin (DOXO) were used as a control. After 48 h incubation, 20 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution is added and the plates are incubated for 4 h [27]. More after, media was discarded from wells and 150 µl dimethyl sulfoxide was added on each well. For determining the optical density (OD), plates are placed in a spectrophotometer at 570 nm wavelength. Experiments were carried out at least in triplicate.
After OD was determined, cells viability (%) was calculated with the formula below:

%Viable cells: [(absorbance of treated cells)-(absorbance of blank)] / [(absorbance of control)-(absorbance of blank)] X 100 [28**].**

In the end, IC_{50} values calculating by fitting viability (%) data to sigmoidal curve on Graphpad Prism 9 software.

2.2.6 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.

3. Results and Discussion

3.1 Preparation, Droplet Size and Zeta Potential Measurements of Cationic Nanoemulsions

In previous studies, it was shown that the formulation parameter like the type and concentration of the used compounds were more effective than the process parameters like preparation temperature, pressure and duration during the development of NEs were investigated [20-22].

Based on these findings, the CNEs were composed of 0.5% PS (CNE 1 and 3) or PSHCl (CNE 2), 5 % (CNE 3) and 20% OD (CNE 1) and 2% lecithin and 2% Tween 80, respectively [23, 29].

Previously, Peceol was used to solve PS and prepare CNEs. Unfortunately, phase separation occurred after storage of 1 month at 40 °C. Additionally, the ZP significantly decreased from +48.7 mV at the beginning to +12.8 mV after storage of 6 months at 25 °C, showing that this

formulation was not appropriate to form a complex with pDNA in the long term. An appropriate delivery system for nucleic acids should have a long-term ZP >+30 mV enabling its use in due time. Otherwise, the formulation must be prepared every time freshly, which could be interpreted as waste. Providing a DS <300 nm and PDI <0.3, they come second, so the ZP, caused by the cationic agents PS and PSHCl, is more crucial for complexation and delivering plasmid DNA. The effects of the used compounds and of the microfluidization duration from 1-10 minutes on DS, PDI and ZP were studied to optimize the preparation of the CNEs.

With increasing the microfluidization duration from 1 to 10 minutes, the DS of CNE 1 (Figure 2a) decreased, from 159 nm (1 min) to 114 nm (10 min), just like the PDI slightly decreased from 0.09 (1 min) to 0.08 (10 min) (Figure 2a), and the ZP decreased from +43.2 mV (1 min) to +36.6 mV (10 min) (Figure 2b). Increasing the microfluidization duration resulted in a decrease of DS, PDI and ZP, but not all of these effects are desired. Due to the fact that the ZP is more crucial than DS and PDI, a microfluidization duration of 1 minute is appropriate. Similar results were observed by Isar et al. 2020 [23].

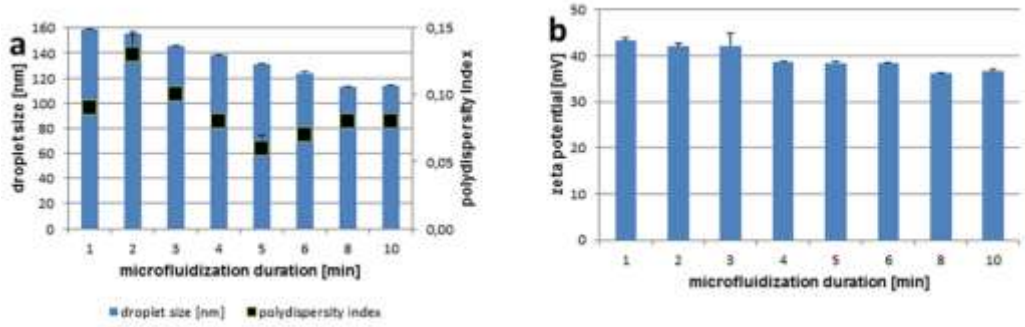

Figure 2 The droplet size, polydispersity index, and zeta potential results of CNE 1 with increasing microfluidization duration.

The DS of CNE 2 decreased from 113 nm (1 min) to 87 nm (10 min) (Figure 3a), the PDI decreased from 0.12 (1 min) to 0.08 (10 min) (Figure 3a), and the ZP first increased from from +42.8 mV (1 min) to +50.1 mV (2 and 3 min), then decreased to +43.6 mV (10 min) (Figure 3b). Due to the fact that the highest ZP was obtained after a microfluidization duration of 2 minutes (+50.1 mV), with a DS of 102 nm and a PDI of 0.09, this is an appropriate microfluidization duration for CNE 2.

