Original Study Article

DOI: https://doi.org/10.17816/MAJ641910 EDN: HYPFPI

Exosomes Facilitate mRNA and siRNA Delivery Using Cationic Liposomes 2X3-DOPE to Rat Heart Mesenchymal Cells *in vitro*

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ABSTRACT

BACKGROUND: The delivery of nucleic acids to mesenchymal stem cells, which are used as model objects in *in vitro* experiments or as therapeutic agents in regenerative medicine and oncology, is an actively developing area of research. Existing non-viral delivery systems either have low effectiveness or highly toxic to mesenchymal stem cells. Therefore, the development of new carriers has become an urgent priority.

AIM: To demonstrate the feasibility of delivering model messenger RNA and small interfering RNA to rat heart mesenchymal stem cells (MSCs) *in vitro* using original cationic liposomes 2X3-DOPE (1:3 molar ratio) and to evaluate the influence of exosomes incorporated into hybrid nanoparticles with 2X3-DOPE on the efficiency of RNA delivery.

METHODS: Exosomes were isolated using a standard ultracentrifugation technique followed by characterization of the obtained vesicles through Western blotting, transmission electron microscopy, atomic force microscopy, and hydrodynamic diameter measurement using dynamic light scattering. Small interfering RNA was chemically synthesized; whereas messenger RNA was obtained by *in vitro* transcription. Complexes of liposomes or hybrid nanoparticles with RNA were prepared by mixing; the properties of the resulting particles were assessed using dynamic light scattering and atomic force microscopy. To evaluate the efficiency of RNA delivery to rat heart mesenchymal stem cells derived from both healthy and ischemic myocardium, we used fluorescence microscopy, laser scanning confocal microscopy, and flow cytometry.

RESULTS: Complexes of cationic liposomes 2X3-DOPE (1:3 molar ratio) with messenger RNA and 2X3-DOPE modified with DSPE-PEG_{2000} (0.62 mol%) complexed with small interfering RNA were successfully prepared and characterized. It was demonstrated that 2X3-DOPE is ineffective for messenger RNA delivery to rat cardiac mesenchymal stem cells; whereas hybrid nanoparticles incorporating exosomes based on these liposomes exhibited up to 40% transfection efficiency. In addition, 2X3-DOPE modified with DSPE-PEG_{2000} (0.62 mol%) was effective for small interfering RNA delivery to rat cardiac mesenchymal stem cells, achieving up to 90% transfection efficiency; whereas the use of hybrid nanoparticles based on this formulation resulted in 100% transfected cells with more than a twofold increase in small interfering RNA in the cells as indicated by the average fluorescence intensity.

CONCLUSION: Cationic liposomes 2X3-DOPE (1:3 molar ratio) modified with DSPE-PEG_{2000} (0.62 mol%) are promising vehicles for small interfering RNA delivery to mesenchymal stem cells, both independently and in combination with exosomes. Exosomes integrated in hybrid nanoparticles based on 2X3-DOPE improve the transfection efficiency of both messenger RNA and small interfering RNA in rat cardiac mesenchymal stem cells *in vitro*.

Keywords: exosomes; hybrid nanoparticles; cationic liposomes; non-viral delivery systems; messenger RNA; small interfering RNA; cardiac mesenchymal cells.

To cite this article

Dovbysh OV, Vysochinskaya VV, Gavrilova NV, Docshin PM, Nikitina EG, Klochev AS, Elpaeva EA, Dobrovolskaya OA, Shmendel EV, Maslov MA, Zabrodskaya YaA. Exosomes Facilitate mRNA and siRNA Delivery Using Cationic Liposomes 2X3-DOPE to Rat Heart Mesenchymal Cells *in vitro*. *Medical Academic Journal.* 2025;25(2):55–67. DOI: 10.17816/MAJ641910 EDN: HYPFPI

Submitted: 29.11.2024 **Accepted:** 10.12.2024 **Published online:** 30.06.2025

Оригинальное исследование

DOI: https://doi.org/10.17816/MAJ641910 EDN: HYPFPI

Экзосомы способствуют доставке мРНК и миРНК с помощью катионных липосом 2X3-DOPE в мезенхимные клетки сердца крыс *in vitro*

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АННОТАЦИЯ

Обоснование. Доставка нуклеиновых кислот в мезенхимные стволовые клетки, используемые в качестве модельных объектов в экспериментах *in vitro* или терапевтических средств в регенеративной медицине и онкологии, — активно разрабатываемая задача. Существующие невирусные системы доставки либо недостаточно высокоэффективны, либо высокотоксичны для клеток, что требует разработки новых носителей для трансфекции мезенхимных стволовых клеток.
Цель — показать возможность доставки модельных матричных РНК и малых интерферирующих РНК в мезенхимные стволовые клетки сердца крысы *in vitro* при помощи оригинальных катионных липосом 2X3-DOPE (1:3 мольн.), а также оценить влияние экзосом в составе гибридных наночастиц с 2X3-DOPE на эффективность доставки РНК.
Методы. Для выделения экзосом использовали стандартную методику ультрацентрифугирования с последующей характеристикой полученных везикул методами вестерн-блоттинга, просвечивающей электронной и атомно-силовой микроскопии, измерение гидродинамического диаметра методом динамического рассеяния света. Малые интерферирующие РНК были получены в ходе химического синтеза, для получения матричных РНК использовали метод *in vitro* транскрипции. Комплексы липосом или гибридных наночастиц с РНК формировали путем смешивания компонентов, параметры полученных частиц оценивали методами динамического рассеяния света и атомно-силовой микроскопии. Для оценки эффективности доставки РНК в мезенхимные стволовые клетки сердца крысы из здорового и ишемизированного миокарда использовали флуоресцентную микроскопию, лазерную сканирующую конфокальную микроскопию, а также проточную цитофлуориметрию.
Результаты. Были получены и охарактеризованы комплексы катионных липосом 2X3-DOPE (1:3 мольн.) с матричной РНК и 2X3-DOPE, содержащими DSPE-PEG_{2000} (0,62 моль%), с малыми интерферирующими РНК, а также комплексы соответствующих гибридных наночастиц с матричной РНК или малыми интерферирующими РНК. Было показано, что катионные липосомы 2X3-DOPE малоэффективны для доставки матричной РНК в мезенхимные стволовые клетки сердца крысы, в то время как гибридные наночастицы с экзосомами на их основе демонстрируют до 40% трансфицированных клеток. Катионные липосомы 2X3-DOPE, содержащие DSPE-PEG_{2000}, эффективны для доставки малых интерферирующих РНК в мезенхимные стволовые клетки сердца крысы (до 90% трансфицированных клеток), в то время как использование гибридных наночастиц позволяет достичь 100% трансфицированных клеток, а также более чем в два раза увеличивает содержание малых интерферирующих РНК в клетках при оценке средней интенсивности флуоресценции.
Заключение. Катионные липосомы 2X3-DOPE (1:3 мольн.), модифицированные DSPE-PEG_{2000} (0,62 моль%), можно рассматривать как перспективное средство доставки малых интерферирующих РНК в мезенхимные стволовые клетки как сами по себе, так и в комплексе с экзосомами. Присутствие экзосом в составе гибридных наночастиц увеличивает эффективность трансфекции мезенхимных стволовых клеток сердца крысы матричными РНК и малыми интерферирующими РНК *in vitro*.

Ключевые слова: экзосомы; гибридные наночастицы; катионные липосомы; невирусные системы доставки; матричные РНК; малые интерферирующие РНК; мезенхимные клетки сердца.

