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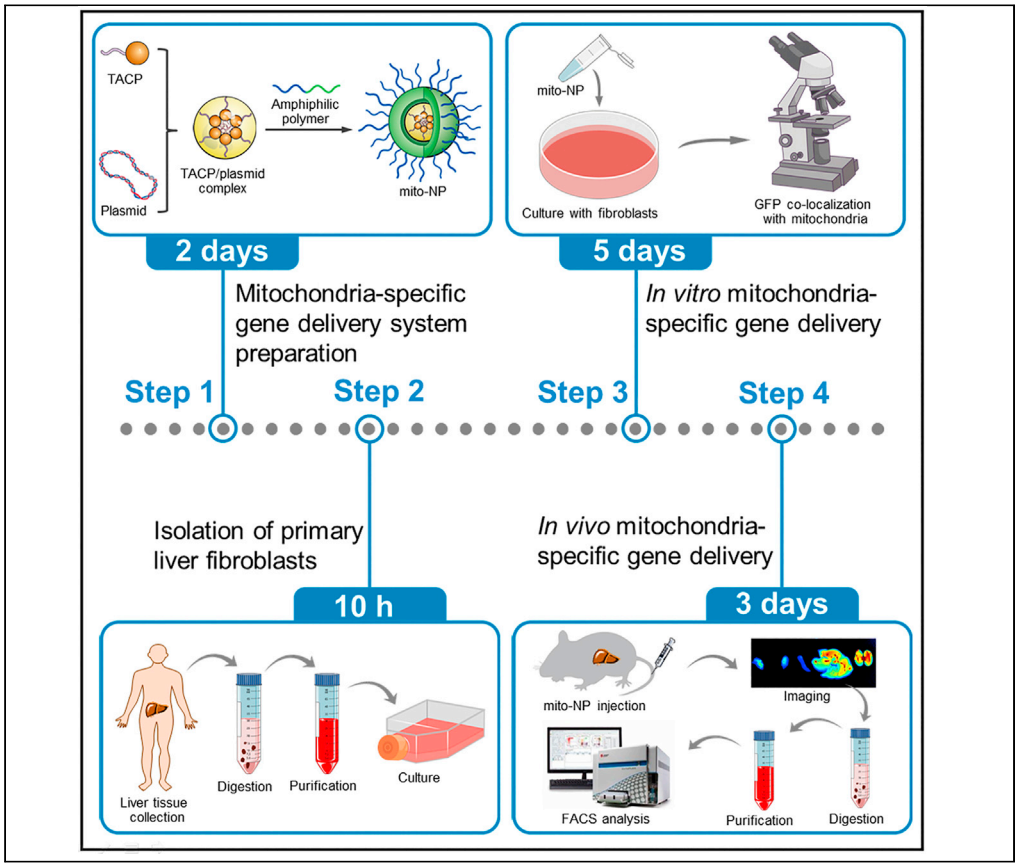
Mitochondria-specific delivery system for targeted regulation of mitochondrial gene expression



Targeted regulation of mitochondrial gene expression is challenging due to the lack of a mitochondria-specific delivery system. We have previously developed various stimuli-responsive nanoparticle (NP)-based delivery systems to transport nucleic acids for regulation of target gene expression. This protocol describes the design and preparation of an NP platform for mitochondria-specific gene delivery (mito-NP). We use mito-NP in primary liver fibroblasts that are transplanted into mice. Mito-NP can be used to deliver various nucleic acid therapeutics and to treat mitochondria-regulated diseases.

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HIGHLIGHTS

A protocol for mitochondria-specific gene delivery system is described

The delivery system achieves targeted regulation of mitochondrial gene expression

This protocol is applicable to mitochondria-specific delivery of various nucleic acids

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Mitochondria-specific delivery system for targeted regulation of mitochondrial gene expression

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SUMMARY

Targeted regulation of mitochondrial gene expression is challenging due to the lack of a mitochondria-specific delivery system. We have previously developed various stimuli-responsive nanoparticle (NP)-based delivery systems to transport nucleic acids for regulation of target gene expression. This protocol describes the design and preparation of an NP platform for mitochondria-specific gene delivery (mito-NP). We use mito-NP in primary liver fibroblasts that are transplanted into mice. Mito-NP can be used to deliver various nucleic acid therapeutics and to treat mitochondria-regulated diseases.