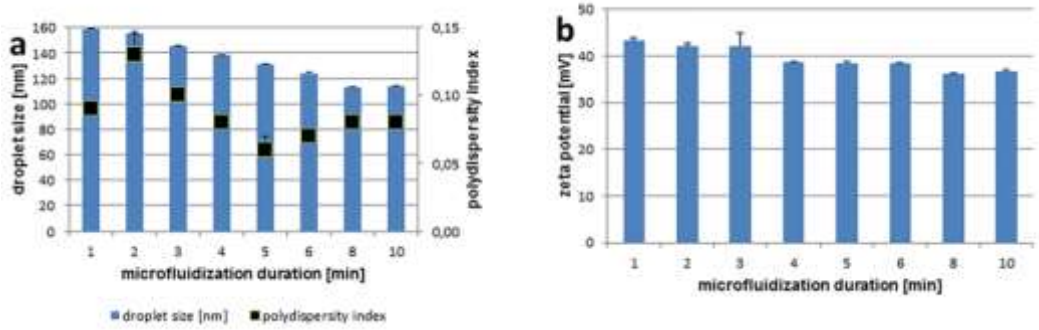

Figure 3: The droplet size, polydispersity index, and zeta potential results of CNE 2 with increasing microfluidization duration.

For CNE 3, the DS decreased from 188 nm (1 min) to 151 nm (10 min) (Figure 4a), but the PDI increased from 0.19 (1 min) to 0.23 (10 min) (Figure 4a), just like the ZP increased from +31.7 mV (1 min) to +35.4 mV (10 min) (Figure 4b). Here, a microfluidization duration of 1 minute is not sufficient to obtain a formulation with a maximum ZP. Increasing the microfluidization duration to 2 minutes resulted in a ZP of +34.1 mV.

A higher ZP was obtained after a microfluidization duration of 2 minutes (+34.1 mV) with a DS of 184 nm and a PDI of 0.19, this is the optimal duration. Further increase of the microfluidization duration did not result in ZPs with significant differences, so a microfluidization duration of 2 minutes is appropriate.

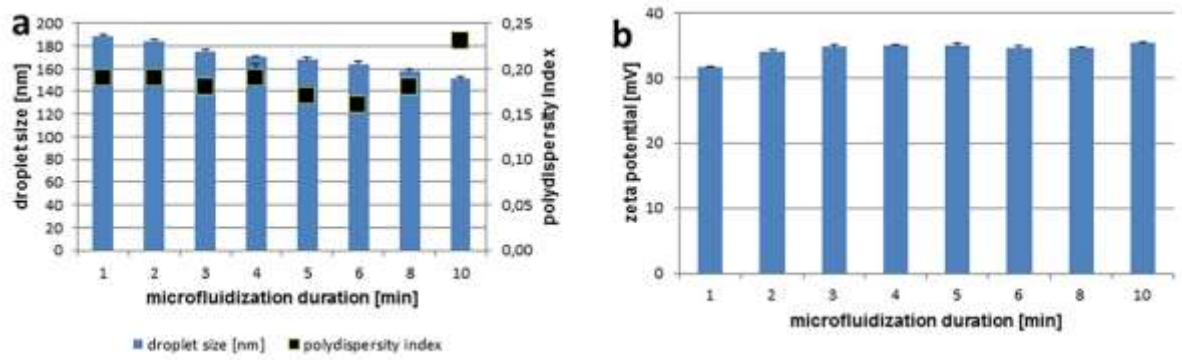

Figure 4: The droplet size, polydispersity index, and zeta potential results of CNE 3 with increasing microfluidization duration.

Comparing the formulation properties of the CNEs showed that for CNE 1 the complexation agent PS was dissolved in the inner oil phase, additionally containing OD and lecithin, whereas for CNE 2, the complexation agent PSHCl was used in the outer aqueous phase, containing Tween 80. The difference between CNE 1 and CNE is based on the concentration of the inner oil phase. CNE 1 contained 20 % OD and 5% OD was used for CNE 3, leading to a formulation with a smaller DS (159 nm) and PDI (0.09) but higher ZP (+43.2 mV) when 20% OD was used, compared to CNE 3, with a DS of 184 nm, a PDI of 0.19 and a ZP of +34.1 mV.

