Video Article

Generation of Cationic Nanoliposomes for the Efficient Delivery of *In Vitro* Transcribed Messenger RNA

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URL: https://www.jove.com/video/58444
DOI: doi:10.3791/58444

Keywords: mRNA, nanoliposomes, DOPE, DC-cholesterol, encapsulation, transfection, delivery, therapeutics

Date Published: 8/13/2018

Citation: Michel, T., Link, A., Abraham, M., Schlensak, C., Peter, K., Wendel, H.P., Wang, X., Krajewski, S. Generation of Cationic Nanoliposomes for the Efficient Delivery of *In Vitro* Transcribed Messenger RNA. *J. Vis. Exp.* (), e58444, doi:10.3791/58444 (2018).

Abstract

The development of messenger RNA (mRNA)-based therapeutics for the treatment of various diseases becomes more and more important because of the positive properties of *in vitro* transcribed (IVT) mRNA. With the help of IVT mRNA, the *de novo* synthesis of a desired protein can be induced without changing the physiological state of the target cell. Moreover, protein biosynthesis can be precisely controlled due to the transient effect of IVT mRNA.

For the efficient transfection of cells, nanoliposomes (NLps) may represent a safe and efficient delivery vehicle for therapeutic mRNA. This study describes a protocol to generate safe and efficient cationic NLps consisting of DC-cholesterol and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) as a delivery vector for IVT mRNA. NLps having a defined size, a homogeneous distribution, and a high complexation capacity, and can be produced using the dry-film method. Moreover, we present different test systems to analyze their complexation and transfection efficacies using synthetic enhanced green fluorescent protein (eGFP) mRNA, as well as their effect on cell viability. Overall, the presented protocol provides an effective and safe approach for mRNA complexation, which may advance and improve the administration of therapeutic mRNA.

Introduction

The use of modified mRNA for therapeutic applications has shown great potential in the last couple of years. In cardiovascular, inflammatory, and monogenetic diseases, as well as in developing vaccines, mRNA is a promising therapeutic agent[1].

Protein replacement therapy with mRNA offers several advantages over the classical gene therapy, which is based on DNA transfection into the target cells[2]. The mRNA function initiates directly in the cytosol. Although the plasmid DNA (pDNA), a construct of double-stranded, circular DNA containing a promoter region and a gene sequence encoding the therapeutic protein[3], also acts in cytosol, it can only be incorporated into cells which are going through mitosis at the time of transfection. This reduces the number of transfected cells in the tissue[1,4]. Specifically, the transfection of tissues with weak mitosis activity, such as cardiac cells, is difficult[5]. In contrast to pDNA, the transfection and translation of mRNA occur in mitotic and non-mitotic cells in the tissue[1,6]. The viral integration of DNA into the host genome may come with mutagenic effects or immune reactions[7,8], but after the transfection of cells with a protein-encoding mRNA, the *de novo* synthesis of the desired protein starts autonomously[9,10]. Moreover, the protein synthesis can be adjusted precisely to the patient's need through individual doses, without interfering with the genome and risking mutagenic effects[11]. The immune-activating potential of synthetically generated mRNA could be dramatically lowered by using pseudo-uridine and 5'-methylcytidine instead of uridine and cytidine[12]. Pseudo-uridine modified mRNA has also been shown to have an increased biological stability and a significantly higher translational capacity[13].

To be able to benefit from the promising properties of mRNA-based therapy in clinical applications, it is essential to create a suitable vehicle for the transport of mRNA into the cell. This vehicle should bear non-toxic properties *in vitro* and *in vivo*, protect the mRNA against nuclease-degradation, and provide sufficient cellular uptake for a prolonged availability and translation of the mRNA[14].

Among all possible carrier types for *in vivo* drug delivery, such as carbon nanotubes, quantum dots, and liposomes, the latter have been studied the most[15,16]. Liposomes are vesicles consisting of a lipid bilayer[10]. They are amphiphilic with a hydrophobic and a hydrophilic section, and through the self-arrangement of these molecules, a spherical double layer is formed[17]. Inside the liposomes, therapeutic agents or drugs can be encapsulated and, thus, protected from enzymatic degradation[18]. Liposomes containing N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA)[19], [1,2-bis(oleoyloxy)-3-(trimethylammonio)propane] (DOTAP)[20], and dioctadecylamidoglycylspermine (DOGS)[21], or DC-cholesterol[22], are well characterized and frequently used for cellular transfection with DNA or RNA.