Как цитировать
Довбыш О.В., Высочинская В.В., Гаврилова Н.В., Докшин П.М., Никитина Е.Г., Клочев А.С., Елпаева Е.А., Добровольская О.А., Шмендель Е.В., Маслов М.А., Забродская Я.А. Экзосомы способствуют доставке мРНК и миРНК с помощью катионных липосом 2X3-DOPE в мезенхимные клетки сердца крыс *in vitro* // Медицинский академический журнал. 2025. Т. 25. № 2. С. 55–67. DOI: 10.17816/MAJ641910 EDN: HYPFPI

Рукопись получена: 29.11.2024 **Рукопись одобрена:** 10.12.2024 **Опубликована online:** 30.06.2025

BACKGROUND

Exosomes are membrane extracellular vesicles ranging from 30 to 150 nm in size. They are secreted by all types of cells. The primary function of exosomes is to facilitate intercellular communication under both normal and pathological conditions by transferring mRNA, microRNA, DNA, and proteins between cells [1–3]. The composition of exosomes is actively studied to identify potential diagnostic markers of various pathological conditions, including the diagnosis and prognosis of cardiovascular diseases [4–6], early diagnosis and progression analysis of neurodegenerative diseases [7], the diagnosis and prevention of human immunodeficiency virus penetration [8–10], and the investigation of influenza virus pathogenesis mechanisms [11].

Today, a promising area is the use of exosomes to deliver therapeutic molecules as these vesicles have a native composition, largely undetected by the immune system, are nanosized, and can carry various exogenous cargo, including the ability to cross the blood-brain barrier [12]. There are several methods for loading extracellular vesicles with therapeutic molecules, including:

1. Co-incubation of therapeutic molecules with exosomes or exosome donor cells to facilitate the packaging of hydrophobic molecules [13–15] and nucleic acids [16] with modifications to improve loading efficiency, such as cholesterol incorporation [17, 18];
2. Transfection of exosome donor cells with plasmid DNA to overexpress a specific gene, allowing for the loading of mRNA, siRNA, or therapeutic molecules [19, 20] or inserting specific sequences to enable targeted packaging by the cell machinery during exosome biogenesis [21–23];
3. Physical methods, such as electroporation [24, 25], sonication [26], and dialysis [27];
4. The use of exosome-liposome hybrids combining the clear and effective packaging of therapeutic molecules, mRNA, siRNA, and proteins from liposomes with the native composition and receptor specificity of exosomes [28–30].

In this study, we explored the approach involving the use of exosome-liposome hybrid vehicles for the delivery of model RNAs (mRNA encoding green fluorescent protein and fluorescently labeled JOE siRNA) to rat cardiac mesenchymal stem cells (MSCs) *in vitro*. Today, MSCs are widely used in both *in vitro* research and direct applications in regenerative medicine and cancer therapy [31]. However, MSCs are difficult to transfect using non-viral delivery systems, necessitating the development of systems that would effectively deliver nucleic acids without being toxic to MSCs or altering their properties [32, 33]. One of such approaches involves the use of exosome-liposome hybrid nanoparticles, which can successfully transfect MSCs resistant to transfection by liposomes alone [34].

We have previously showed that cationic liposomes, specifically 2X3-DOPE (1:3 molar ratio), allow to efficiently transfect cells with mRNA [35, 36]; when modified with $DSPE\text{-}PEG_{2000}$ (0.62 mol%), they can effectively deliver siRNA [37] to continuous cell lines *in vitro* with the delivered molecules exerting a biological effect.

Thus, the **aim of this study** was to show the feasibility of delivering model mRNA and siRNA to rat cardiac MSCs *in vitro* using cationic liposomes 2X3-DOPE (1:3 molar ratio) and to improve transfection efficiency through the use of hybrids of cationic liposomes 2X3-DOPE and exosomes isolated from blood serum.

METHODS

Isolation of exosomes. Exosomes were isolated from an 8 ml human serum sample as described in [38]. Briefly, the serum was diluted 4-fold and centrifuged for 30 minutes at 3000 g and 8 °C. The supernatant was collected and centrifuged again for 30 minutes at 10,000 g and 8 °C. The supernatant was collected once more and subjected to ultracentrifugation in a Beckman ultracentrifuge for 2 hours at 110,000 g and 4 °C. The pellet was resuspended in 300 µl phosphate-buffered saline (PBS) (Sigma Aldrich, USA). To confirm the presence of exosomes in the sample, Western blotting with an exosomal marker protein (see *Methods*, sec. 2) and transmission electron microscopy (see *Methods*, sec. 3) were performed. The protein concentration in the exosomes was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA) according to the manufacturer's instructions.

Western blotting. Proteins were separated by polyacrylamide gel electrophoresis (PAGE) using the Laemmli method [39] under reducing conditions over beta-mercaptoethanol. The sample was mixed with 4-fold Laemmli buffer (250 mM Tris-HCl, pH 6.8, 1.43 M beta-mercaptoethanol, 8% (w/v) SDS, 0.04% (w/v) bromophenol blue, and 44% (w/v) glycerol); the proteins were denatured at 95 °C in a solid-state thermostat GNOM (DNA-Technologies, Russia) for 5 minutes. Approximately 10 µg protein was then loaded into a well of a 10-well gradient gel (8–17%). Precision Plus Protein WesternC Standards (BioRad, USA) were used as molecular weight markers. Electrophoretic separation was performed in a Mini-PROTEAN Tetra Vertical Electrophoresis Cell (BioRad).

For semi-dry transfer, transfer paper (Servicebio, China), a 0.22 µm nitrocellulose membrane (BioRad), and the gel post-PAGE were incubated in transfer buffer (47.9 mM Tris base, 38.6 mM glycine, 0.0375% SDS, and 20% (v/v) ethanol) for 15 minutes. The transfer was conducted using a Trans-blot Turbo Transfer System (BioRad) following the manufacturer's standard protocol.

After the transfer, the membrane was washed twice in phosphate-buffered saline with Tween® detergent (PBST) (0.1% Tween® 20 (BioRad) in PBS) for 10 minutes. Then, it was incubated in a blocking solution (5% Blotting-Grade Blocker (BioRad) in PBST) overnight at +4 °C followed by washing with PBST as described above. Primary mouse monoclonal HLA class I antibodies (Abcam, UK) were diluted in the blocking

solution according to the manufacturer's instructions and incubated with the membrane for 2 hours. After washing, the primary antibodies were detected using GAM-HRP (BioRad) in a blocking solution for 1 hour along with StrepTactin-HRP (BioRad) for subsequent visualization of the molecular weight marker. After additional washing, the proteins and the marker were detected using the commercial Clarity Max Western ECL Substrate kit (BioRad). Chemiluminescence was recorded using a ChemiDoc MP gel imaging system (BioRad).

Transmission electron microscopy (TEM). To verify the morphology of exosomes, such as size and shape, we performed transmission electron microscopy using negative staining. A 200-mesh copper microscopy grid (Electron Microscopy Science (EMS), USA) coated with collodion (Sigma-Aldrich) was placed on a 10 µl drop of the exosome sample for 1 minute. After washing twice with distilled water, the sample was stained with a 2% aqueous solution of sodium phosphotungstate hydrate (Sigma Aldrich) for 2 minutes. After the staining, excess moisture was removed using a paper filter and the grid was dried at room temperature for 10 minutes. For all observations, we used a JEOL JEM 1100 electron microscope (JEOL, Japan) with an accelerating voltage of 80 kV.

Nucleic acids. Two model RNAs were used, messenger RNA encoding enhanced green fluorescent protein (mRNA-eGFP) and small interfering RNA carrying the fluorescent label JOE at the 3′-end of the antisense strand (siRNA-JOE).

To produce mRNA, a plasmid encoding the *eGFP* gene in the pJet1.2 vector containing the T7 promoter was obtained. The enhanced green fluorescent protein variant eGFP C5MKY7 (UniProt) was used as the initial reference sequence. For this, the *eGFP* gene was provided as an expression vector by the museum of the Smorodintsev Research Institute of Influenza. The *eGFP* gene was cloned into the pJet1.2 vector at the XbaI and XhoI sites. The pJet1.2-eGFP genetic construct was analyzed using the Vector NTI 10 Advance software package (Invitrogen, USA). Restriction products were purified from agarose gel using a cleanup kit (Evrogen, Russia) and subsequently ligated using T4 DNA ligase (Thermo Scientific). Transformation was performed by electroporation into *Escherichia coli DH5α* strain cells.