The smallest DS of 102 nm, lowest PDI of 0.09 and highest ZP of +50.1 mV were observed for CNE 2, where PSHCl was used as complexation agent in the outer aqueous phase. Since the particle properties of CNE 1 and CNE 2 seem to be more beneficial compared to CNE 3, it can be supposed that CNE 3 is probably not suitable for our purpose of complexation pDNA.

CNE 1 showed after storage of up to 12 months at 25 and 40 °C no significant changes of DS and PDI (Table 2). The DS of 159 nm at the day of preparation increased to 176 nm and 180 nm after storage of 12 months at 25 and 40 °C, respectively.

Table 2: The droplet size, polydispersity index, and zeta potential results of cationic nanoemulsion 1 after storage of 12 months at 25 and 40 °C.

storage time and temperature	DS [nm ± SD]	PDI [± SD]	ZP [mV± SD]
d0	159 ± 1.3	0.09 ± 0.01	43.2 ± 1.3
d7, 25 °C	160 ± 2.1	0.12 ± 0.05	39.6 ± 1.2
d7, 40 °C	159 ± 1.7	0.13 ± 0.03	38.2 ± 1.7
d14, 25 °C	168 ± 2.5	0.08 ± 0.02	36.0 ± 1.9
d14, 40 °C	165 ± 0.6	0.09 ± 0.01	32.9 ± 0.3
d28, 25 °C	164 ± 1.2	0.10 ± 0.01	39.9 ± 1.3
d28, 40 °C	161 ± 0.3	0.10 ± 0.01	19.6 ± 0.4
m3, 25 °C	169 ± 3.2	0.09 ± 0.02	33.9 ± 0.9
m3, 40 °C	182 ± 5.3	0.06 ± 0.02	20.1 ± 0.2
m6, 25 °C	176 ± 3.1	0.09 ± 0.01	32.4 ± 0.7
m6, 40 °C	185 ± 3.5	0.04 ± 0.01	19.4 ± 0.4
m9, 25 °C	171 ± 2.8	0.12 ± 0.01	35.4 ± 0.8
m9, 40 °C	180 ± 1.7	0.07 ± 0.03	19.9 ± 0.4
m12, 25 °C	176 ± 2.7	0.09 ± 0.05	33.8 ± 0.8
m12, 40 °C	180 ± 1.7	0.07 ± 0.03	19.9 ± 0.4

The ZP decreased from 43.2 mV to 33.8 mV after storage of 12 months at 25 °C, still more than the aimed 30 mV, and to 19.9 mV after storage of 12 months at 40 °C (Table 2). Thus, even after storage at 25 °C, CNE 1 is a ready to use formulation for further studies, without the need for fresh preparation.

The DS of CNE 2 showed no significant changes over 28 days, however storage at 40 °C caused an increase from 102 nm to 160 nm (Table 3). Finally, DSs of 179 nm and 203 nm were observed after storage of six and nine months at 25 °C, respectively. After storage of six months at 40 °C phase separation was observed. The PDI was very constant over nine months, at 25 °C. Just like the DS, the ZP was stable after storage of 28 days at 25 °C with values of 50.1 mV at the beginning and 48.3 mV after 28 days. Unfortunately, the ZP decreased after storage of 28 days at 40 °C to 39 mV, but remained between 38.3 and 41.8 mV, even after storage of three months at 25 and 40 °C, six and nine months at 25 °C (Table 3). The stability studies for storage of 12 months are ongoing.

Table 3: The droplet size, polydispersity index, and zeta potential results of cationic nanoemulsion 2 after storage of nine months at 25 °C and three months at 40 °C.

storage time and temperature	DS [nm ± SD]	PDI [± SD]	ZP [mV± SD]
d0	102 ± 0.4	0.09 ± 0.01	50.1 ± 0.4
d7, 25 °C	124 ± 1.1	0.09 ± 0.03	52.6 ± 2.3
d7, 40 °C	123 ± 0.7	0.07 ± 0.01	50.1 ± 1.3
d14, 25 °C	122 ± 1.8	0.09 ± 0.03	50.3 ± 1.8
d14, 40 °C	125 ± 0.6	0.07 ± 0.01	47.7 ± 0.2
d28, 25 °C	131 ± 1.9	0.10 ± 0.01	48.3 ± 0.5
d28, 40 °C	160 ± 2.6	0.05 ± 0.02	39.0 ± 0.5
m3, 25 °C	155 ± 4.1	0.08 ± 0.02	38.6 ± 0.8
m3, 40 °C	155 ± 2.0	0.07 ± 0.03	41.8 ± 0.3
m6, 25 °C	179 ± 2.7	0.08 ± 0.02	40.0 ± 1.2
m9, 25 °C	203 ± 5.1	0.09 ± 0.02	38.3 ± 0.3