For complete details on the use and execution of this protocol, please refer to Zhao et al. (2020).

BEFORE YOU BEGIN

Prior to the preparation of the mitochondria-specific targeting nanoplatform (denoted mito-NP), a triphenylphosphonium (TPP)-decorated amphiphilic cationic peptide ($C_{17}H_{35}$CONH-GR_6GGGK (TPP)G-OH, denoted TACP) and an endosomal pH-responsive polymer, methoxyl-poly(ethylene glycol)-*b*-poly(2-(diisopropylamino)ethyl methacrylate) (denoted Meo-PEG-*b*-PDPA) were required. The design principle and chemical structure are provided as below.

Design of Meo-PEG-*b*-PDPA

The endosomal pH-responsive polymer, Meo-PEG-*b*-PDPA, is an amphiphilic block copolymer with a hydrophilic PEG segment and a hydrophobic PDPA segment. In aqueous solution, this polymer could spontaneously self-assemble into stable nanoparticles (NPs) with PEG chain as hydrophilic outer shell and PDPA chain as hydrophobic inner core. The PEG outer shell could improve the stability of NPs and prolong their half-life by minimizing their non-specific interactions with serum components (Knop et al., 2010). The PDPA inner core could be used to encapsulate hydrophobic therapeutics (e.g., small molecule inhibitors, chemotherapeutic drugs) or the complexes of cationic lipid and nucleic acid. More importantly, because the Meo-PEG-*b*-PDPA polymer shows a sharp pH-responsive characteristic with a pK_a (~6.35) close to the endosomal pH (6.0–6.5) (Wang et al., 2014), the PDPA segment could be protonated in the endosomal microenvironment to induce the endosomal swelling via the "proton sponge" effect (Won et al., 2009), thereby improving the endosomal escape ability of the encapsulated therapeutics. The chemical structure of Meo-PEG-*b*-PDPA polymer is shown in Figure 1 and the detailed synthesis of this polymer has been reported in detail in our previous studies (Xu et al., 2017; Xu et al., 2016).