Cationic liposomes comprise a positively charged lipid and an uncharged phospholipid[23]. Transfection *via* cationic liposomes is one of the most common methods for the transport of nucleic acids into cells[24,25]. The cationic lipid particles form complexes with the negatively charged

phosphate groups in the backbone of nucleic acid molecules[26]. These so-called lipoplexes attach to the surface of the cell membrane and enter the cell through endocytosis or endocytosis-like-mechanisms[27].

In 1989, Malone *et al*. successfully described cationic lipid-mediated mRNA transfection[28]. However, using a mixture of DOTMA and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), the group found that DOTMA manifested cytotoxic effects[28]. Additionally, Zohra *et al*. showed that DOTAP (1,2-dioleoyloxy-3-trimethylammonium-propane chloride) can be used as an mRNA transfection reagent[29]. However, for the efficient transfection of cells, DOTAP should be used in combination with other reagents, such as fibronectin[29] or DOPE[30]. So far, DOTMA was the first cationic lipid on the market used for the gene delivery[31]. Other lipids are used as therapeutic carriers or are being tested in different stages of clinical trials, (e.g., EndoTAG-I, containing DOTAP as a lipid carrier), is currently being investigated in a phase-II clinical trial[32].

This work describes a protocol for the generation of NLps containing DC-cholesterol and DOPE. This method is easy to perform and allows the generation of NLps of different sizes. The general goal of NLp generation using the dry-film method is to create liposomes for mRNA complexation, thus allowing efficient and biocompatible cell transfection *in vitro*[14,33].

Protocol

1. Generation of Cationic Nanoliposomes (Figure 1)

1. Dissolve the lipids DC-cholesterol (3β-[N-(N′,N′-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride) and DOPE (dioleoyl phosphatidylethanolamine), delivered as a powder, in chloroform to achieve a final concentration of 25 mg/mL.
   Note: Store the dissolved lipids at -20 °C.
2. Work with 25 mg/mL stock solution of both lipids. Mix 40 µL of the dissolved DC-cholesterol and 80 µL of the dissolved DOPE in a glass flask.
   Note: The total lipid amount is 3 mg. To avoid fast evaporation of the chloroform, place the lipids on ice during pipetting.
3. Vaporize the chloroform for 15 min under an argon gas flow. Subsequently, fill a desiccator with silica gel, place the open glass flask inside, and apply a vacuum overnight to make sure that the remaining chloroform is evaporated, and a lipid film is formed inside the glass flask.
   Note: Work fast and avoid unnecessary O_2 contact.
4. Rehydrate the formed lipid film with 1 mL of nuclease-free water and vortex the suspension for 15 min (**Figure 2A**). Afterward, place the suspension into a sonication bath for 1 h (**Figure 2B**).
   Note: The suspension will be slightly cloudy.
5. Assemble the mini extruder according to the manufacturer's instructions, fill a syringe with the lipid suspension, and place the filled syringe and an empty syringe on both sides of the extruder. Press the lipid suspension through the membrane from one syringe to the other, 20x – 25x, to extrude the suspension (**Figure 2C**).
   Note: The size of the NLps is determined by the pore size of the membrane used.
6. Store the NLps in a glass flask at 4 °C until further use.
   Note: After prolonged storage time, the NLps should be placed in the sonication bath again for 15 min by 35 kHz to circumvent complex formation.