Storage of CNE 3 at 25 and 40 °C over a period of three months had no negative effect on DS, which was 184 nm at the beginning and 164 nm and 171 nm after three months (Table 4). A decrease of the PDI from 0.19 to 0.07 and 0.06, respectively, after storage three months at 25 and 40 °C was observed. The reason for that decrease is probably based on a more compact structure and size distribution with increasing storage time. A slight decrease of DS is an indicator for this, too. The ZP revealed stable values of 34.1 mV at the beginning and 34.4 mV after storage of three months at 25 °C. Unfortunately, storage at 40 °C had non-beneficial effects on ZP, resulting in a decrease from 34.1 mV to 12.4 mV (Table 4), which is not appropriate. The stability studies for storage of 6 months and further are ongoing.

Table 4: The droplet size, polydispersity index, and zeta potential results of cationic nanoemulsion 3 after storage of three months at 25 and 40 °C.

storage time and temperature	DS [nm ± SD]	PDI [± SD]	ZP [mV± SD]
d0	187 ± 3.3	0.19 ± 0.02	34.1 ± 0.7
d7, 25 °C	179 ± 4.0	0.20 ± 0.04	32.7 ± 1.5
d7, 40 °C	173 ± 4.5	0.17 ± 0.01	27.2 ± 1.8
d14, 25 °C	179 ± 3.1	0.19 ± 0.03	34.8 ± 0.3
d14, 40 °C	171 ± 0.4	0.21 ± 0.01	24.0 ± 2.4
d28, 25 °C	178 ± 2.3	0.19 ± 0.02	32.8 ± 0.2
d28, 40 °C	164 ± 1.6	0.21 ± 0.01	26.8 ± 0.4
m3, 25 °C	164 ± 1.7	0.07 ± 0.03	34.4 ± 0.4
m3, 40 °C	171 ± 2.6	0.06 ± 0.02	12.4 ± 0.5

CNE 1 and 3 showed appropriate DSs, PDIs, and ZPs after storage of 12 months for CNE 1 and three months for CNE 3 at 25 °C. Storage at 40 °C had disadvantageous effects on ZP, which significantly decreased to <30 mV. On the contrary to these CNEs, the DS of CNE 2 increased, but is still <300 nm, the PDI is constant and the ZP only slightly decreased from 50.1 mV to 38.3 mV after storage of nine months at 25 °C. The fact that phase separation was observed for CNE 2 is a

disadvantage in terms of usability for further studies. On the other hand, the ZPs of CNE 1 and 3 are significantly decreasing but still <30 mV, which is also a drawback. It can be pointed out that the CNEs showed stable DS, PDI and ZPs after storage at 25 °C, except for CNE 2 with increasing DS. Here, it can be stated that using PSHCl for the outer aqueous phase has no beneficial effects on particle properties compared to PS used in the inner oil phase. If intended to use a freshly prepared formulation, CNE 2 seemed to be more appropriate due to the highest ZP of the CNEs. However, this was revised after the stability studies due to decreased ZP and increased DS results.

Further studies about complexation with pDNA, SDS release and cytotoxicity will help to find out the best possible formulation for delivering pDNA.

3.2 Gel Retardation Assay of Cationic Nanoemulsion-pDNA Complexes

Our supposition that CNE 3 is not suitable for complexation pDNA was right, and a CNE3-pDNA complex was not obtained probably due to the ZP. According to the CNEs-pDNA complexation results, the complex of 1 µl plasmid and 5 µl CNE 1 (Figure 5a) and 1 µl plasmid and 0.5 µl CNE 2 (Figure 5b), respectively, (1:10 diluted) were appropriate for further studies. For CNE 3 it was not possible to form a suitable complex with pDNA, so this CNE was rated as not appropriate for our purposes to form an appropriate complex with pDNA.

