Journal of Visualized Experiments

Generation of Cationic Nanoliposomes for Efficient Delivery of in vitro transcribed messenger RNA

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58444R2
Full Title:	Generation of Cationic Nanoliposomes for Efficient Delivery of in vitro transcribed messenger RNA
Keywords:	mRNA, nanoliposomes, DOPE, DC-cholesterol, encapsulation, transfection, delivery, therapeutics
Corresponding Author:	S Dr. Krajewski University Hospital Tuebingen Tuebingen, na GERMANY
Corresponding Author's Institution:	University Hospital Tuebingen
Corresponding Author E-Mail:	stefanie.krajewski@uni-tuebingen.de
Order of Authors:	Tatjana Michel
	Antonia Link
	Meike Abraham
	Christian Schlensak
	Karlheinz Peter
	Hans-Peter Wendel
	Xiaowei Wang
	S Dr. Krajewski
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Department of Thoracic, Cardiac and Vascular Surgery University of Tuebingen Calwerstr. 7/1 72076 Tuebingen Germany

TITLE:

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

AUTHORS AND AFFILIATIONS:

Tatjana Michel[1], Antonia Link[1], Meike-Kristin Abraham[1,2], Christian Schlensak[1], Karlheinz Peter[2,3], Hans-Peter Wendel[1], Xiaowei Wang[2,3], Stefanie Krajewski[1]

[1]Department of Thoracic and Cardiovascular Surgery, Clinical Research Laboratory, University Medical Center, Tübingen, Germany

[2]Atherothrombosis and Vascular Biology, Baker Heart & Diabetes Institute, Melbourne, Victoria, Australia

[3]Department of Medicine, Monash University, Melbourne, Victoria, Australia

Corresponding Author:

Stefanie Krajewski (stefanie.krajewski@uni-tuebingen.de)

E-mail Addresses of the Co-authors:

Tatjana Michel (tatjana.michel@uni-tuebingen.de)
Antonia Link (antonia_link@web.de)
Meike-Kristin Abraham (meike-kristin.abraham@hotmail.de)
Christian Schlensak (christian.schlensak@med.uni-tuebingen.de)
Karlheinz Peter (Karlheinz.Peter@baker.edu.au)
Hans-Peter Wendel (hans-peter.wendel@med.uni-tuebingen.de)
Xiaowei Wang (Xiaowei.Wang@baker.edu.au)

KEYWORDS:

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

SHORT ABSTRACT:

Here we describe a protocol for the generation of cationic nanoliposomes, which is based on the dry-film method and can be used for the safe and efficient delivery of *in vitro* transcribed messenger RNA.

LONG 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)

1.1. 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.

1.2. 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.

1.3. 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.

1.4. 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.

1.5. 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.

1.6. 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

2.1. Prepare the eGFP-encoding mRNA following the protocol published earlier[34].

2.2. 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.

2.2.1. 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.

2.2.2. 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.

2.2.3. Add nuclease-free H_2O to the mixture, up to a volume of 100 µL.

2.2.4. Run the PCR cycles in the thermocycler following the protocol in **Table 1**.

2.3. Purify the eGFP-encoding DNA sequence with the PCR purification kit.

2.3.1. Therefore, mix the PCR solution with 500 µL of binding buffer from the kit and use it to fill purification columns.

2.3.2. Centrifuge the columns at maximum speed for 1 min and discard the filtrate.

2.3.4. Add 750 µL of wash buffer I to the column, centrifuge again at the maximum speed for 1 min, and discard the filtrate.

2.3.5. Repeat the centrifuge step 1x to remove the buffer from the column filter.

2.3.6. Transfer the column to a fresh 1.5-mL tube.

2.3.7. 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.

2.3.8. Measure the concentration of the DNA with a photometer and store it at -20 °C.

2.3.9. 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.

2.3.10. 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.

2.3.11. 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.

2.3.12. Analyze the gel using a gel-analyzing station under UV light.

2.4. 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.

2.4.1. 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.

2.4.2. Incubate the IVT reaction mix for 4 h at 37 °C.

2.4.3. 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.

2.5. To purify the mRNA, use the RNA clean-up kit.

2.5.1. Fill up the IVT mix to the volume of 100 µL with nuclease-free H_2O.

2.5.2. Add 350 µL of lysis buffer and mix by pipetting up and down.

2.5.3. Add 250 µL of 100% ethanol and mix again for 1x. Pipette the mix into the cleanup columns.

2.5.4. Centrifuge for 15 s at 8,000 x *g* and discard the filtrate.

2.5.5. Add 500 µL of the washing buffer into a column, centrifuge again for 15 s at 8,000 x *g*, and remove the filtrate.

2.5.6. Wash the column 1x with 500 µL of wash buffer and centrifuge for 2 min at 8,000 x *g*.

2.5.7. 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.

2.6. 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.

2.7. Purify the mRNA again, following steps 2.5.1 - 2.5.7.

2.8. Measure the mRNA concertation with a photometer.

2.9. 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.

2.9.1. 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.

2.9.2. 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.

2.9.3. Analyze the gel using a gel doc station with UV light.

3. Complexation of Synthetic mRNA

3.1. Thaw the synthetic mRNA on ice, vortex it, and centrifuge shortly before opening the tube.

3.2. 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.