2. *In Vitro* Transcription of Synthetic mRNA

1. Prepare the eGFP-encoding mRNA following the protocol published earlier[34].
2. Amplify the eGFP sequence from the plasmid with 0.7 µM of the forward (5'-TTG GAC CCT CGT ACA GAA GCT AAT ACG-3') and reverse (5'-T_{120} CTT CTT ACT CAG GCT TTA TTC AAA GAC CA-3') primers and the polymerase kit with PCR.
   1. Mix 20 µL of a commercial buffer solution which changes the melting behavior of DNA (**Table of Materials**), as well as 20 µL of the 5x mix from the polymerase kit. Add 7 µL of each (forward and reverse) primer.
   2. Add 25 ng of the eGFP plasmid to the mixture and 2 µL of polymerase from the polymerase kit.
      Note: Keep the polymerase on ice before pipetting it to the solution.
   3. Add nuclease-free H_2O to the mixture, up to a volume of 100 µL.
   4. Run the PCR cycles in the thermocycler following the protocol in **Table 1**.
3. Purify the eGFP-encoding DNA sequence with the PCR purification kit.
   1. Therefore, mix the PCR solution with 500 µL of binding buffer from the kit and use it to fill purification columns.
   2. Centrifuge the columns at maximum speed for 1 min and discard the filtrate.
   3. Add 750 µL of wash buffer I to the column, centrifuge again at the maximum speed for 1 min, and discard the filtrate.
   4. Repeat the centrifuge step 1x to remove the buffer from the column filter.
   5. Transfer the column to a fresh 1.5-mL tube.
   6. Add 20 µL of nuclease-free H_2O to the column, incubate for 1 min, and centrifuge for 1 min at maximum speed. Repeat this step 1x.
   7. Measure the concentration of the DNA with a photometer and store it at -20 °C.
   8. Perform the DNA quality analyses using gel electrophoresis. Add 0.5 g of agarose in Tris-Borate-EDTA (TBE) buffer and heat it up in the microwave on high heat until the agarose is completely dissolved.
   9. Add 5 µL of gel-staining solution to the liquid agarose, fill the solution into the gel chamber, and wait until the gel is polymerized.
   10. Mix 200 ng of DNA with 2 µL of 6x loading dye and fill it up to an end volume of 12 µL. Pipette a DNA ladder, as well as the DNA sample, into the gel wells and run the electrophoresis for 1 h at 100 V.
   11. Analyze the gel using a gel-analyzing station under UV light.
4. Run the *in vitro* transcription to generate mRNA from the DNA sequence with an *in vitro* transcription kit containing T7-polymerase and substitute the modified nucleotides of UTP and CTP with Ψ-UTP and methyl-CTP.
   1. For the *in vitro* transcription, mix the ingredients following **Table 2**.

Note: Replace 1.5 µL of methyl-CTP with 1:10 Cy3-CTP diluted in nuclease-free H_2O during the *in vitro* transcription of eGFP mRNA to achieve Cy3-labeling of the mRNA.
2. Incubate the IVT reaction mix for 4 h at 37 °C.
3. Add 1 µL of DNase I from the T7 polymerase kit and incubate it for 15 min by 37 °C to digest the DNA template.

5. To purify the mRNA, use the RNA clean-up kit.
   1. Fill up the IVT mix to the volume of 100 µL with nuclease-free H_2O.
   2. Add 350 µL of lysis buffer and mix by pipetting up and down.
   3. Add 250 µL of 100% ethanol and mix again for 1x. Pipette the mix into the cleanup columns.
   4. Centrifuge for 15 s at 8,000 x *g* and discard the filtrate.
   5. Add 500 µL of the washing buffer into a column, centrifuge again for 15 s at 8,000 x *g*, and remove the filtrate.
   6. Wash the column 1x with 500 µL of wash buffer and centrifuge for 2 min at 8,000 x *g*.
   7. Move the column into a fresh 1.5-mL reaction tube. Elute the mRNA 2x by a 1-min incubation of 20 µL of nuclease-free H_2O on the column membrane, followed by a 1-min centrifugation at maximum speed.
6. Remove the phosphate groups from the mRNA using a dephosphorylation kit. Add 4.5 µL of 10x phosphatase buffer and 1 µL of phosphatase to the mRNA and incubate for 1 h at 37 °C.
7. Purify the mRNA again, following steps 2.5.1 - 2.5.7.
8. Measure the mRNA concertation with a photometer.
9. Use gel electrophoresis to analyze the purity and the size of the mRNA. Therefore, prepare an agarose gel as described in steps 2.3.9 - 2.3.10.
   1. Mix 3.3 µL of formamide, 1 µL of 37% formaldehyde, 1 µL of 10x MEN, and 1.7 µL of 6x loading dye with 200 ng of mRNA and fill it up to 10 µL with nuclease-free H_2O for each sample and RNA marker.
   2. Incubate the mix for 10 min at 65 °C for mRNA denaturation. Load the wells of the gel with the mRNA and RNA marker and run the gel for 1 h at 100 V.
   3. Analyze the gel using a gel doc station with UV light.

3. Complexation of Synthetic mRNA

1. Thaw the synthetic mRNA on ice, vortex it, and centrifuge shortly before opening the tube.
2. Mix 10 µL of synthetic mRNA (mRNA concentration is 100 ng/µL) with 1 µL, 2.5 µL, 5 µL, 10 µL, or 20 µL of NLp suspension (NLp concentration is 3 mg/mL). Centrifuge briefly and incubate for 20 min at room temperature (RT) for nanolipoplex formation.
   Note: Do not mix by pipetting. This can lead to the loss of volume. Vortex shortly for a thorough mixing.
3. Add 1 mL of regular cell medium to the nanolipoplexes and mix them by pipetting up and down.