For the *in vitro* transcription (IVT), reagents and kits by Jena Bioscience (Germany) were used along with modified nucleotides 5-methylcytidine (5-Methyl-CTP, m5C) and pseudouridine (Pseudo-UTP, Ψ) by Biolabmix LLC (Russia). To remove the template plasmid DNA, the sample containing the reaction mixture post-IVT was treated with DNase (Thermo Scientific). The Poly(A) Tailing Enzyme Test Kit (Jena Bioscience) was used for the polyadenylation of the obtained mRNA. The resulting mRNA was purified using the RNA Clean & Concentrator kit (Zymo Research, USA). The concentration of the purified RNA was measured using a Qubit-4 fluorimeter (Thermo Scientific). The obtained mRNA was verified by electrophoretic separation under denaturing conditions.

The model 21 bp siRNA-JOE (scrambled siRNA not complementary to any gene in the target organism) with sense strand 5′-CCGGUGUGCUUCGACAACUdTdT and antisense strand 5′-AGUUGUCGAAGCACACCGGdTdT [37] sequences was obtained by annealing synthetic oligonucleotides (DNA Synthesis, Russia) using a T100 Thermal Cycler (BioRad) as follows: 90 °C for 1 minute, then 2 minutes each at 80 °C, 70 °C, 60 °C, 50 °C, and 40 °C followed by 1 hour at 37 °C and hold at 4 °C indefinitely. The annealing efficiency was assessed via 12% PAGE in 0.5 M TBE buffer at 170 V.

Cationic liposomes. Cationic liposomes based on the cationic amphiphile 2X3 [40] and the helper lipid DOPE (Avanti Polar Lipids, USA) were prepared at MIREA – Russian Technological University using previously described methods [35, 37]. Two types of cationic liposomes were produced, 2X3-DOPE in a 1:3 molar ratio ("2X3-DOPE") and 2X3-DOPE with DSPE-PEG_{2000} (0.62 mol%) ("2X3-DOPE-PEG").

Preparation of 2X3-DOPE/mRNA complexes. The 2X3-DOPE/mRNA complexes were prepared in serum-free DMEM/F-12 medium (1:1, Gibco, USA) by adding 5 µl mRNA solution (100 ng) to 0.73 nmol cationic liposomes solution in the final volume of 5 µl at a nitrogen-to-phosphorous (N/P) ratio of 10/1 (cationic lipid/RNA charge ratio). The mixture was then vigorously mixed for 10 seconds and incubated at room temperature for 15 minutes [35].

Preparation of exosome-liposome hybrid complexes with mRNA. The exosome-cationic liposome hybrids (EXO-2X3-DOPE) were prepared in serum-free DMEM/F-12 medium (1:1, Gibco) immediately prior to complex formation by mixing 15 µg exosomes with 0.73 nmol cationic liposomes in the final volume of 5 µl followed by vigorous mixing for 10 seconds and incubation at room temperature for 15 minutes.

The EXO-2X3-DOPE/mRNA complexes were prepared in serum-free DMEM/F-12 medium (1:1, Gibco) by adding 5 µl mRNA solution (100 ng) to 5 µl exosome-cationic liposome hybrid solution at an N/P ratio of 10/1 (specifying the charge ratio of nitrogen atoms in the cationic liposomes to negatively charged phosphate groups from the RNA, excluding exosomes). The mixture was then vigorously mixed for 10 seconds and incubated at room temperature for 15 minutes.

Preparation of 2X3-DOPE-PEG/siRNA complexes. The 2X3-DOPE-PEG/siRNA complexes were prepared in serum-free DMEM/F-12 medium (1:1, Gibco) by adding 5 µl siRNA solution (5 pmol) to 0.42 nmol of cationic liposome solution in the final volume of 5 µl at an N/P ratio of 8/1. The mixture was then vigorously mixed for 10 seconds and incubated at room temperature for 15 minutes [37].

For the experiment assessing the qualitative internalization of the complexes using confocal microscopy, the complexes were prepared in serum-free DMEM/ F-12 medium (1:1, Gibco) by adding 25 µl siRNA solution (25 pmol) to 25 µl cationic liposome solution at an N/P ratio of 8/1. The mixture was vigorously mixed for 10 seconds and incubated at room temperature for 15 minutes.

DOI: https://doi.org/10.17816/MAJ641910

Preparation of exosome-liposome hybrid complexes with siRNA. The exosome-liposome hybrids (EXO-2X3-DOPE-PEG) were prepared immediately prior to complex formation by mixing 15 μg exosomes with 0.42 nmol cationic liposome solution in the final volume 5 μl followed by vigorous mixing for 10 seconds and incubation at room temperature for 15 minutes.

The EXO-2X3-DOPE-PEG/siRNA complexes were prepared in serum-free DMEM/F-12 medium (1:1, Gibco) by adding 5 μl siRNA solution (5 pmol) to 5 μl exosome-cationic liposome hybrid solution at an N/P ratio of 8/1 (for cationic liposomes and siRNA, excluding exosomes). The mixture was then vigorously mixed for 10 seconds and incubated at room temperature for 15 minutes. For the qualitative assessment of complex internalization using confocal microscopy, the complexes were similarly prepared to achieve a final volume of 50 μl.

Dynamic Light Scattering (DLS). The hydrodynamic diameters of the studied complexes were determined using a Zetasizer Nano-ZS system (Malvern Instruments, UK). Fresh complexes were diluted with indicator-free medium filtered through a 0.2 μm syringe filter (Jet Biofil, China) to a final volume of 50 μl (ZEN0118 cuvette, Malvern Instruments). Measurements were conducted by backscattering at an angle of 173°.

Atomic force microscopy (AFM). Thirty microliters of distilled water were added to 5 μl sample prepared in the medium, which was then applied to freshly cleaved mica (SPI Supplies) and incubated for 1 minute. The sample was subsequently removed and the substrate was washed three times with 50 μl distilled water followed by drying in a SpeedVac vacuum concentrator (Eppendorf) for 15 minutes. The sample surface topography was measured using a SolverNEXT scanning probe microscope (NT-MDT, Russia) in semi-contact mode and an NSG-03 probe (NT-MDT). For each sample, images of several scanning fields measuring 20 × 20 μm and 5 × 5 μm were made. The images were processed using the Gwyddion software [41].

Primary cell cultures. In this study, two types of cardiac mesenchymal cells were used, post-infarction cardiac mesenchymal cells (iCMC) isolated from the infarction zone of the ischemic myocardium in Wistar rats 24 hours after surgically induced myocardial infarction and healthy cardiac mesenchymal cells (hCMC) drawn from the myocardium of rats following sham surgery. The methods for cell isolation and phenotypic characterization are described in detail in our previous publication [42, 43]. Cells from the ischemic myocardium were isolated by mechanical tissue dissociation followed by enzymatic digestion with a collagenase solution. The resulting cell suspension was centrifuged and cultured at 37 °C in an atmosphere of 5% CO_2 and 99% humidity. We used the DMEM/F-12 (Invitrogen, USA) culture medium with added 20% ECM endothelial medium (Invitrogen), 10% fetal bovine serum (HyClone, USA), 100 mM essential amino acids (MEM NEAA, Gibco), 2 mM L-glutamine (Gibco), and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Gibco). From the second day of cultivation, the medium was replaced daily. Large tissue fragments were removed on the third day. By the third passage, both cell populations consisted of homogeneous cultures that did not contain living cardiomyocytes. Mesenchymal cells were identified based on their phenotypic characteristics, including adhesion to plastic, expression of CD90 and CD166 markers, and the absence of endothelial and hematopoietic markers (CD45, CD31, CD34).

Transfection of cells with the studied complexes. The day before transfection, iCMC and hCMC cells were seeded at a density of 10^4 cells per well in a 96-well plate and incubated at 37 °C with 5% CO_2. On the day of transfection, once the cells reached 90% confluency, 10 μl of the solutions containing the studied complexes were added to each well (100 ng of mRNA or 5 pmol of siRNA per well) and incubated for 24 hours at 37 °C with 5% CO_2.

For experiments assessing the qualitative internalization of the complexes using confocal microscopy, the day before transfection, cells were seeded onto a coverslip placed in a well of a 24-well plate at a density of 6×10^4 cells per well and incubated at 37°C with 5% CO_2. On the day of transfection, once the cells reached 90% confluency, 50 μl of the solutions containing the studied complexes with siRNA (25 pmol per well) were added to the cells and incubated for 24 hours at 37 °C with 5% CO_2.