3.3. 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

4.1. To perform the encapsulation experiments, use the RNA quantification kit.

4.2. 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.

4.3. 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.

4.4. Use **Table 3** for the preparation of high-range standard (20 ng/mL - 1 µg/mL).

4.5. 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**.

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.

4.7. Add 1 mL of nuclease-free H_2O to form nanolipoplexes and mix by pipetting up and down.

4.8. 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.

4.9. 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

5.1. Plate 1.5 x 10^5 A549 cells/well of a 12-well plate 1 d prior to transfection.

5.2. 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

6.1. Wash the prepared cells 1x with 1 mL/well PBS (without Ca^{2+}/Mg^{2+}).

6.2. 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.

6.3. 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

7.1. Remove the supernatant and wash the cells with 1 mL/well PBS (without Ca^{2+}/Mg^{2+}) to remove the remaining NLps.

7.2. Prepare the cells for flow cytometry.

7.2.1. 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.

7.2.2. Centrifuge the cells for 5 min at 400 x *g* and carefully remove the supernatant without touching the cell pellet.

7.2.3. Wash the cells with 1 mL of PBS (without Ca^{2+}/Mg^{2+}).

7.2.4. Resuspend the cells in 300 µL of 1x fixation solution and transfer the cells into flow cytometry tubes.

7.2.5. Analyze the cells at 488 nm in a flow cytometer (**Figure 4A**).

Note: Vortex the cells 1x directly before measuring.

7.3. Prepare the cells for fluorescence microscopy.

7.3.1. Fix the cells with 1 mL/well 100% methanol, which was previously stored at -20 °C.

7.3.2. 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.

7.3.3. Remove the DAPI solution and wash the cells again with 100% methanol stored at -20 °C.

7.3.4. 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

8.1. 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.

8.2. 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.

8.3. Add 500 µL/well of 1:10 diluted MTT solution to the cells and incubate for 4 h at 37 °C.

8.4. 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.

8.5. Pipette the DMSO solution in triplets into a clear-bottom 96-well plate and measure the adsorption at 540 nm using a microplate reader.

8.6. 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 AND TABLE LEGENDS:

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.

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.

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).

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).

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).

Table 1: PCR cycle protocol for DNA amplification.

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

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

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[50-52], 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.

ACKNOWLEDGEMENTS:
None

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Sahin, U., Kariko, K., Tureci, O. mRNA-based therapeutics--developing a new class of drugs. *Nature Reviews Drug Discovery.* **13** (10), 759-780 (2014).

2. Yamamoto, A., Kormann, M., Rosenecker, J., Rudolph, C. Current prospoects for mRNA gene delivery. *European Journal of Pharmaceutics and Biopharmaceutics.* **71** (3), 484-489 (2009).

3. Williams, P. D., Kingston, P. A. Plasmid-mediated gene therapy for cardiovascular disease. *Cardiovascular Research.* **91** (4), 565-576 (2011).

4. Devoldere, J., Dewitte, H., De Smedt, S. C., Remaut, K. Evading innate immunity in nonviral mRNA delivery: don't shoot the messenger. *Drug Discovery Today.* **21** (1), 11-25 (2016).

5. Laguens, R. P., Crottogini, A. J. Cardiac regeneration: the gene therapy approach. *Expert Opinion on Biological Therapy.* **9** (4), 411-425 (2009).

6. Hadas, Y., Katz, M. G., Bridges, C. R., Zangi, L. Modified mRNA as a therapeutic tool to induce cardiac regeneration in ischemic heart disease. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine.* **9** (1) (2017).

7. Zohra, F. T., Chowdhury, E. H., Akaike, T. High performance mRNA transfection through carbonate apatite-cationic liposome conjugates. *Biomaterials.* **30** (23-24), 4006-4013 (2009).

8. Youn, H., Chung, J.-K. Modified mRNA as an alternative to plasmid DNA (pDNA) for transcript replacement and vaccination therapy. *Expert Opinion on Biological Therapy.* **15** (9), 1337-1348 (2015).

9. Avci-Adali, M. *et al.* Optimized conditions for successful transfection of human endothelial cells with in vitro synthesized and modified mRNA for induction of protein expression. *Journal of Biological Engineering.* **8** (1), 8-8 (2014).

10. Akbarzadeh, A. *et al.* Liposome: classification, preparation, and applications. *Nanoscale Research Letters.* **8** (1), 102 (2013).

11. Michel, T., Wendel, H.-P., Krajewski, S. Next-generation Therapeutics: mRNA as a Novel Therapeutic Option for Single-gene Disorders. In: *Modern Tools for Genetic Engineering*. Edited by Kormann, M., 3-20, IntechOpen (2016).

12. Karikó, K. *et al.* Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. *Molecular Therapy: The Journal of the American Society of Gene Therapy.* **16** (11), 1833-1840 (2008).

13. Anderson, B. R. *et al.* Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation. *Nucleic Acids Research.* **38** (17), 5884-5892 (2010).

14. Michel, T. *et al.* Cationic Nanoliposomes Meet mRNA: Efficient Delivery of Modified mRNA Using Hemocompatible and Stable Vectors for Therapeutic Applications. *Molecular Therapy - Nucleic Acids.* **8** (September), 459-468 (2017).