4. Analysis of the Encapsulation Efficiency of Nanoliposomes

1. To perform the encapsulation experiments, use the RNA quantification kit.
2. Prepare the working solution by diluting the fluorescent dye 1:200 for a high-range and 1:2,000 for a low-range assay.
   Note: Thaw the fluorescent dye on ice. Prepare the working solution directly before use.
3. Prepare the high-range and low-range standard curves using 1 mL of a 2 µg/mL stock solution of eGFP mRNA in nuclease-free H_2O.
   Note: For the standard curves, use the mRNA that will be used in the encapsulation experiments.
4. Use **Table 3** for the preparation of high-range standard (20 ng/mL - 1 µg/mL).
5. For the low-range standard, dilute the 2 µg/mL eGFP mRNA stock solution 1:20 to achieve a final concentration of 100 ng/mL. Prepare a low-range standard (1 ng/mL - 50 ng/mL) as described in **Table 4**.
6. Combine 1 µg of eGFP mRNA (10 µL) and 7.5 µg of NLps (2.5 µL) and incubate for 20 min at RT.
   Note: Keep the mRNA on ice to avoid degradation.
7. Add 1 mL of nuclease-free H_2O to form nanolipoplexes and mix by pipetting up and down.
8. Add 1 mL of 1:200 or 1:2,000 RNA fluorescent dye working solution to the encapsulated samples and standards and incubate for 5 min at RT in the dark.
9. Pipette the standards and samples in duplicates into a black 96-well plate and measure the fluorescence at 530 nm on a microplate reader (**Figure 3**).

5. Preparation of the Cells for Transfection

1. Plate 1.5 x 10^5 A549 cells/well of a 12-well plate 1 d prior to transfection.
2. Incubate the cells at 37 °C and 5% CO_2 in regular cell medium (DMEM/high glucose with 10% fetal bovine serum [FBS], 2 mM L-glutamine, 1% penicillin/streptomycin) for 24 h before transfection.

6. Transfection of the Cells

1. Wash the prepared cells 1x with 1 mL/well PBS (without Ca^{2+}/Mg^{2+}).
2. Add 1 mL of prepared nanolipoplex mixture, containing 1 µg of eGFP mRNA encapsulated into 1-µL, 2.5-µL, 5-µL, 10-µL, or 20-µL NLps, to one well of the prepared plate with A549 cells.
3. Add the nanolipoplex suspension to the cells and incubate at regular conditions for 24 h to analyze transfection efficacy, or for 24 h and 72 h to analyze the cell viability after transfection.

Note: Transfection with NLps does not require a medium change.

7. Analysis of Cell Transfection Efficacy Using Flow Cytometry and Fluorescence Microscopy

1. Remove the supernatant and wash the cells with 1 mL/well PBS (without Ca^{2+}/Mg^{2+}) to remove the remaining NLps.
2. Prepare the cells for flow cytometry.
    1. Trypsinize the cells with 500 µL/well trypsin/EDTA (0.05%) at 37 °C for 3 min. Stop the process and inactivate the trypsin by adding the same amount (500 µL/well) of regular FBS-containing medium.
    2. Centrifuge the cells for 5 min at 400 x *g* and carefully remove the supernatant without touching the cell pellet.
    3. Wash the cells with 1 mL of PBS (without Ca^{2+}/Mg^{2+}).
    4. Resuspend the cells in 300 µL of 1x fixation solution and transfer the cells into flow cytometry tubes.
    5. Analyze the cells at 488 nm in a flow cytometer (**Figure 4A**).
    Note: Vortex the cells 1x directly before measuring.

3. Prepare the cells for fluorescence microscopy.
    1. Fix the cells with 1 mL/well 100% methanol, which was previously stored at -20 °C.
    2. Add 500 µL/well 300 nM DAPI (4′,6-diamidino-2-phenylindole) dissolved in PBS (without Ca^{2+}/Mg^{2+}) and incubate for 5 min in the dark.
    3. Remove the DAPI solution and wash the cells again with 100% methanol stored at -20 °C.
    4. Analyze the cells using a fluorescence microscope (**Figure 4B**).
    Note: Use the following excitation/emission wavelengths: eGFP 488/509 nm, DAPI 358/461 nm, and Cy3 550/570 nm.