Flow cytometry. Quantitative evaluation of the transfection efficiency of iCMC and hCMC cells with the studied complexes containing mRNA-eGFP and siRNA-JOE was performed using a CytoFLEX (Beckman Coulter, USA) 24 hours after cell transfection. Prior to analysis, the cells were stained with NucBlue Live ReadyProbe Reagent (Hoechst 33342) (Thermo Scientific) according to the manufacturer's instructions. The cells were then washed with PBS, removed using trypsin, centrifuged at 400 g for 5 minutes, and re-dissolved in 100 μl PBS.

Transfection efficiency was assessed by the percentage of transfected cells and by the mean fluorescence intensity (MFI) of the cells. mRNA-eGFP complexes were detected using the eGFP fluorescent signal; whereas siRNA complexes were detected using the JOE fluorescent signal. At least 10,000 events were measured for each sample. The results were analyzed using Kaluza Analysis software with statistical analysis performed in GraphPad Prism 10 software.

Fluorescence microscopy. Qualitative assessment of the efficiency of iCMC and hCMC cell transfection with the studied complexes containing siRNA-JOE was performed using the Cytell Cell Imaging System (GE Healthcare, USA) 24 hours after transfection. NucBlue Live ReadyProbe Reagent (Hoechst 33342) was used for intravital imaging of cell nuclei.

Laser scanning confocal microscopy. Qualitative assessment of the internalization of the studied complexes with siRNA-JOE was conducted using a Leica TCS SP8

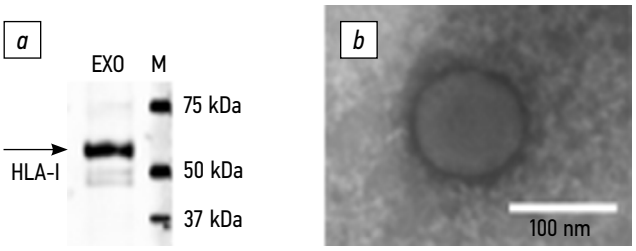

Fig. 1. Characterization of extracellular vesicles isolated from human serum. *a*, Western blot analysis using antibodies against the specific exosome marker protein HLA class I. M, the molecular weight marker with electrophoretic mobility in kDa shown on the right. EXO, the sample under investigation; the arrow indicates the position of the detected protein band. *b*, Electron micrograph of a sample of extracellular vesicles. The scale bar is 100 nm long.

Рис. 1. Характеристика внеклеточных везикул, выделенных из сыворотки крови человека: *a* — вестерн-блоттинг с антителами к специфическому маркерному белку экзосом HLA-I; M — маркер молекулярных масс, справа подписана электрофоретическая подвижность в кДа; EXO — исследуемый образец, стрелкой отмечено положение выявленной зоны белка; *b* — электронная микрофотография образца внеклеточных везикул. Длина масштабного отрезка 100 нм.

confocal microscope (Leica Microsystems, Germany) equipped with an oil immersion objective with a magnification of 60X and a numerical aperture of 1.25.

24 hours post-transfection, the cells were fixed in 4% paraformaldehyde (PFA) and permeabilized with 0.1% Triton X-100. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and the actin cytoskeleton was stained with Alexa Fluor 680 Phalloidin (Thermo Scientific).

RESULTS AND DISCUSSION

Isolation and characterization of exosomes. Exosomes were isolated from human serum using a standard method involving sequential centrifugation and ultracentrifugation of the sample. To confirm the presence of exosomes in the isolated extracellular vesicles from blood serum, they were characterized for marker proteins using Western blotting; their size and morphology were assessed using transmission electron microscopy (TEM) (see Fig. 1).

It was shown that the sample is positive for the exosomal marker protein HLA class I (see Fig. 1, *a*) and the vesicles in the sample are spherical with a size of approximately 100 nm (see Fig. 1, *b*), which is consistent with exosomes. Additionally, the hydrodynamic diameter of the vesicles determined by DLS was 178.5 ± 3.9 nm (see Table 1). Thus, we can conclude that exosomes are present in the extracellular vesicle samples isolated from blood serum.

Preparation and characterization of complexes with mRNA and siRNA. To study the efficiency of RNA molecule delivery to the cells under investigation, we obtained hybrid nanoparticles containing exosomes from blood serum and 2X3-DOPE liposomes able to form complexes when mixed with RNA. The formation of these hybrid nanoparticles can be detected by a change in the hydrodynamic diameter (D_H) relative to the original exosomes and liposomes [34].

Cationic liposomes and their complexes prepared in RNase-free water and the optimal N/P ratios for complex formation were previously characterized in our publications [35] (for 2X3-DOPE and mRNA complexes) and [37] (for 2X3-DOPE-PEG and siRNA complexes). However, the physiological internal composition of exosomes isolated from blood serum does not allow for DLS measurements in water. Therefore, at the first stage of the study, cationic liposomes (2X3-DOPE and 2X3-DOPE-PEG), exosomes (EXO), hybrid nanoparticles (EXO-2X3-DOPE and EXO-2X3-DOPE-PEG), and complexes with mRNA and siRNA (2X3-DOPE/mRNA, EXO-2X3-DOPE/mRNA, 2X3-DOPE-PEG/siRNA, and EXO-2X3-DOPE-PEG/siRNA) were characterized using the DLS method in cell culture medium without serum (see Table 1). The hydrodynamic diameter (D_H, nm) is also shown in Fig. 2.

The study showed that the hybrid EXO-2X3-DOPE nanoparticles had a higher D_H of 376 ± 8 nm compared to the studied EXO exosomes (178 ± 4 nm) and a lower D_H than that of the cationic 2X3-DOPE liposomes (998 ± 62 nm). This may indicate the formation of a new structure during the preparation of the hybrid nanoparticles. With added mRNA, the particle size differed significantly from that of the original liposomes and hybrid nanoparticles, reaching 700–800 nm, which indicates the successful formation of the complexes. It is worth noting that previous measurements of liposomes and their complexes with mRNA in water showed D_H values of approximately 70 nm and 100–150 nm [35], respectively, suggesting a trend to form associates in a buffer solution driven by weak interactions.

In samples containing 2X3-DOPE-PEG, there was no significant change in size neither upon the formation of hybrid nanoparticles nor upon the formation of complexes with siRNA and the hybrid nanoparticles; D_H values in all cases were

Table 1. Determination of the hydrodynamic diameter (D_H, nm) and polydispersity index (PDI) of cationic liposomes, exosomes, hybrid nanoparticles, and their complexes with RNA by the DLS method. The results are shown as the mean ± standard deviation for three measurements

Таблица 1. Определение гидродинамического диаметра (D_H, нм) и индекса полидисперсности (PDI) катионных липосом, экзосом, гибридных наночастиц и их комплексов с РНК методом динамического рассеяния света. Результаты представлены как среднее значение ± стандартное отклонение по трем измерениям

Sample	D_H, nm	PDI
EXO	178 ± 4	0.46 ± 0.01
2X3-DOPE	998 ± 62*	0.12 ± 0.05
EXO-2X3-DOPE	376 ± 8	0.45 ± 0.07
2X3-DOPE/mRNA	790 ± 51	0.52 ± 0.04
EXO-2X3-DOPE/mRNA	674 ± 6	0.52 ± 0.07
2X3-DOPE-PEG	161 ± 1**	0.09 ± 0.01
EXO-2X3-DOPE-PEG	184 ± 4	0.38 ± 0.05
2X3-DOPE-PEG/siRNA	390 ± 7	0.17 ± 0.03
EXO-2X3-DOPE-PEG/siRNA	211 ± 3	0.27 ± 0.01

Note: *D_H of 2X3-DOPE nanoparticles in water was 64.7 ± 0.5 nm, corresponding to [35]; **D_H of 2X3-DOPE-PEG nanoparticles in water was 49.4 ± 1.3 nm, corresponding to [37].

around 150–200 nm. In contrast, the formation of complexes between siRNA and liposomes resulted in a 2.5-fold increase in particle size (from 161 ± 1 nm to 390 ± 7 nm). It should be noted that previously determined characteristics of complexes 2X3-DOPE-PEG with siRNA in RNase-free water showed similar changes, albeit with slightly smaller sizes (59 ± 1 nm and 218 ± 7 nm, respectively) [37], indicating greater stability of the PEGylated form of liposomes in buffer solution.