15. Tran, M. A., Watts, R. J., Robertson, G. P. Use of Liposomes as Drug Delivery Vehicles for Treatment of Melanoma. *Pigment Cell & Melanoma Research.* **22** (4), 388-399 (2009).

16. Immordino, M. L., Dosio, F., Cattel, L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. *International Journal of Nanomedicine.* **1** (3), 297-315 (2006).

17. Ross, P. C., Hui, S. W. Lipoplex size is a major determinant of in vitro lipofection efficiency. *Gene Therapy.* **6** (4), 651-659 (1999).

18. Xing, H., Hwang, K., Lu, Y. Recent Developments of Liposomes as Nanocarriers for Theranostic Applications. *Theranostics.* **6** (9), 1336-1352 (2016).

19. Felgner, P. L. *et al.* Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proceedings of the National Academy of Sciences of the United States of America*. **84** (21), 7413-7417 (1987).

20. Leventis, R., Silvius, J. R. Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochimica et Biophysica Acta.* **1023** (1), 124-132 (1990).

21. Behr, J. P., Demeneix, B., Loeffler, J. P., Perez-Mutul, J. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proceedings of the National Academy of Sciences of the United States of America.* **86** (18), 6982-6986 (1989).

22. Gao, X., Huang, L. A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochemical and Biophysical Research Communications.* **179** (1), 280-285 (1991).

23. Martin, B. *et al.* The design of cationic lipids for gene delivery. *Current Pharmaceutical Design.*

11 (3), 375-394 (2005).

24. Yang, S. Y. *et al.* Comprehensive study of cationic liposomes composed of DC-Chol and cholesterol with different mole ratios for gene transfection. *Colloids and Surfaces B: Biointerfaces.* **101**, 6-13 (2013).

25. Bennett, M. J., Nantz, M. H., Balasubramaniam, R. P., Gruenert, D. C., Malone, R. W. Cholesterol enhances cationic liposome-mediated DNA transfection of human respiratory epithelial cells. *Bioscience Reports.* **15** (1), 47-53 (1995).

26. Son, K. K., Patel, D. H., Tkach, D., Park, A. Cationic liposome and plasmid DNA complexes formed in serum-free medium under optimum transfection condition are negatively charged. *Biochimica et Biophysica Acta.* **1466** (1-2), 11-15 (2000).

27. Lonez, C., Vandenbranden, M., Ruysschaert, J. M. Cationic lipids activate intracellular signaling pathways. *Advanced Drug Delivery Reviews.* **64** (15), 1749-1758 (2012).

28. Malone, R. W., Felgner, P. L., Verma, I. M. Cationic liposome-mediated RNA transfection. *Proceedings of the National Academy of Sciences of the United States of America.* **86** (16), 6077-6081 (1989).

29. Zohra, F. T., Maitani, Y., Akaike, T. mRNA delivery through fibronectin associated liposome-apatite particles: a new approach for enhanced mRNA transfection to mammalian cell. *Biological and Pharmaceutical Bulletin.* **35** (1), 111-115 (2012).

30. Rejman, J., Tavernier, G., Bavarsad, N., Demeester, J., De Smedt, S. C. mRNA transfection of cervical carcinoma and mesenchymal stem cells mediated by cationic carriers. *Journal of Controlled Release.* **147** (3), 385-391 (2010).

31. Balazs, D. A., Godbey, W. Liposomes for use in gene delivery. *Journal of Drug Delivery.* **2011**, 326497 (2011).

32. Bulbake, U., Doppalapudi, S., Kommineni, N., Khan, W. Liposomal Formulations in Clinical Use: An Updated Review. *Pharmaceutics.* **9** (2) (2017).

33. Abraham, M. K. *et al.* Nanoliposomes for Safe and Efficient Therapeutic mRNA Delivery: A Step Toward Nanotheranostics in Inflammatory and Cardiovascular Diseases as well as Cancer. *Nanotheranostics.* **1** (2), 154-165 (2017).

34. Avci-Adali, M. *et al.* In vitro synthesis of modified mRNA for induction of protein expression in human cells. *Journal of Visualized Experiments.* (93), e51943 (2014).

35. Yang, S., Chen, J., Zhao, D., Han, D., Chen, X. Comparative study on preparative methods of DC-Chol/DOPE liposomes and formulation optimization by determining encapsulation efficiency.

International Journal of Pharmaceutics. **434** (1-2), 155-160 (2012).

36. Caracciolo, G., Amenitsch, H. Cationic liposome/DNA complexes: from structure to interactions with cellular membranes. *European Biophysics Journal.* **41** (10), 815-829 (2012).

37. Farhood, H., Serbina, N., Huang, L. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochimica et Biophysica Acta.* **1235** (2), 289-295 (1995).

38. Zhang, Y. *et al.* DC-Chol/DOPE cationic liposomes: a comparative study of the influence factors on plasmid pDNA and siRNA gene delivery. *International Journal of Pharmaceutics.* **390** (2), 198-207 (2010).

39. Klein, R. A. The detection of oxidation in liposome preparations. *Biochimica et Biophysica Acta.* **210** (3), 486-489 (1970).

40. Ming-Ren Toh, G. N. C. C. Liposomes as sterile preparations and limitations of sterilisation techniques in liposomal manufacturing. *Asian Journal of Pharmaceutical Sciences.* **8** (2), 88-95 (2013).