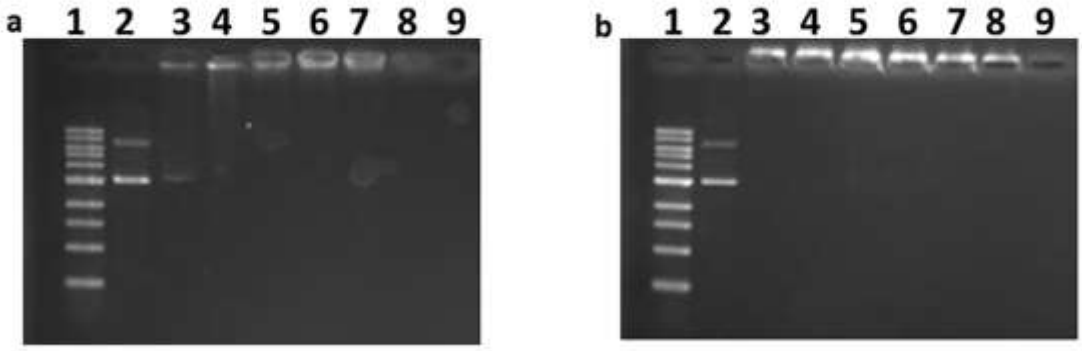

Figure 5: Gel electrophoresis image of the complexation assay between the constant amount of pEGFP-C1 with the increasing amount of CNE 1 (a) or CNE 2 (b). (1:1Kb plus DNA ladder, 2: Naked DNA as positive control, 3,4,5,6,7,8: pDNA:CNE complexes for the ratio of 1:0.5, 1:1, 1:2, 1:3, 1:4, 1:5 (v/v),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

3.3 Particle Characterization of Cationic Nanoemulsion-pDNA Complex Formulations

For CNE 1, the DS only slightly decreased from 192 nm to 181 nm after complexation with pDNA, the PDI slightly decreased from 0.13 to 0.15, but the ZP decreased from +46.6 mV to +29.5 mV, still around the required value of 30 mV for stable nanoparticles (Figure 6). The formation of a CNE 2-pDNA complex resulted in a significant increase of the DS from 144 nm to 288 nm, an increase of the PDI from 0.14 to 0.21, and a significant decrease of the ZP from +47.9 mV to +5.1 mV. Comparing both CNE-pDNA complex formulations revealed that the DS of CNE 1-pDNA is smaller (181 nm) than of CNE 2-pDNA (288 nm), the size distribution of CNE 1-pDNA is more narrow (PDI of 0.15) compared to CNE 2-pDNA (PDI of 0.21) and the ZPs of approximately +30 mV (CNE 1-pDNA) vs +5.1 mV (CNE 1-pDNA). These results can be explained with the different CNE:pDNA ratios. The ratio CNE 1:pDNA was 5:1, while for CNE 2:pDNA (0.5:1) the amount of CNE was lower, resulting in a significant increase of DS and decrease of ZP. 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 study results using HEK293 cells will give more insights.

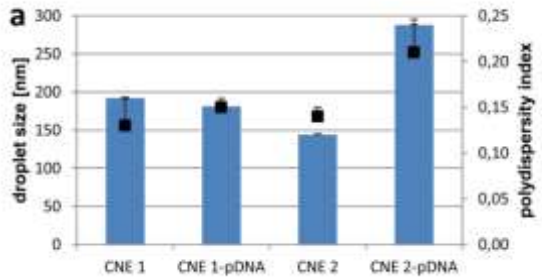

Figure 6: The droplet size, polydispersity index, and zeta potential results of the cationic nanoemulsions 1 and 2 before and after complexation with pDNA.

3.4 SDS Release Studies of pDNA from Cationic Nanoemulsion-pDNA Complexes

To investigate the SDS induced pDNA release studies from CNE-pDNA complexes, these were prepared in six different CNE:pDNA ratios from 0.5:1 to 5:1 (v/v). It could be shown that 1% SDS was appropriate for the release of pDNA, independent from the CNE-pDNA ratio (Figure 7).