The larger particle size upon the formation of a complex with 2X3-DOPE-PEG and its relative stability in complexes with hybrid nanoparticles may suggest more efficient compaction of siRNA in conjunction with the latter. Importantly, all samples with PEGylated cationic liposomes had a low polydispersity index (PDI), indicating greater stability in the culture medium compared to their non-PEGylated counterparts.

Further optimization of the liposome/exosome ratio in hybrid nanoparticles could enhance the stability of the resulting complexes in both the culture medium and the *in vivo* environment. In addition, the formation of liposome complexes with nucleic acids and exosome-liposome hybrids with nucleic acids was visualized using atomic force microscopy (AFM) (see Fig. 3).

It was found that liposome complexes with nucleic acids form particles that are similar to those previously described in [35] and [37] for the 2X3-DOPE/mRNA and 2X3-DOPE-PEG/siRNA complexes, respectively. However, when complexes are formed in the presence of exosomes with sizes correlating with the TEM data (see Fig. 1, *b*), qualitatively new, larger structures are produced (see Fig. 3, right column).

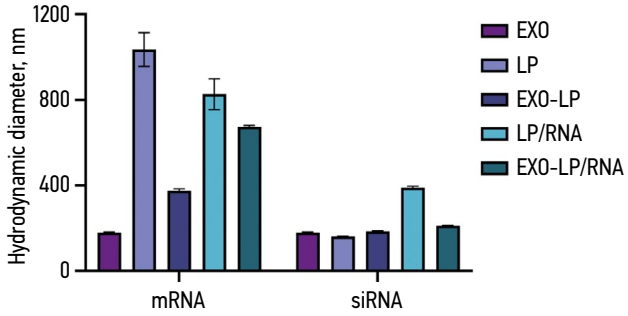

Fig. 2. Hydrodynamic diameter of the studied samples determined by the DLS method. EXO, exosomes; LP, cationic liposomes (2X3-DOPE or 2X3-DOPE-PEG for mRNA or siRNA, respectively); EXO-LP, hybrid nanoparticles containing 2X3-DOPE or 2X3-DOPE-PEG; LP/RNA, complexes of liposomes with RNA; EXO-LP/RNA, complexes of hybrid nanoparticles with RNA.

Рис. 2. Гидродинамический диаметр исследуемых образцов, полученный методом динамического рассеяния света. EXO — экзосомы; LP — катионные липосомы 2X3-DOPE или 2X3-DOPE-PEG для матричных (мРНК) или малых интерферирующих (миРНК) соответственно; EXO-LP — гибридные наночастицы с 2X3-DOPE или 2X3-DOPE-PEG; LP/RNA — комплексы липосом и РНК; EXO-LP/RNA — комплексы гибридных наночастиц с РНК.

Thus, based on the DLS and AFM data, it can be concluded that in the presence of exosomes, complexes are formed that differ from the original 2X3-DOPE/mRNA and 2X3-DOPE-PEG/siRNA. This suggests the formation of exosome-liposome hybrids that carry mRNA and siRNA. It is important to note that future studies should focus on optimizing the conditions for the preparation of exosome-liposome hybrids and a de-

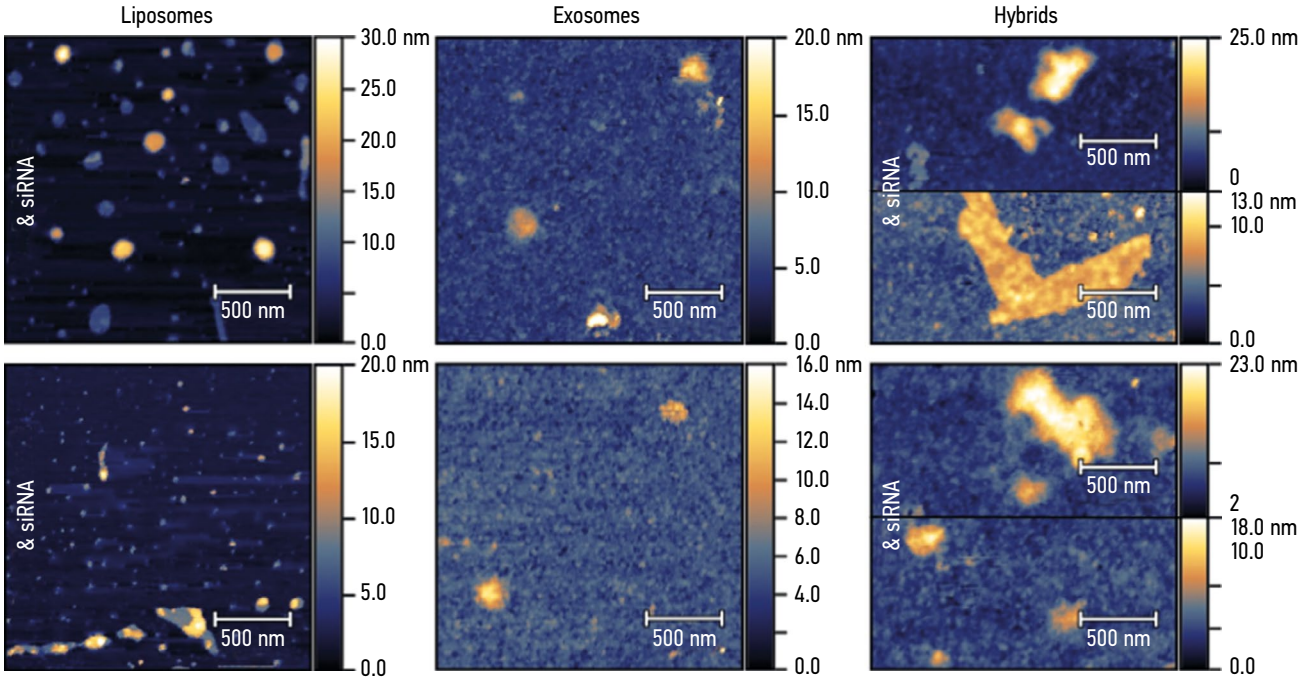

Fig. 3. Surface topography of samples containing 2X3-DOPE/mRNA and 2X3-DOPE-PEG/siRNA complexes (left column), exosomes (middle column), and their hybrids (right column) visualized by atomic force microscopy. A pseudo-color scale bar reflecting the particle height in nanometers (nm) is shown to the right of all images. The scale bar in each image is 500 nm long.

Рис. 3. Топография поверхности образцов, содержащих комплексы 2X3-DOPE/мРНК и 2X3-DOPE-PEG/миРНК (левый столбец), экзосомы (средний столбец) и их гибриды (правый столбец), полученные методом атомно-силовой микроскопии. Справа от всех рисунков изображена линейка псевдоцвета, отражающая высоту частиц в нм. Длина масштабного отрезка составляет 500 нм.