8. Cell Viability Assay

1. Dissolve 5 mg of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in 1 mL of RPMI (without phenol red).
Note: The dissolved MTT must be further diluted 1:10 (final concentration: 0.5 mg/mL) in RPMI before use.
2. Wash the transfected cells 3x with 1 mL/well PBS (without Ca^{2+}/Mg^{2}).
Note: The remains of the regular cell medium should be completely removed.
3. Add 500 µL/well of 1:10 diluted MTT solution to the cells and incubate for 4 h at 37 °C.
4. Remove the MTT solution from the cells after incubation and add 500 µL/well DMSO (dimethyl sulfoxide). Incubate again for 10 min at 37 °C.
5. Pipette the DMSO solution in triplets into a clear-bottom 96-well plate and measure the adsorption at 540 nm using a microplate reader.
6. Set the viability of the untreated cell on 100% and calculate the cell viability of the other groups in comparison to the untreated control cells (**Figure 5**).

Representative Results

Using the protocol as described, NLps consisting of the lipids DC-cholesterol and DOPE were prepared using the dry-film method (**Figure 1**). During the preparation, the nanoliposome solution shows different stages in turbidity (**Figure 2**).

The encapsulation efficacy of the NLps can then be analyzed after the encapsulation of 1 µg of eGFP-encoding mRNA by analyzing the free amount of mRNA, which was not encapsulated, using the RNA quantification kit (**Figure 3**).

After the encapsulation of eGFP mRNA in different amounts of NLps, the formed nanolipoplexes can be incubated with cells *in vitro* and the percentage of eGFP-expressing cells can be analyzed using flow cytometry 24 h posttransfection (**Figure 4A**). Even 1 µL of the nanoliposome solution is sufficient to achieve a high transfection of the cells *in vitro*. When the cells are transfected with NLps containing Cy3-labelled eGFP mRNA, the presence of the eGFP mRNA in the cytoplasm (red fluorescence), as well as the already produced eGFP protein (green fluorescence) can be visualized (**Figure 4B**).

Since the transfection of cells using nanolipoplexes can have adverse effects on cells, the viability of the cells was tested 24 h (**Figure 5A**) and 72 h (**Figure 5B**) posttransfection. No effects on cell viability could be detected when the cells were treated with 2.5 or 5 µL of NLps or nanolipoplexes, respectively.

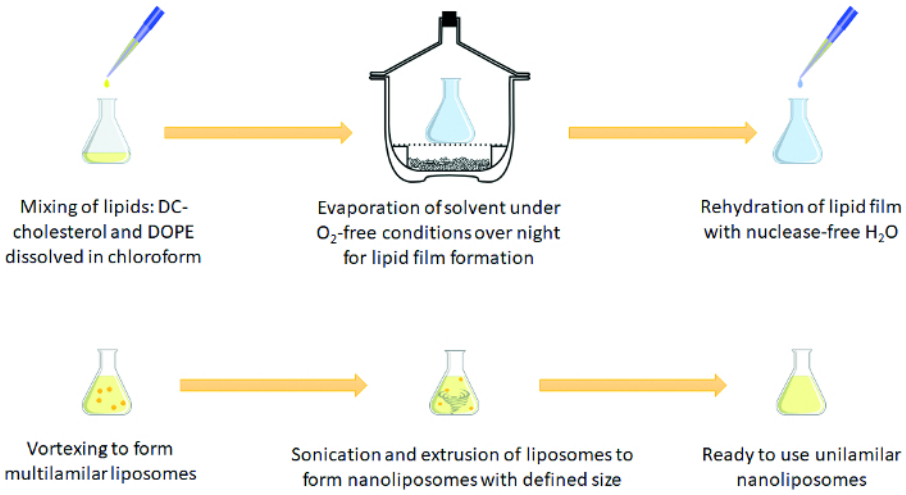

Figure 1: Schematic overview of the manufacturing process of cationic nanoliposomes. First, the dissolved lipids DC-cholesterol and DOPE should be mixed together in a glass flask. Second, the visible chloroform liquid should be evaporated under argon or nitrogen gas flow and the chloroform leftovers should be allowed to evaporate overnight in vacuum. Third, the formed lipid film on the bottom of the glass flask should be rehydrated with nuclease-free H_2O, followed by vortexing to form multilamellar liposomes. Through the sonication and extrusion of the liposome solution, the ready-to-use unilamellar NLps are produced. Please click here to view a larger version of this figure.