41. Torchilin, V. P., Omelyanenko, V. G., Lukyanov, A. N. Temperature-dependent aggregation of pH-sensitive phosphatidyl ethanolamine-oleic acid-cholesterol liposomes as measured by fluorescent spectroscopy. *Analytical Biochemistry.* **207** (1), 109-113 (1992).

42. Rabinovich, P. M., Weissman, S. M. Cell engineering with synthetic messenger RNA. *Methods in Molecular Biology.* **969**, 3-28 (2013).

43. Ishida, T., Harashima, H., Kiwada, H. Liposome clearance. *Bioscience Reports.* **22** (2), 197-224 (2002).

44. Freise, J., Muller, W. H., Brolsch, C., Schmidt, F. W. "In vivo" distribution of liposomes between parenchymal and non parenchymal cells in rat liver. *Biomedicine.* **32** (3), 118-123 (1980).

45. Roerdink, F., Dijkstra, J., Hartman, G., Bolscher, B., Scherphof, G. The involvement of parenchymal, Kupffer and endothelial liver cells in the hepatic uptake of intravenously injected liposomes. Effects of lanthanum and gadolinium salts. *Biochimica et Biophysica Acta.* **677** (1), 79-89 (1981).

46. Wang, X. *et al.* Dual-Targeted Theranostic Delivery of miRs Arrests Abdominal Aortic Aneurysm Development. *Molecular Therapy.* **26** (4), 1056-1065 (2018).

47. Flierl, U. *et al.* Phosphorothioate backbone modifications of nucleotide-based drugs are potent platelet activators. *Journal of Experimental Medicine.* **212** (2), 129-137 (2015).

48. Woodle, M. C. Controlling liposome blood clearance by surface-grafted polymers. *Advanced*

Drug Delivery Reviews. **32** (1-2), 139-152 (1998).

49. Sawant, R. R., Torchilin, V. P. Challenges in development of targeted liposomal therapeutics. *The AAPS Journal.* **14** (2), 303-315 (2012).

50. Ewert, K. K., Evans, H. M., Bouxsein, N. F., Safinya, C. R. Dendritic cationic lipids with highly charged headgroups for efficient gene delivery. *Bioconjugate Chemistry.* **17** (4), 877-888 (2006).

51. Elouahabi, A., Ruysschaert, J. M. Formation and intracellular trafficking of lipoplexes and polyplexes. *Molecular Therapy.* **11** (3), 336-347 (2005).

52. Hoekstra, D., Rejman, J., Wasungu, L., Shi, F., Zuhorn, I. Gene delivery by cationic lipids: in and out of an endosome. *Biochemical Society Transactions.* **35** (Pt 1), 68-71 (2007).

53. Lonez, C., Vandenbranden, M., Ruysschaert, J. M. Cationic liposomal lipids: from gene carriers to cell signaling. *Progress in Lipid Research.* **47** (5), 340-347 (2008).

54. Filion, M. C., Phillips, N. C. Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. *Biochimica et Biophysica Acta.* **1329** (2), 345-356 (1997).

55. Takano, S., Aramaki, Y., Tsuchiya, S. Physicochemical properties of liposomes affecting apoptosis induced by cationic liposomes in macrophages. *Pharmaceutical Research.* **20** (7), 962-968 (2003).

56. Ciani, L. *et al.* DOTAP/DOPE and DC-Chol/DOPE lipoplexes for gene delivery studied by circular dichroism and other biophysical techniques. *Biophysical Chemistry.* **127** (3), 213-220 (2007).

57. Benson, H. A. Elastic Liposomes for Topical and Transdermal Drug Delivery. *Methods in Molecular Biology.* **1522**, 107-117 (2017).

58. Johler, S. M., Rejman, J., Guan, S., Rosenecker, J. Nebulisation of IVT mRNA Complexes for Intrapulmonary Administration. *PLoS One.* **10** (9), e0137504 (2015).

59. Mays, L. E. *et al.* Modified Foxp3 mRNA protects against asthma through an IL-10-dependent mechanism. *Journal of Clinical Investigation.* **123** (3), 1216-1228 (2013).

60. Kormann, M. S. *et al.* Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. *Nature Biotechnology.* **29** (2), 154-157 (2011).