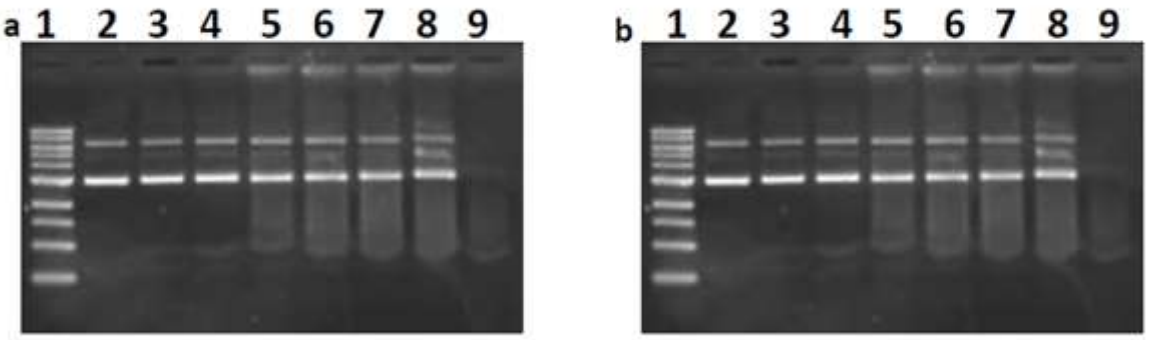

Figure 7: SDS release of pDNA from CNE 1-pDNA (a) and CNE 2-pDNA (b) complexes at six different ratios; lane 1: MW; lane 2: pDNA as control; 3-8: CNE-pDNA ratios from 0.5:1 - 5:1; lane 9: blank CNE.

3.5 Cytotoxicity

The cytotoxicity study results of the CNEs, CNE-pDNA complexes, OD-NE, PS, PSHCl and DOXO using HEK 293 revealed that with increasing the applied dose from 0.25 µg/ml to 25 µg/mL, the viability of the HEK 293 cells decreased from 78% to 14% for CNE 1, from 73% to 12% for CNE 2, from 71% to 16% for CNE 3 (Figure 8a). This decreasing effect of the viability of CNE 1 and CNE 2 is not desired on non-cancerous cells. For the CNE-pDNA complexes, the viability decreased from 99% to 77 % for CNE 1-pDNA and from 96% to 79% for CNE 2-pDNA (Figure 8a). There are no significant differences in the viabilities between the CNE-pDNA complexes. Thus, complexation with pDNA resulted in increased viability.

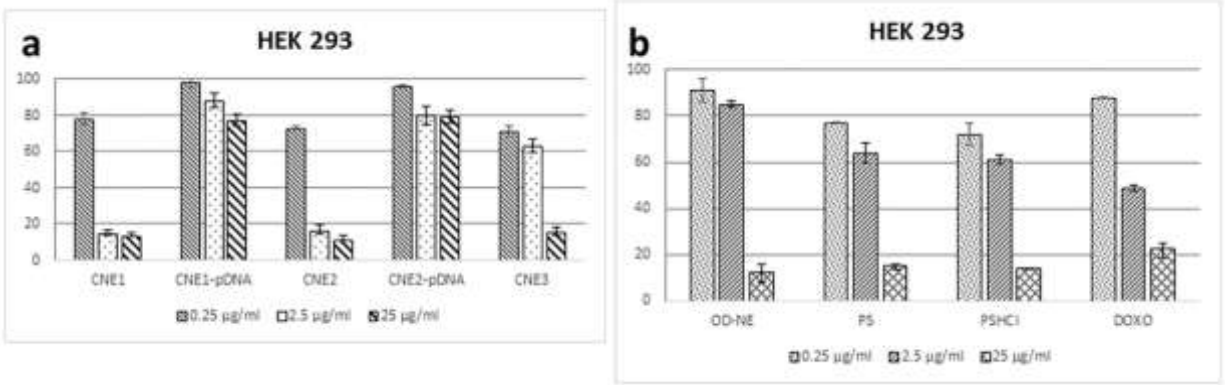

Figure 8: Cytotoxicity study results of CNEs, CNE-pDNA complexes, OD-NE, PS, PSHCL and DOXO on HEK 293 cells.

The viability of OD-NE decreased from 81% to 14% with increasing applied doses (Figure 8b). Thus, a distinctive effect of OD as the oil component on the viability of HEK 293 cells was observed.

Another factor influencing the cytotoxicity of the CNEs is the ZP. Applicating a concentration of 2.5 µg/mL resulted in viabilities of 15% for CNE 1, 17% for CNE 2 and 63% for CNE 3, respectively. Comparing the ZPs of the CNEs showed results of 46.6 mV for CNE 1, 47.9 mV for CNE 2 and 34.1 mV for CNE 3. Thus, the higher the ZP, the more decreased viabilities were observed. A compromise of a ZP of approximately about 30 mV is indicated as appropriate for delivering nucleic acids like pDNA.

The viabilities decreased from 77% to 15 % for PS, from 72% to 14 % for PSHCl and from 88% to 22 % for DOXO as positive control (Figure 8b). The viability results can be summarized in three groups: The first group with similar viabilities of CNE 1, CNE 2 and OD-NE. The second group with CNE 3, PS and PSHCl. And the third group with the CNE-pDNA complexes.