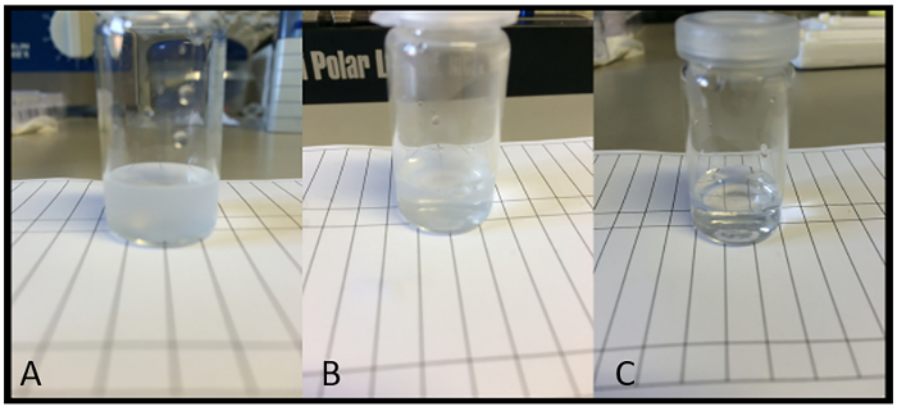

Figure 2: The nanoliposome solution in different stages during manufacturing. (**A**) This figure shows the liposome solution directly after rehydration of the lipid film and vortexing for 15 min, as well as (**B**) after 1 h in a sonication bath (**C**) followed by 25 cycles of extrusion. Please click here to view a larger version of this figure.

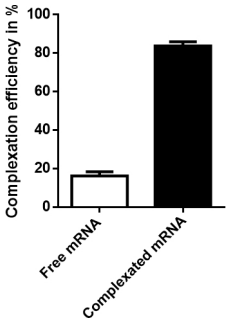

Figure 3: Encapsulation efficacy of the nanoliposomes. This panel shows the quantification of free eGFP mRNA after encapsulation in NLps. The results are presented as means ± SEM (*n* = 3). Please click here to view a larger version of this figure.

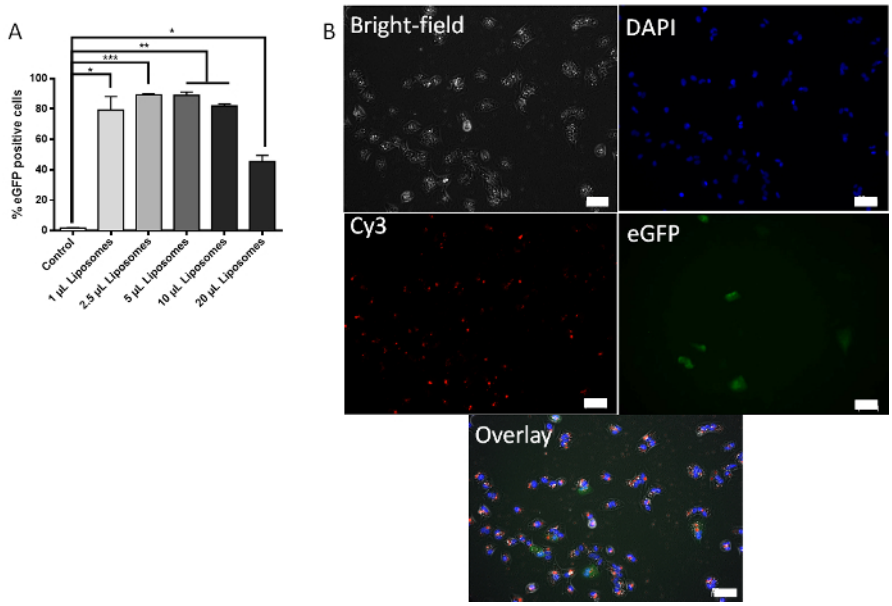

Figure 4: Transfection efficacy of the nanolipoplexes. (**A**) This panel shows the determination of the best mRNA/nanoliposome ratio for transfection using different amounts of NLps to encapsulate 1 µg of eGFP mRNA 24 h posttransfection. (**B**) This panel shows the detection of Cy3-labeled synthetic mRNA encapsulated in 2.5 µL NLps and the eGFP expression in the cells 24 h posttransfection (the scale bar = 50 µm). The results are presented as means ± SEM (*n* = 3). Please click here to view a larger version of this figure.