Figure 1

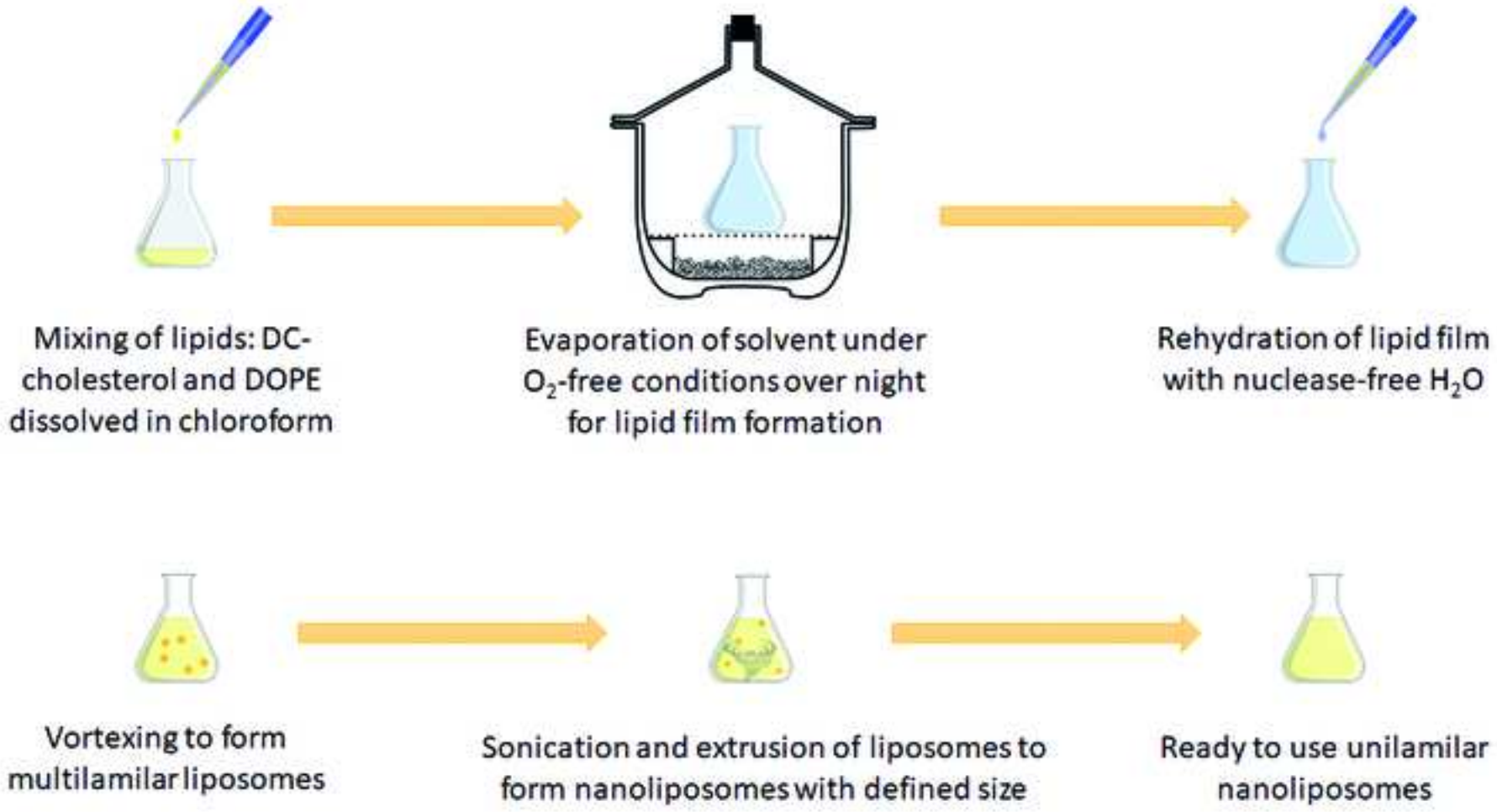

Figure 2

Click here to access/download;Figure;figure 2.tif

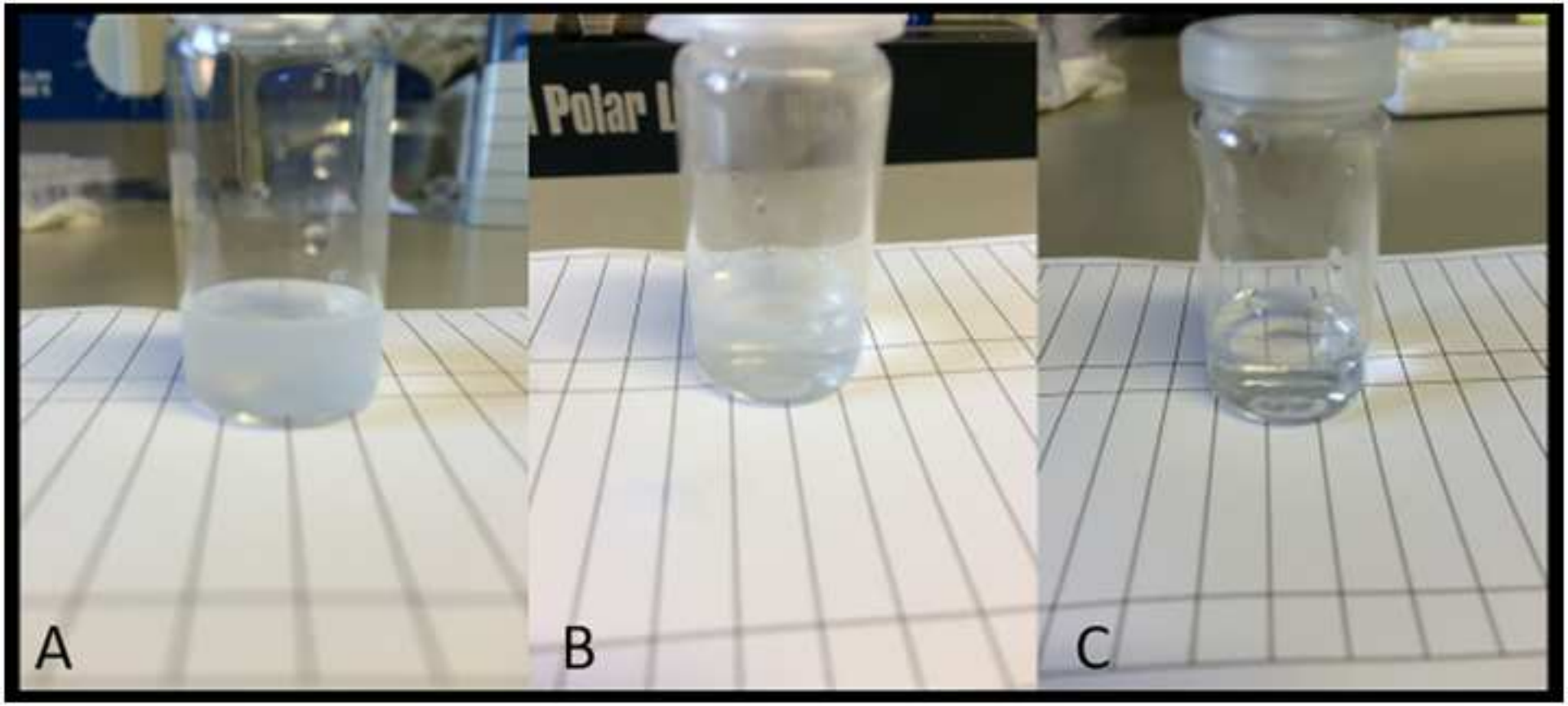

Figure 3

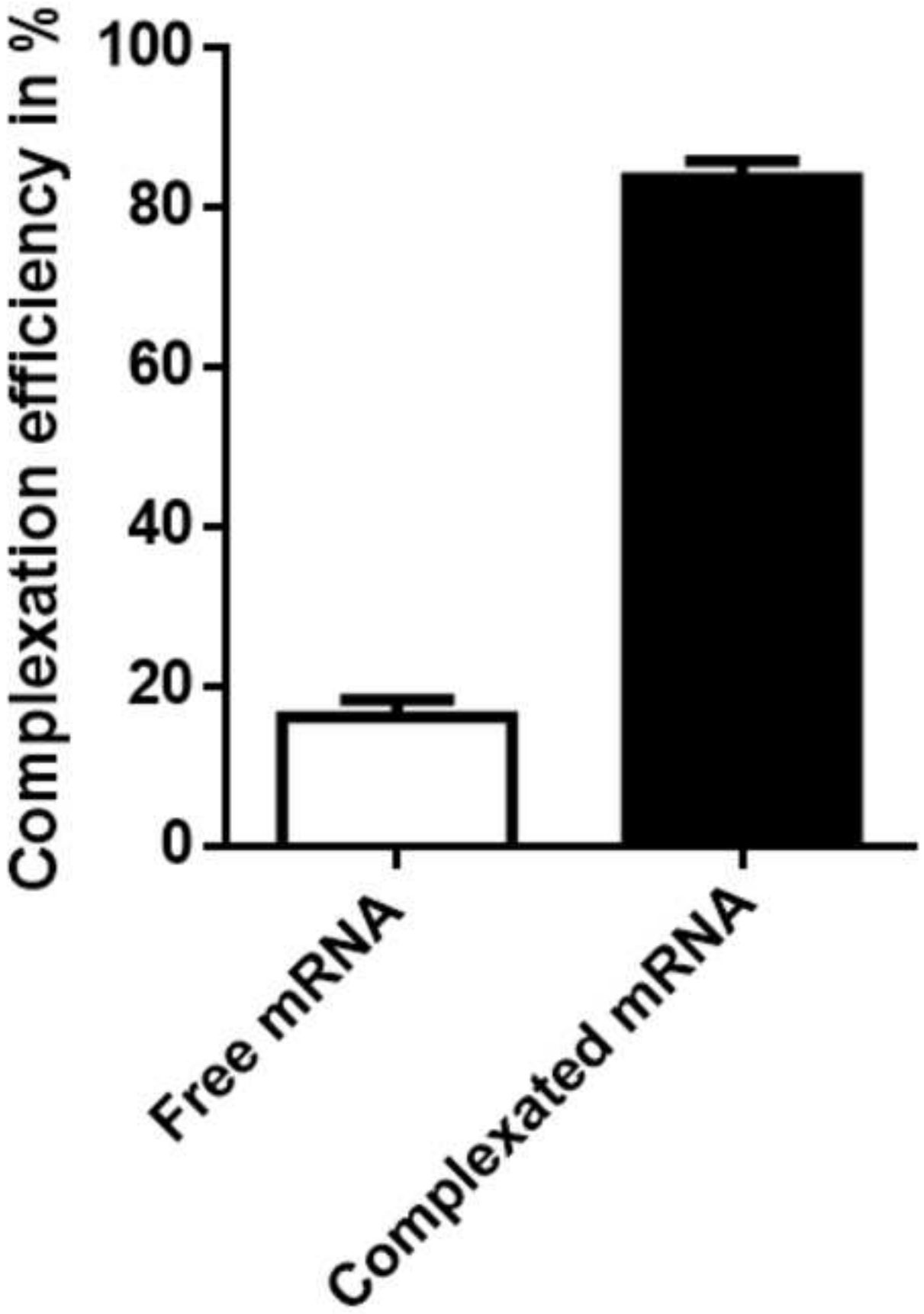

Figure 4

Click here to access/download;Figure;figure 4.tif

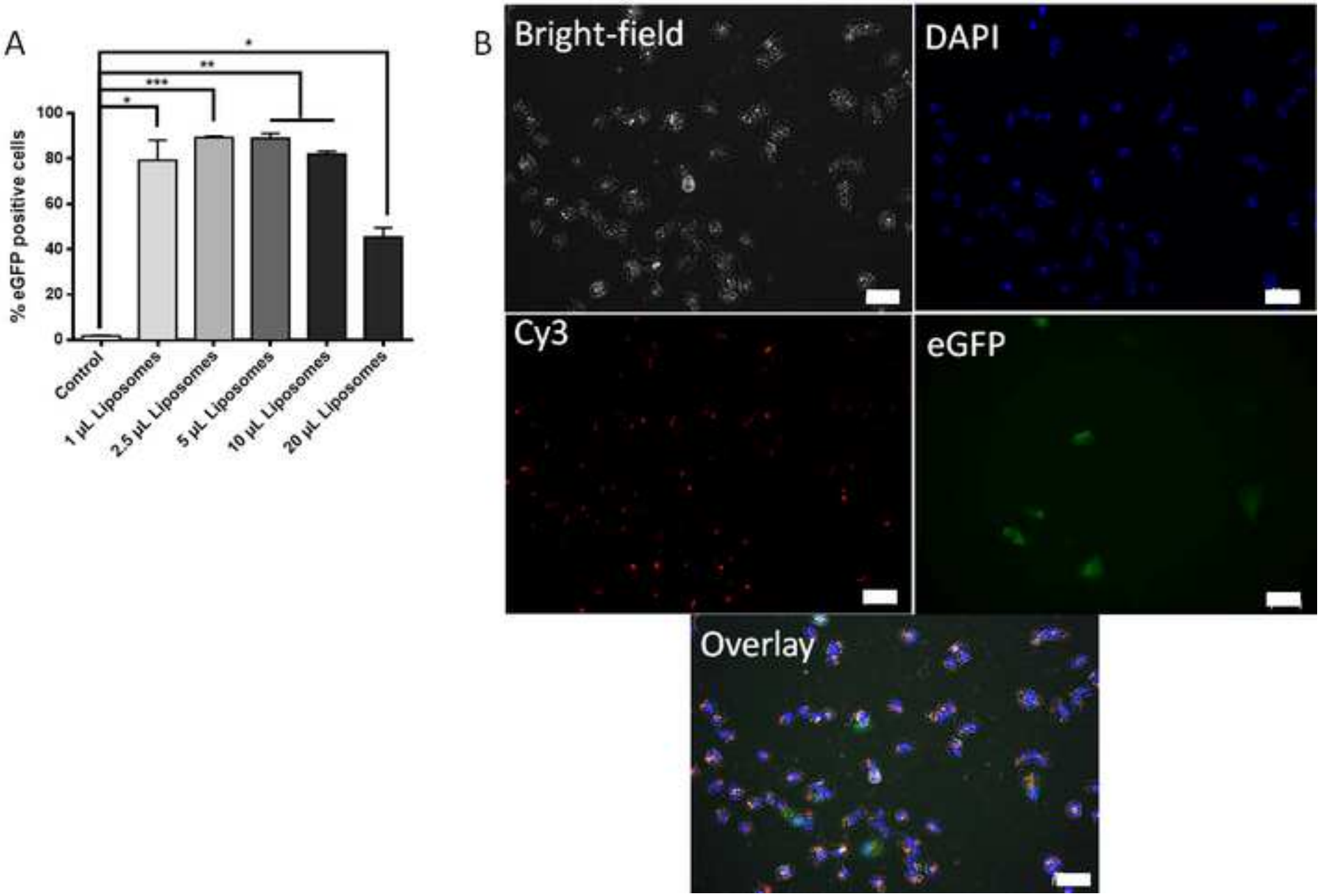

Figure 5

Click here to access/download;Figure;figure 5.tif

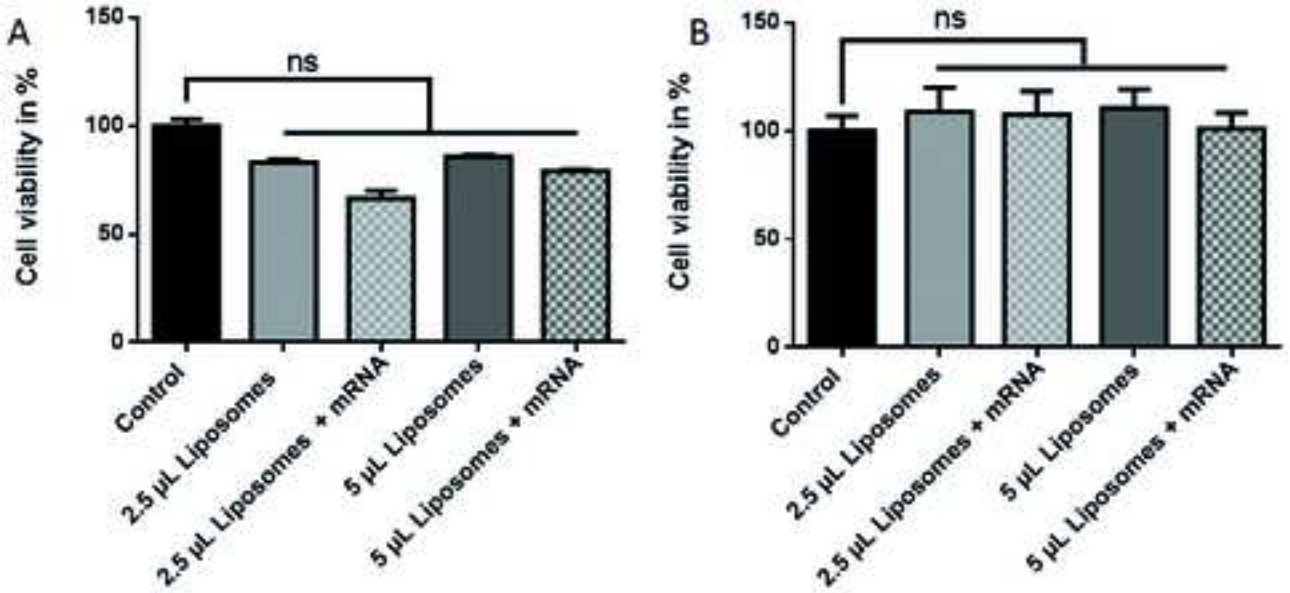

Table 1

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 2

	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 3

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 4

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

Name of Material/ Equipment	Company
(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	AppliChem, Darmstadt, Germany
(3β-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride	Avanti, Alabama, USA
4 ',6-diamidino-2-phenylindole (DAPI)	Thermo Fisher Scientific, Darmstadt, Germany
BD FACScan system	BD Biosciences, Heidelberg, Germany
Cell Fix (10x)	BD Biosciences, Heidelberg, Germany