Regarding the cytotoxicity study results on HEK 293 it can be stated that all three CNE-pDNA complexes showed suitable viabilities. However, from the CNEs, CNE 3 seems to be more appropriate than CNE 1 and 2.

The cytotoxicity study results of the CNEs, CNE-pDNA complexes, OD-NE, PS, PSHCl and DOXO using MDA-MB-231 cells showed that with increasing the applied dose from 0.25 µg/ml to 25 µg/mL, the viability of the MDA-MB-231 cells decreased from 97% to 25% for CNE 1, from 95% to 10% for CNE 2, from 102% to 40% for CNE 3 (Figure 9a). The highest decrease in the viability to 10% was observed for CNE 2, containing PSHCl in the outer aqueous phase. The difference between CNE 1 and CNE 2 is the use of the cationic agents PS in the inner oil phase and PSHCl in the outer aqueous phase. Complexation of the CNEs with pDNA resulted in increased viabilities for all CNE-pDNA complexes, as expected (Figure 9a). The viability decreased from 98% to 26 % for OD-EA. For PS, the viability decreased from 95% to 13%, from 103% to 12% for PSHCl and from 98% to 30 % for DOXO as positive control (Figure 9b).

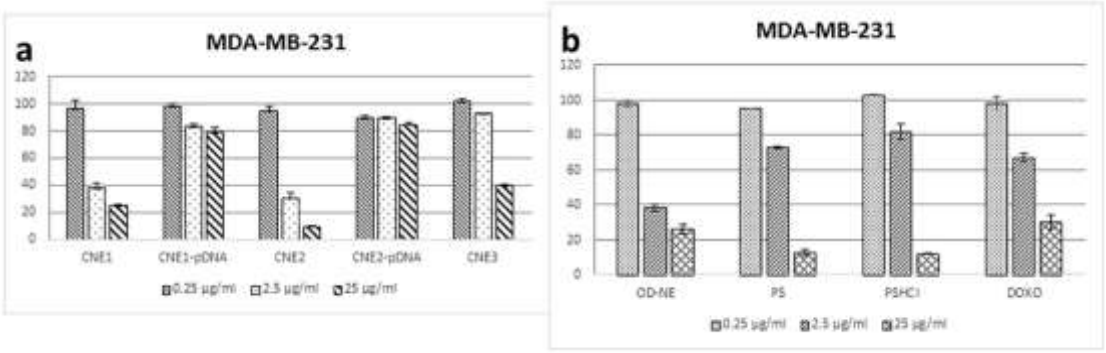

Figure 9: Cytotoxicity study results of CNEs, CNE-pDNA complexes, OD-NE, PS, PSHCL and DOXO on MDA-MB-231 cells.

Interestingly, the decrease of the viabilities on breast cancer cells is more distinctive for PS (13 %) and PSHCl (12 %), compared to DOXO (30 %). Comparing CNE 1 and CNE 3 revealed that the concentration of the oil compound has a distinctive effect on cytotoxicity. The higher the oil concentration, the higher decrease in the viability was observed.

Regarding these results showed that CNE 2, showing the highest decrease in viability, is superior to CNE 1 and 3. These results can be explained with the DS and ZP results of the CNEs. The smallest DS and highest ZP were obtained for CNE 2 (144 nm and 47.9 mV). Thus, CNE 2 seems to be more appropriate.

With increasing the applied dose of CNEs, CNE-pDNA complexes, OD-NE, PS, PSHCl and DOXO from 0.25 µg/ml to 25 µg/mL, the viability of the MCF-7 cells decreased from 77% to 48 % for CNE 1, from 85% to 13% for CNE 2 and from 107% to 54% for CNE 3 (Figure 10a). The highest decrease in the viability to 13% was observed for CNE 2, containing PSHCl in the outer aqueous phase with the highest ZP of 47.9 mV. Complexation of the CNE 1 and 2 with pDNA resulted in increased viability of 76 % for CNE 1-pDNA and 86 % for CNE 2-pDNA, compared to CNE 1 and 2 with viabilities of 48 % and 13 %, respectively, with an applied concentration of 25 µg/mL. For the OD-NE, the viability decreased from 92% to 50 % (Figure 10b).