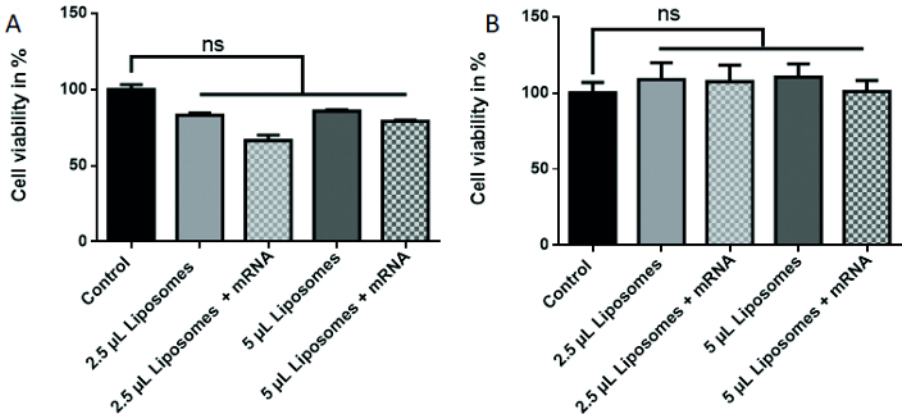

Figure 5: Cell viability after the transfection with nanoliposomes and encapsulated mRNA. This panel shows the measurement of cell viability using an MTT assay (**A**) 24 h and (**B**) 72 h posttransfection. The results are presented as means ± SEM (*n* = 3). Please click here to view a larger version of this figure.

Step	Temperature in °C	Duration
1	95	5 min
2	94	15 s
3	55	1 min
4	72	1 min
5	Go to 2 30 x	
6	72	10 min
7	4	hold

Table 1: PCR cycle protocol for DNA amplification.

	Concentration	Amount
ATP	7,5 mM	4 µL
GTP	1,875 mM	1 µL
5'-Methylcytidin	7,5 mM	3 µL
Ψ	7,5 mM	3 µL
ARCA	2,5 mM	10 µL
DNA Template	1.5 µg	
Buffer mix	1x	4 µL
T7 enzyme mix		4µL
RNase-Inhibitor		1 µL
Nuclease-free water	fill up to 40 µL	

Table 2: Mix for the *in vitro* transcription of DNA to mRNA.

Volume of nuclease-free H_2O (µL)	Volume of eGFP mRNA high-range stock solution (µL)	Volume of 1:200 fluorescent dye (µL)	Final concentration (ng/mL)
0	100	100	1000
50	50	100	500
90	10	100	100
98	2	100	20
100	0	100	blank

Table 3: Protocol for a high-range standard curve.

Volume of nuclease-free H_2O (µL)	Volume of eGFP mRNA low-range stock solution (µL)	Volume of 1:2000 fluorescent dye (µL)	Final concentration (ng/mL)
0	100	100	50
50	50	100	25
90	10	100	5
98	2	100	1
100	0	100	blank

Table 4: Protocol for a low-range standard curve.

Discussion

The presented protocol describes the generation of NLps with high encapsulation efficacy for synthetically modified mRNA, as well as the reliable transfection of cells *in vitro*. Moreover, the NLps guarantee the release of mRNA, which in turn, is translated into a functional protein inside the cells. Additionally, the transfections using NLps can be performed in regular cell medium, resulting in high cell viabilities during transfection, and last up to three days after transfection.

To use mRNA as a therapeutic, self-assembling system for its delivery is preferred. The most common transfection reagents include cationic lipids, including liposomes. As liposomes are positively charged, negatively charged nucleic acids can be encapsulated in them, thereby allowing the electrostatic repulsion of cell membranes to be overcome[35]. The cationic lipid DC-cholesterol has already been described in earlier studies as a stable and biocompatible vehicle[36]. The addition of the neutral lipid DOPE leads to an enhanced transfection efficacy[37]. Considering the outlined advantages mentioned above, these lipids were chosen for the preparation of NLps. In addition, previous studies have demonstrated that the use of these two lipids is preferable over others due to an increased cellular transfection rate with negatively charged nucleic acids[38].

For the successful generation of NLps and nanolipoplexes, it is critical to pay extra attention to some of the steps. The procedure of nanoliposome generation should be carried out under O_2-free conditions. The presence of O_2 during the NLp generation can lead to the degradation of phospholipids and reduced reproducibility[39]. Moreover, despite modifications, mRNA is very sensitive with regard to degradation through nucleases. Hence, for the rehydration of the lipid film, the use of RNase-free H_2O is strongly recommended to prevent mRNA degradation during the complexation. Also, RNase-free conditions should be ensured for the storage of nanolipoplexes.