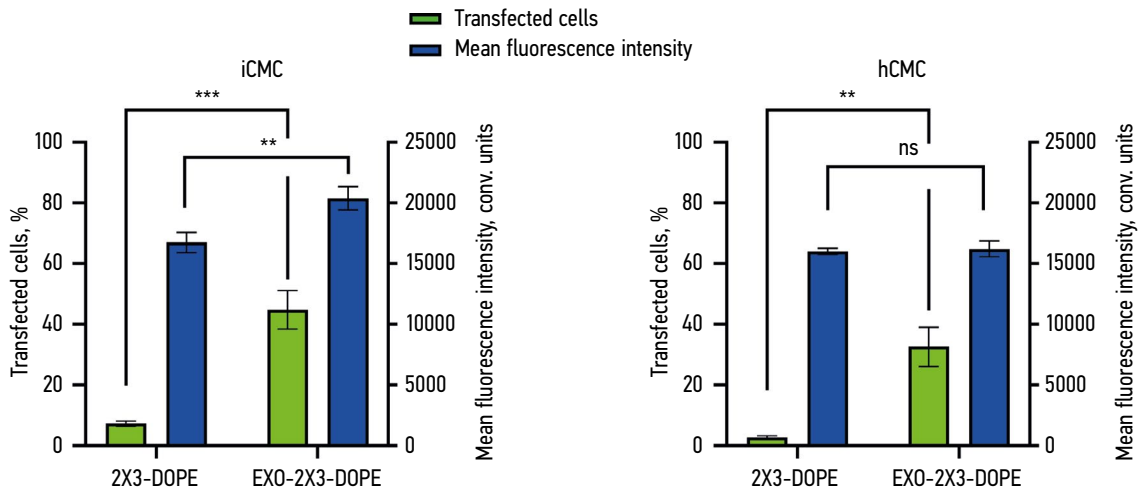

Fig. 4. Transfection efficiency of iCMC and hCMC with complexes of cationic liposome 2X3-DOPE with mRNA-eGFP and complexes of hybrid nanoparticles EXO-2X3-DOPE with mRNA-eGFP, 24 hours post-transfection, assessed by flow cytometry. The statistical significance of differences was determined using one-way ANOVA adjusted for multiple comparisons (Tukey test) (***$p < 0.0002$, **$p < 0.0021$).

Рис. 4. Эффективность трансфекции клеток iCMC и hCMC комплексами катионных липосом 2X3-DOPE с мРНК-eGFP и комплексами гибридных наночастиц EXO-2X3-DOPE с мРНК-eGFP через 24 ч с момента трансфекции, выполненное методом проточной цитофлуориметрии. Статистическая значимость различий была определена с помощью однофакторного дисперсионного анализа с поправкой на множественное сравнение (тест Тьюки) (***p <0,0002, **p <0,0021).

tailed characterization of their physicochemical properties. Such efforts will help determine the mechanisms of their formation and potentially improve the efficiency of encapsulation and delivery of nucleic acids.

Evaluation of the efficiency of cell transfection with complexes containing model mRNA and siRNA. To evaluate the efficacy of EXO-2X3-DOPE and EXO-2X3-DOPE-PEG hybrids as RNA delivery vehicles in iCMC and hCMC cells, we used model RNAs, including mRNA encoding the green fluorescent protein eGFP (mRNA-eGFP) and siRNA without a target gene labeled with the fluorescent tag JOE (siRNA-JOE).

The efficiency of mRNA-eGFP delivery to iCMC and hCMC cells using cationic liposomes 2X3-DOPE and EXO-2X3-DOPE-PEG hybrid nanoparticles was assessed by flow cytometry. It is only possible to detect the fluorescent signal from eGFP with the successful intracellular delivery of mRNA-eGFP and subsequent protein translation.

It was shown that the use of EXO-2X3-DOPE hybrid nanoparticles increased the proportion of transfected iCMC from 7.3 ± 0.9% to 44.8 ± 6.3% and hCMC from 2.7 ± 0.5% to 32.6 ± 6.5% compared to the delivery of mRNA-eGFP with cationic liposomes 2X3-DOPE (see Fig. 4, green bars).

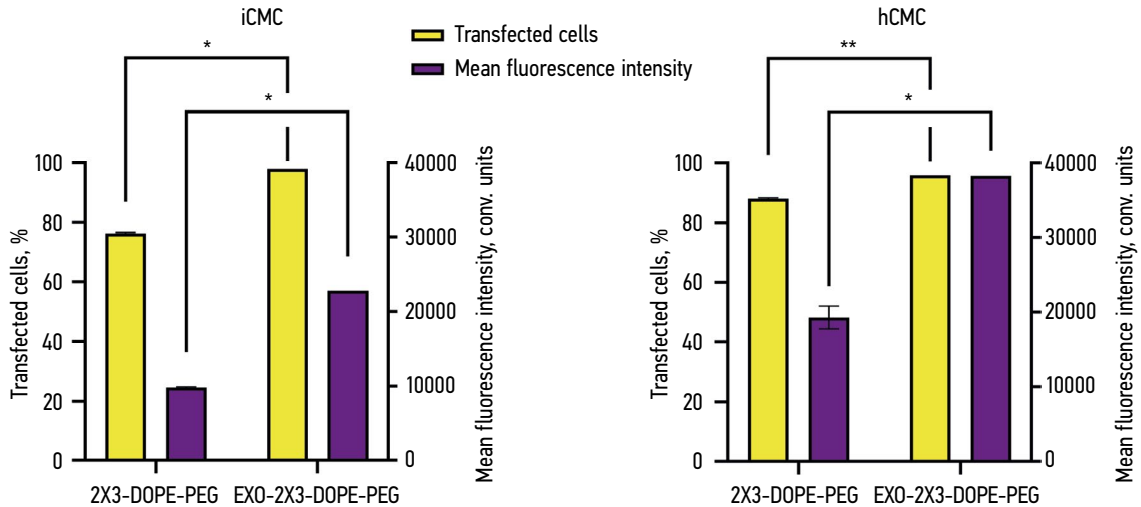

Fig. 5. Transfection efficiency of iCMC and hCMC with cationic liposome complexes 2X3-DOPE-PEG containing siRNA-JOE and hybrid nanoparticle complexes EXO-2X3-DOPE-PEG with siRNA-JOE, 24 hours post-transfection, assessed by flow cytometry. Statistical significance of the differences was determined using a one-way ANOVA adjusted for multiple comparisons (Tukey test) (**$p < 0.0021$, *$p < 0.0332$).

Рис. 5. Эффективность трансфекции клеток iCMC и hCMC комплексами катионных липосом 2X3-DOPE-PEG с миРНК-JOE и комплексами гибридных наночастиц EXO-2X3-DOPE-PEG с миРНК-JOE через 24 ч с момента трансфекции, выполненное методом проточной цитофлуориметрии. Статистическая значимость различий была определена с помощью однофакторного дисперсионного анализа с поправкой на множественное сравнение (тест Тьюки) (**p <0,0021, *p <0,0332).

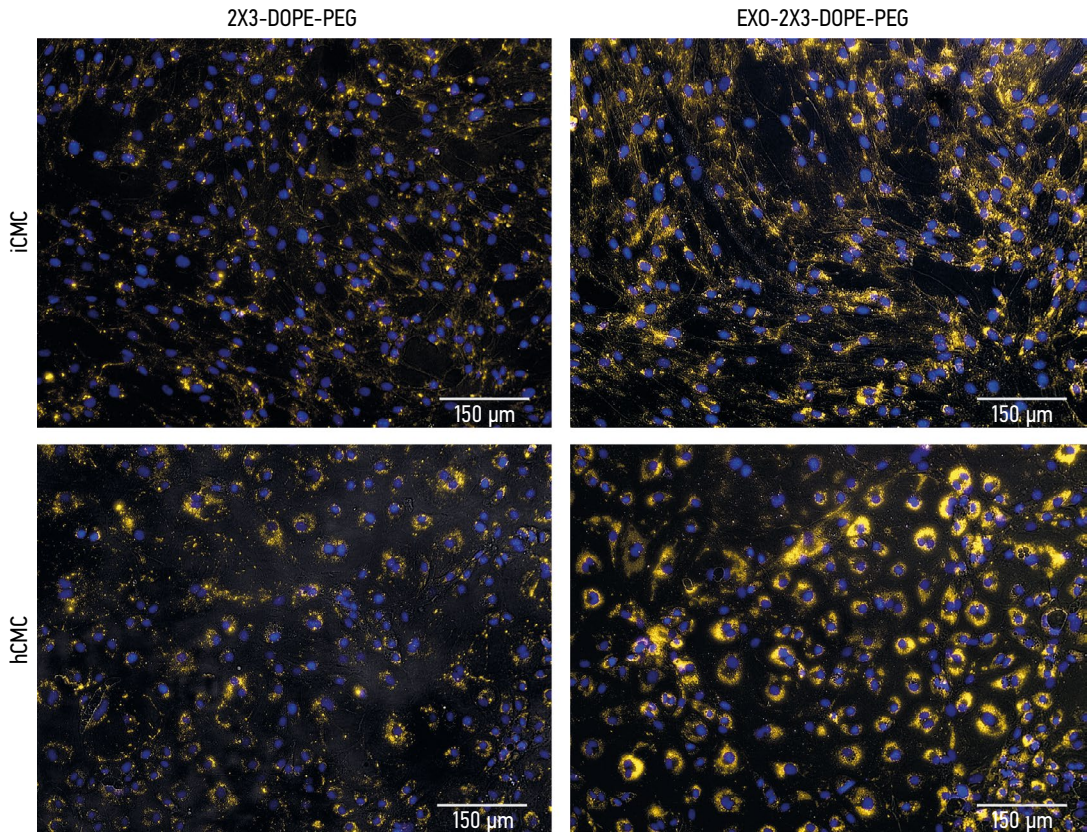

Fig. 6. Transfection efficiency of iCMC (top) and hCMC (bottom) with cationic liposome complexes 2X3-DOPE-PEG containing siRNA-JOE (left) and hybrid nanoparticle complexes EXO-2X3-DOPE-PEG with siRNA-JOE (right), 24 hours post-transfection, assessed by fluorescence microscopy. Cell nuclei are stained with Hoechst 33342 (blue); siRNA is labeled with JOE (yellow). The scale bar is 150 μm long.