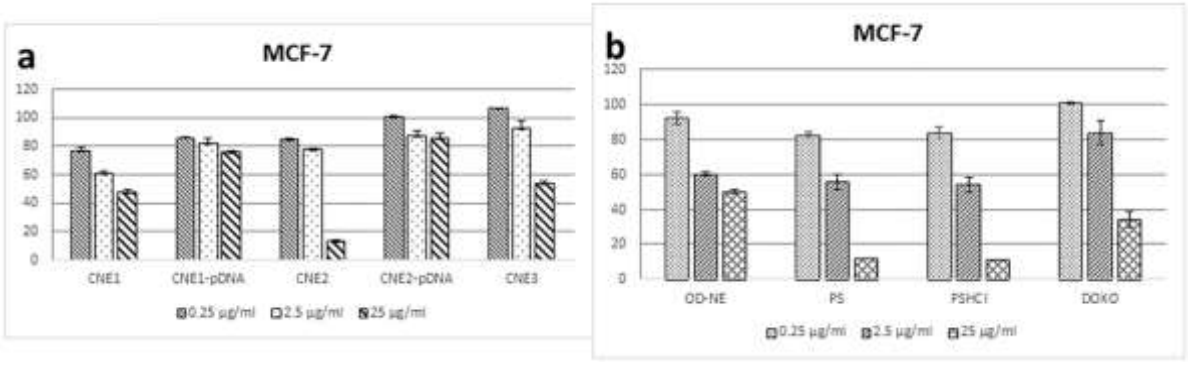

Figure 10: Cytotoxicity study results of CNEs, CNE-pDNA complexes, OD-NE, PS, PSHCL and DOXO on MCF-7 cells

The viability of MCF-7 cells decreased from 83 % to 12 % for PS, from 84 % to 11 % for PSHCl and from 101 % to 34 % for DOXO as positive control (Figure 10b). Again, the decrease of the viability is more distinctive for PS and PHCl, compared to DOXO, with viabilitiesof 12 % (PS), 11 % (PSHCl) vs. 34% (DOXO). Interestingly, incorporation of PSHCl into the CNE 2 has no effect on the viability, reflected as viability of 11 % for PSHCl compared to CNE 2 with a viability of 13 %. In addition, there is not a significant difference in the viabilities of CNE 1 (48 %) and CNE 3 (54 %) and OD-NE (50 %). Thus, the cytotoxic effect of CNE 1 and 3 is probably not based on PS, but on the CNE itself.

Evaluation of zeta potential in gene delivery systems is crucial in terms of electrostatic interaction with genetic materials and interaction with the negatively charged cell membrane, but also in terms of cytotoxicity. The substance that provides the cationic charge in these systems is also the main source of the cytotoxicity [30,31]. Therefore, the evaluation of zeta potential also reflects the evaluation of the cationic content in the formulation as a whole.

Based on these results it can be stated that CNE 2 seems to be superior compared to CNE 1 and 3.

4. Conclusion

Three CNEs were prepared by microfluidization and characterized in terms of DS, PDI, ZP, long-term stability after storage at 25 and 40 °C, complexation with pDNA, SDS induced release and particle characterization of CNE-pDNA complexes and cytotoxicity on breast cancer cells MDA-MB-231 and MCF-7. The prepared CNEs showed appropriate properties like a small DS of <200 nm, a narrow size distribution, expressed as PDI <0.3, and a high zeta potential of >+30 mV. Long-term stability studies after storage at 25 and 40 °C have shown that after storage of six months at 40 °C phase separation was observed for CNE 2. Thus, CNE 2 seems to be not appropriate in terms of stability. Unfortunately, only CNE 1 and 2 were able to form complexes with pDNA. CNE 1-pDNA had the smallest DS, a PDI of 0.15 and a ZP of still 29.5 mV, and released pDNA. On the other hand, the DS of CNE 2 increased twofold, the PDI increased significantly and the ZP decreased significantly. The highest decrease in the viabilities of MDA-MB-231 and MCF-7 cells to 10 % and 13 %, respectively, was observed for CNE 2.

Although the CNEs are not ideal and each showed some disadvantages, CNE 1 seems to be more appropriate than CNE 2 and CNE 3.

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Author's Contributions

Selen Isar: Drafted and performed some of the experiments like preparation, droplet size and zeta potential measurements.
Yiğit Şahin: Performed the cytotoxicity studies.
Hasan Akbaba: Drafted and performed some of the experiments like complexation studies, SDS release studies and performed the statistical analysis.
Ayşe Nalbantsoy: Drafted and supervised the cytotoxicity studies and analyzed their results.
Gülşah Erel Akbaba: Drafted some of the experiments, wrote and revised the manuscript,
Yücel Başpınar: Drafted and wrote the manuscript, performed some of the experiments and analyzed all results.

Ethics

There are no ethical issues after the publication of this manuscript.

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