Furthermore, NLps can form aggregates over time because of the unstable thermodynamic system. The influence parameters include storage temperature and surface charge of the liposomes[40]. NLp aggregation may result in the destabilization of the liposome membrane and the risk of undesirable mRNA release[41], leading to poor transfection efficacy and mRNA degradation in the extracellular space. However, as previously shown, nanolipoplexes can be stored at 4 °C for up to six months without aggregation or loss of transfection efficacy[35].

By using liposomes as a drug carrier system, two of the key problems of drug delivery can be solved. Liposomes protect the encapsulated drug from degradation and are able to passively target tissues that have a discontinuous endothelium, such as the liver or bone marrow[16]. For the delivery of therapeutic nucleic acids, parameters such as particle size and encapsulation capacity are critical for the evaluation and cellular uptake of liposomal vehicles. Particularly, the size of the liposomes in the nanometer scale allows an interaction with the cell membrane[42] and is, thereby, important for the *in vivo* use later. First, the liposomes should be small enough to avoid clearance through the renal and hepatic system[43]. Second, the liposome size should help to overcome the blood vessels' barrier to target the cells of the desired organ. It was reported that liposomes in the size range of 100 - 300 nm were able to efficiently transfect hepatocytes[44]; however, large-sized liposomes (*e.g.*, 400 nm) were not able to overcome the endothelial barrier[45].

Although the described method has been established for mRNA delivery, it can also be implemented for other nucleic acid therapeutics, such as microRNA. In a recent study, we demonstrated that microRNA 126 can be selectively targeted and, therefore, the development of abdominal aortic aneurysms could be effectively prevented[46]. As DNA/RNA therapeutics can cause side effects, such as platelet activation, when they come into direct contact with cells, packaging within liposomes can avoid this, thus rendering it further advantageous[47]. Therefore, the method presented here is highly versatile and can be used for designing drug delivery for many diseases. The established protocol not only allows the fast and cost-effective generation of an efficient mRNA carrier with a defined size but also offers the possibility to customize the lipid formulation according to the needs of a particular application: (1) the size of the liposomes can easily be altered by changing the filters; (2) the surface of the positively charged liposomes could be modified by using, for example, polyethylene glycol, to increase stability and delay blood clearance during *in vivo* application[48]. The binding of specific antibodies to the liposomes allows the targeted delivery of the encapsulated drug[49]. With further examination and a more detailed insight into the physical properties, the protocol might still be improved upon.

For the generation of liposomes, three common methods are available: the dry-film, the ethanol injection, and the reverse-phase evaporation. In the Yang *et al*. study, these three manufacturing techniques were compared[35]. It was found that liposomes with a defined size and an equal distribution in the solution can be generated using the dry-film method. Furthermore, the dry-film procedure conducted in this study resulted in the production of NLps with a defined size of 200 nm, a homogeneous distribution, and a high encapsulation capacity.

On one hand, the positive charge of the liposomes leads to an increased encapsulation capacity and better cell surface fusion[50,51,52], but on the other hand, it may destabilize the cell membrane and activate different immune activation pathways and cell death[27,53]. However, the apoptotic properties of cationic lipids can be minimized by using the helper lipid DOPE[54,55]. In the study by Zhang *et al*., it was found that a 1:2 ratio of DC-cholesterol and DOPE during liposome generation leads to the most efficient cellular transfection using nucleic acids[38]. In the method implemented in the present study, the lipid ratio of 1:2 DC-cholesterol and DOPE was used in the mixed lipid suspension during the lipid film preparation, and the prepared liposomes led to high transfection efficacy and, simultaneously, high cell viability. Similar results were also found by other researchers, such as Ciani[56] and Farhood[37].

Overall, liposomes have been used for years in clinical trials, showing great biocompatibility and low toxicity *in vivo*. In combination with mRNA, NLps could be used for the efficient delivery of mRNA to cells or organs *in vitro* and *in vivo*, to induce *de novo* synthesis of a desired protein. With regard to therapeutic applications, nanolipoplexes could be used, for example, in wound healing patches for transdermal mRNA delivery[57] to activate cell regeneration, or as a spray for the nebulization of mRNA[58] for the cure of lung diseases[59,60] such as cystic fibrosis.

The presented protocol guarantees an easy and accessible way for the generation of NLps using the dry-film method, which can then be used for the efficient encapsulation of *in vitro* transcribed mRNA and the safe transfection of cells.

Disclosures

The authors have nothing to disclose.

Acknowledgements

None

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