Рис. 6. Оценка эффективности трансфекции клеток iCMC (сверху) и hCMC (снизу) комплексами катионных липосом 2X3-DOPE-PEG с миРНК-JOE (слева) и комплексами гибридных наночастиц EXO-2X3-DOPE-PEG с миРНК-JOE (справа) через 24 ч с момента трансфекции, выполненное методом флуоресцентной микроскопии. Ядра клеток окрашены Hoechst 33342 (синий), миРНК мечена JOE (желтый). Длина масштабного отрезка составляет 150 мкм.

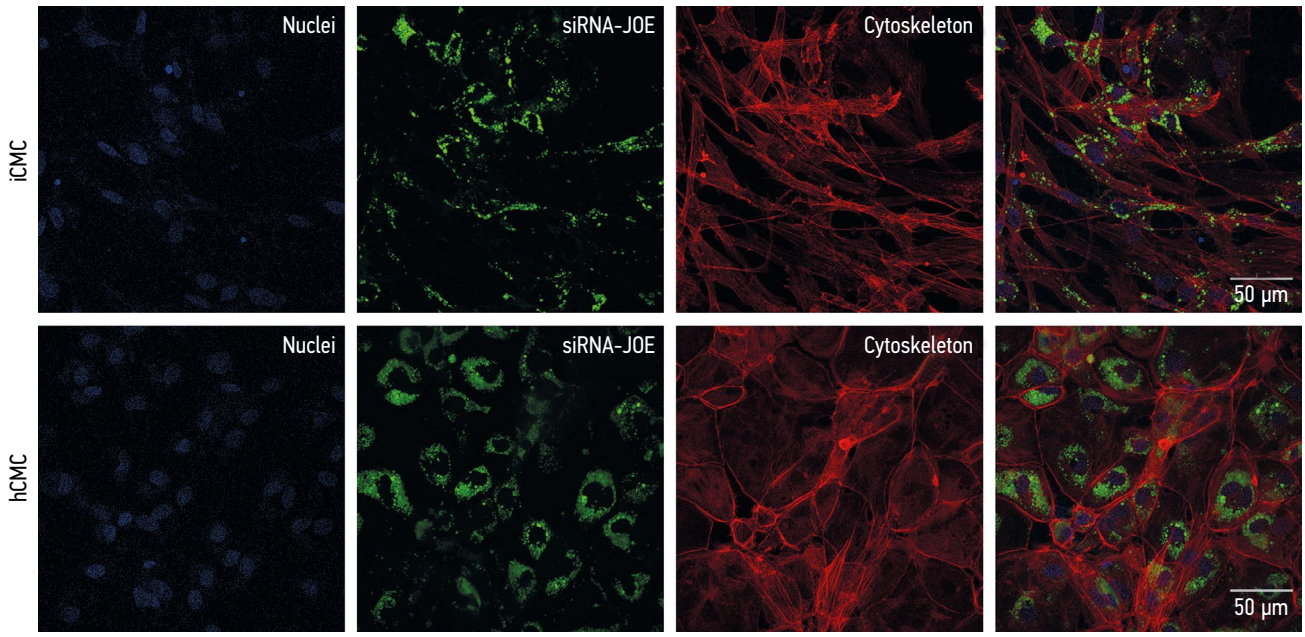

Fig. 7. Evaluation of internalization of the studied complexes in iCMC (top) and hCMC (bottom), 24 hours post-transfection, by laser scanning confocal microscopy. Nuclei are stained with DAPI (blue), siRNA is labeled with JOE (green), and the actin cytoskeleton is stained with Alexa Fluor 680 Phalloidin (red). The scale bar is 50 μm long.

Рис. 7. Оценка интернализации исследуемых комплексов в iCMC (сверху) и hCMC (снизу) через 24 ч с момента трансфекции, выполненное методом лазерной сканирующей конфокальной микроскопии. Ядра окрашены DAPI (синий), миРНК мечена JOE (зеленый), актиновый цитоскелет Alexa Fluor 680 фаллоидин (красный). Длина масштабного отрезка составляет 50 мкм.

Moreover, in iCMC, the use of EXO-2X3-DOPE also increased the average fluorescence intensity from 16,754 ± 832 arbitrary units to 20,402 ± 954 arbitrary units, indicating more efficient translation of mRNA-eGFP (see Fig. 4, blue columns).

Thus, it was demonstrated that cationic liposomes 2X3-DOPE have low efficiency of delivering model mRNA-eGFP to rat cardiac MSCs *in vitro*. However, the modification of liposomes with exosomes increases the efficiency of mRNA delivery by 30–35%.

There are limited studies focused on the development of non-viral mRNA delivery systems for mesenchymal stem cells. For instance, the transfection efficiency of human bone marrow-derived stromal cells (hBMSCs) was reported to be 18.2% when delivering mRNA using a cationic hyperbranched poly(amidoamine)-based nanoparticle system (PAMAM) [44]. In other stem cells, the highest efficiency of mRNA delivery by these nanoparticles was 26.6%. The vast majority of studies on non-viral delivery of mRNA to MSCs use commercial lipid-based transfection reagents, such as Lipofectamine RNAiMAX [45], Lipofectamine MessengerMAX [46], Lipofectamine2000, and Stemfect [47]. However, the use of such carriers *in vivo* is unfeasible due to their high toxicity. Thus, the development of effective and safe vehicles to deliver mRNA to MSCs for potential *in vivo* applications remains a pertinent issue.

The original 2X3-DOPE liposomes used in our study have already been successfully used to deliver model mRNAs *in vivo* [48], making the creation of hybrid nanoparticles based on them a promising area for further development of mRNA carriers. Despite the fact that the transfection efficiency determined in our study is higher than that reported in the literature for original carriers, it remains below 50%. Therefore, further improvement of the technology for preparing hybrid nanoparticles is required to improve the efficiency of cell transfection.

To evaluate the efficiency of siRNA delivery using EXO-2X3-DOPE-PEG hybrid nanoparticles, a quantitative analysis of the percentage of transfected cells was also conducted using flow cytometry. The results showed that the use of EXO-2X3-DOPE-PEG hybrid nanoparticles increased the percentage of transfected iCMC and hCMC from 75–90% to nearly 100% (see Fig. 5, yellow bars). In addition, the application of EXO-2X3-DOPE-PEG hybrid nanoparticles augmented the mean fluorescence intensity by 2.3 times for iCMC and by 2 times for hCMC compared to cationic liposomes (see Fig. 5, purple bars). These findings correlate well with the qualitative assessment of transfection efficiency in iCMC and hCMC performed via fluorescence microscopy (see Fig. 6).

To confirm the internalization of the fluorescently labeled siRNA molecules into iCMC and hCMC, laser scanning confocal microscopy was performed 24 hours after cell transfection with the studied complexes. Representative data are shown in the images of iCMC and hCMC with complexes of cationic liposome 2X3-DOPE-PEG containing siRNA-JOE (see Fig. 7). The results show that the complexes are localized in the focal plane of the nucleus and do not extend beyond the actin cytoskeleton, indicating their successful intracellular localization.