Chloroform	Merck, Darmstadt, Germany
Dimethyl sulfoxid (DMSO)	Serva Electrophoresis GmbH, Heidelberg, Germany
Dioleoyl phosphatidylethanolamine (DOPE)	Avanti, Alabama, USA
Fluorescence microscope	Zeiss Axio, Oberkochen, Germany
Lipofectamine 2000	Thermo Fisher Scientific,
Mini extruder	Avanti, Alabama, USA
Nuclease-free water	Qiagen, Hilden, Germany
Opti-Mem	Thermo Fisher Scientific, Darmstadt, Germany
PBS buffer (w/o Ca^{2+}/Mg^{2+})	Thermo Fisher Scientific,
Quant-iT Ribo Green RNA reagent kit	Thermo Fisher Scientific,

RPMI (w/o phenol red)	Thermo Fisher Scientific, Darmstadt, Germany
Silica gel	Carl Roth, Karlsruhe, Germany
Trypsin/EDTA (0.05%)	Thermo Fisher Scientific, Darmstadt, Germany
HotStar HiFidelity Polymerase Kit	Qiagen, Hilden, Germany
QIAquick PCR Purification Kit	Qiagen, Hilden, Germany
Pseudouridine-5'-Triphosphate (Ψ-UTP)	TriLink Biotechnologies, San Diego, USA
5-Methylcytidine-5'-Triphosphate (Methyl-CTP)	TriLink Biotechnologies, San Diego, USA
Cyanine 3-CTP	PerkinElmer, Baesweiler, Germany
RNeasy Mini Kit	Qiagen, Hilden, Germany
MEGAscript T7 Transcription Kit	Thermo Fisher Scientific, Darmstadt, Germany
3´-O-Me-m^{7}G(5')ppp(5')G RNA Cap Structure Analog	New England Biolabs, Ipswich, USA
Antarctic Phosphatase	New England Biolabs, Ipswich, USA
Agarose	Thermo Fisher Scientific, Darmstadt, Germany
GelRed	Biotium, Fremont, USA
peqGOLD DNA ladder mix	VWR, Pennsylvania, USA

Invitrogen 0.5-10kb RNA ladder	Fisher Scientific, Göteborg, Sweden

Catalog Number	Comments/Description
A2231	
700001	
D1306	
340181	
102445	
20385.02	
850725	
11668019	
129114	
11058021	
70011044	
Q33140	

11835030

P077

25300054

202602

28104

N-1019

N-1014

NEL580001EA

74104

AM1333

S1411L

M0289S

16500-500

41003

25-2040

11528766

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Generation of Cationic Nanoliposomes for Efficient Delivery of in vitro transcribed messenger RNA

Author(s): Michel, Link, Abraham, Schlensak, Peter, Wendel, Wang, Krajewski

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: ☑ Standard Access ☐ Open Access

Item 2 (check one box):

☑ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Stefanie Krajewski
Department:	Department of Thoracic, Cardiac and Vascular Surgery
Institution:	University Hospital Tuebingen
Article Title:	Generation of Cationic Nanoliposomes for Efficient Delivery of in vitro transcribed messenger RNA
Signature:	S. Krajewski
Date:	05/08/18

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

July 24th 2018

RE: Resubmission JoVE58444

Dear Dr. Myers,

please find enclosed our manuscript entitled “Generation of Cationic Nanoliposomes for Efficient Delivery of in vitro transcribed messenger RNA” for resubmission in the Journal of Visualized Experiments.

All changes made during this revision process are highlighted in the revised version of the manuscript. Also, please see our comments in the manuscript.

Yours sincerely,

Dr. Stefanie Krajewski

Department of Thoracic, Cardiac and Vascular Surgery,
Clinical Research Laboratory,
University Hospital Tuebingen,
Germany