Thus, it has been demonstrated that cationic liposomes 2X3-DOPE-PEG are highly efficient for the delivery of model siRNA-JOE to rat heart MSCs *in vitro*, achieving over 75% transfection rate. Furthermore, the modification of liposomes with exosomes increases siRNA delivery efficiency to nearly 100% as indicated by both the proportion of transfected cells and a more than twofold increase in mean fluorescence intensity.

In contrast, siRNA delivery systems in MSCs are actively developed. For instance, lipid-polymer nanoparticles based on poly(lactic-*co*-glycolic) acid (PLGA) combined with DOTAP and DOPE lipids have achieved siRNA delivery efficiency to MSCs of 72.7% [49]. In addition, the use of poly-ε-caprolactone scaffolds functionalized with lipid-polymer nanoparticles (using the commercial transfection reagent TransIT-TKO) resulted in a transfection efficiency of approximately 75% after 48 hours [50]. A different approach involving a polymer carrier pDMAEMA-*b*-p(DMAEMA-*co*-PAA-*co*-BMA) successfully delivered siRNA to 96% MSCs [51]. Our study showed that cationic liposomes 2X3-DOPE-PEG used either independently or as part of hybrid nanoparticles with exosomes are highly efficient in delivering siRNA to MSCs, making them suitable for further studies as siRNA delivery vehicles.

It is important to note that the incorporation of exosomes to improve the efficiency of cell transfection in hybrid nanoparticles both addresses the issue of increasing the percentage of transfected cells and improves the targeting of mRNA and siRNA delivery due to various receptors on the surface of exosomes.

CONCLUSION

The main objectives of this study were, first, to demonstrate that it is possible to deliver model mRNA and siRNA to mesenchymal stem cells *in vitro* by cationic liposomes 2X3-DOPE using rat heart MSCs as an example, and, second, to improve the delivery efficiency by modifying the latter with exosomes and obtaining hybrid nanoparticles. It was shown that cationic liposomes are extremely ineffective in delivering mRNA (at the level of a few percent of transfected cells), but added exosomes increase the percentage of transfected cells to 30–40%. When delivering model siRNA, it was shown that cationic liposomes themselves ensure effective delivery at about 80–90% of transfected cells; whereas exosomes increase this proportion to 100% and the content of siRNA in cells increases by more than 2 times. Thus, the use of exosomes to produce hybrid nanoparticles with cationic liposomes 2X3-DOPE approach for developing non-viral delivery methods of mRNA and siRNA to mesenchymal stem cells with the potential for targeted delivery. The conditions

for producing these hybrid nanoparticles and detailed characterization of their physicochemical properties will be further improved.

ADDITIONAL INFORMATION

Author contributions: O.V. Dovbysh: preparation and physicochemical characterization of complexes, evaluation of transfection efficiency using flow cytometry, fluorescence and laser scanning microscopy, writing—original draft, visualization; V.V. Vysochinskaya: preparation and physicochemical characterization of complexes, evaluation of transfection efficiency using flow cytometry, fluorescence and laser scanning microscopy, writing—original draft; N.V. Gavrilova: exosome isolation, transmission electron microscopy, writing—original draft; P.M. Docshin: rat cardiac mesenchymal cells isolation, writing—original draft; E.G. Nikitina: rat cardiac mesenchymal cells isolation; A.S. Klochev: exosome isolation, western blotting, writing—original draft; E.A. Elpaeva: genetic engineering, writing—original draft; O.A. Dobrovolskaya: mRNA producing using in vitro transcription, writing—original draft; E.V. Shmendel: cationic liposomes formulation; M.A. Maslov: synthesis of 2X3 to liposome formulation, funding acquisition; Ya.A. Zabrodskaya: conceptualization, atomic force microscopy, visualization, writing—original draft, writing—review & editing, supervision, funding acquisition. All authors approved the version of the manuscript to be published, and agreed to be accountable for all aspects of the work, ensuring that issues related to the accuracy or integrity of any part of it are appropriately reviewed and resolved.

Ethics approval: The animal study was approved by the Bioethics Commission of the Saint Petersburg State Chemical and Pharmaceutical University (Minutes No. Rats-MI-SC-2018, dated January 10, 2018).

Funding sources: The study was supported by grant No. 24-15-20026 from the Russian Science Foundation and the St. Petersburg Science Foundation, https://rscf.ru/project/24-15-20026/. Liposome formulation was performed with the financial support of the Ministry of Science and Higher Education of the Russian Federation under the strategic academic leadership program *Priority 2030*, agreement 075-15-2024-221 dated February 2, 2024.

Disclosure of interests: The authors have no relationships, activities, or interests for the last three years related to for-profit or not-for-profit third parties whose interests may be affected by the content of the article.

Statement of originality: No previously obtained or published material (text, images, or data) was used in this study or article.

Data availability statement: All data generated in this study are available in the article. All the data will be available upon reasonable request. Please send your request to the corresponding author.

Generative AI: Generative AI technologies were not used for this article creation.

Provenance and peer-review: This paper was submitted unsolicited and reviewed following the standard procedure. The review process involved an external reviewer and an in-house reviewer.

ДОПОЛНИТЕЛЬНАЯ ИНФОРМАЦИЯ

Вклад авторов. О.В. Довбыш — приготовление и физико-химическая характеристика комплексов, оценка эффективности трансфекции методами проточной цитофлуориметрии, флуоресцентной и лазерной сканирующей микроскопии, написание и перевод статьи, визуализация данных; В.В. Высочинская — приготовление комплексов, оценка эффективности трансфекции методами проточной цитофлуориметрии, флуоресцентной и лазерной сканирующей микроскопии, написание статьи; Н.В. Гаврилова — выделение экзосом, просвечивающая электронная микроскопия, написание статьи; П.М. Докшин — получение мезенхимных клеток сердца крысы, написание статьи; Е.Г. Никитина — получение мезенхимных клеток сердца крысы; А.С. Клочев — выделение экзосом, вестерн-блоттинг, написание статьи; Е.А. Елпаева — генная инженерия, написание статьи; О.А. Добровольская — получение мРНК методом *in vitro* транскрипции, написание статьи; Е.В. Шмендель — получение катионных липосом; М.А. Маслов — получение липида 2X3 для катионных липосом, финансирование; Я.А. Забродская — атомно-силовая микроскопия, общее руководство работой, формулировка идеи, визуализация данных, написание и редактирование статьи, перевод статьи на английский язык, финансирование. Все авторы одобрили рукопись (версию для публикации), а также согласились нести ответственность за все аспекты настоящей работы, гарантируя надлежащее рассмотрение и решение вопросов, связанных с точностью и добросовестностью любой ее части.

Этическая экспертиза. Работа с животными была одобрена биоэтической комиссией Санкт-Петербургского государственного химико-фармацевтического университета (протокол № Rats-MI-SC-2018 от 10.01.2018).

Источники финансирования. Исследование поддержано грантом Российского научного фонда № 24-15-20026, https://rscf.ru/project/24-15-20026/, совместно с грантом Санкт-Петербургского научного фонда. Приготовление липосом выполнено при финансовой поддержке Министерства науки и высшего образования Российской Федерации в рамках программы «Приоритет 2030», соглашение 075-15-2024-221 от 07.02.2024.

Раскрытие интересов. Авторы заявляют об отсутствии отношений, деятельности и интересов за последние три года, связанных с третьими лицами (коммерческими и некоммерческими), интересы которых могут быть затронуты содержанием статьи.

Оригинальность. При создании настоящей работы авторы не использовали ранее опубликованные сведения (текст, иллюстрации, данные).

Доступ к данным. Все данные, полученные в настоящем исследовании, доступны в статье, а также могут быть предоставлены автором, ответственным за переписку, по запросу.

Генеративный искусственный интеллект. При создании настоящей статьи технологии генеративного искусственного интеллекта не использовали.

Рассмотрение и рецензирование. Настоящая работа подана в журнал в инициативном порядке и рассмотрена по обычной процедуре. В рецензировании участвовали внешний и внутренний рецензенты.

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