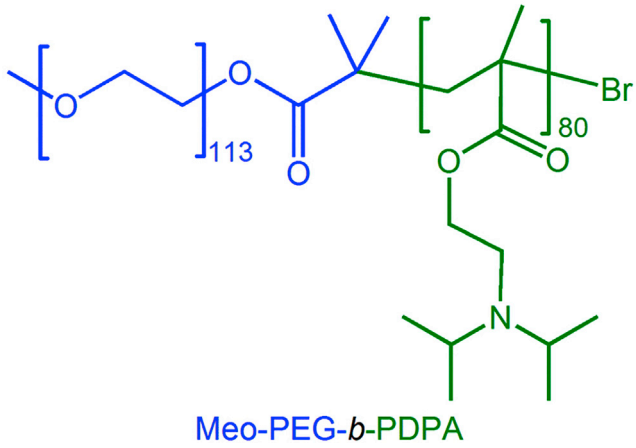

Figure 1. Chemical structure of the amphiphilic block polymer Meo-PEG-*b*-PDPA

Design of TACP

The mitochondria-targeting peptide TACP is an amphiphilic peptide, which is composed of a hydrophobic alkyl chain, an oligoarginine-containing peptide sequence, and a TPP side group. The positively charged oligoarginine sequence could not only complex nucleic acid such as circRNA-expressing vector through electrostatic interaction, but also shows the ability to penetrate membrane including endosomal and mitochondrial membrane (Fuchs and Raines, 2006). The incorporation of TPP group into the TACP structure is mainly due to its unique mitochondria-targeting ability (Smith et al., 2003). When dissolving TACP in dimethyl sulfoxide (DMSO) and then mixing with the aqueous solution of circRNA-expressing vector, the complexes of TACP and vector could be formed with the hydrophobic alkyl chains positioned on the surface of the TACP/vector complexes, which could then be encapsulated into the hydrophobic cores of the NPs made with the Meo-PEG-*b*-PDPA polymer (Xu et al., 2018). In this protocol, we specifically designed the mitochondria-targeting peptide TACP according to our previous studies (Chen et al., 2013; Xu et al., 2012) and this peptide was then synthesized by GL Biochem Ltd. The chemical structure of TACP is shown in Figure 2.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human surgically resected liver samples	The Third Affiliated Hospital, Sun Yat-sen University	N/A
Healthy C57BL/6J mouse liver samples	This paper	N/A
Healthy C57BL/6J mouse spleen samples	This paper	N/A
Healthy C57BL/6J mouse lung samples	This paper	N/A
Healthy C57BL/6J mouse kidney samples	This paper	N/A
Healthy C57BL/6J mouse heart samples	This paper	N/A
Chemicals, peptides, and recombinant proteins		
N,*N*-Dimethylformamide (DMF)	Sigma	Cat# 227056
Dimethyl sulfoxide (DMSO)	Sigma	Cat# 472301
Dulbecco's modified Eagle's medium (DMEM)	Gibco	Cat# 11965092

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fetal bovine serum (FBS)	Gibco	Cat# 16000-044
Hanks' balanced salt solution (HBSS)	Thermo Fisher	Cat# 14025092
Trypsin-EDTA (0.25%), phenol red	Thermo Fisher	Cat# 25200056
Phosphate buffered saline (PBS) (pH 7.4)	Hyclone	Cat# SH30256.01
Hypure water	Hyclone	Cat# SH30538.02
MACS tissue storage solution	Miltenyi Biotech	Cat# 130-100-008
Collagenase, type I	Worthington Biochem	Cat# LS004196
Collagenase, type III	Worthington Biochem	Cat# LS004182
Pronase	Roche	Cat# 10165921001
Hyaluronidase	Sigma	Cat# H1136
OptiPrep	Sigma	Cat# D1556
Hoechst 33342	Invitrogen	Cat# H3570
Lipofectamine 3000 Transfection Reagent	Thermo Fisher	Cat# L3000015
MitoTracker Red FM	Invitrogen	Cat# M7512
Critical commercial assays		
Label IT Tracker Intracellular Nucleic Acid Localization Kits	Mirus	Cat# MIR 7021
Experimental models: cell lines		
Human liver fibroblasts	This paper	N/A
Mouse liver fibroblasts	This paper	N/A
Experimental models: organisms/strains		
Healthy male C57BL/6J mouse (5 weeks old)	Sun Yat-sen University	N/A
Recombinant DNA		
CircRNA-expressing vector	Geneseed, Guangzhou	N/A
pXCX-CMV-EGFP plasmid	Addgene	Cat# 38053
Other		
Cell culture dish (15 mm)	Nest	Cat# 801002
Amicon Ultra 15 mL filters	Millipore	Cat# UFC9100
75 cm^2 flask, canted neck	Corning	Cat# 430720U
15 mL centrifuge tube	Corning	Cat# 430052
50 mL centrifuge tube	Corning	Cat# 430090
70 μm cell strainer	BD Falcon	Cat# 352350
Copper grid	Sigma	Cat# CF150CU50
Insulin syringe with detachable needle	BD	Cat# 329651
Benchtop centrifuge	Eppendorf	N/A
pH meter	N/A	N/A
Biosafety cabinet	N/A	N/A
Surgical scissors and tweezers	Roboz	N/A
Water bath	N/A	N/A
10 mL glass vial	N/A	N/A
20 mL glass vial	N/A	N/A
15 mm × 5 mm stirring bar	N/A	N/A
Ultrasonic bath sonicator	N/A	N/A
Magnetic stirrer	N/A	N/A
Confocal laser-scanning microscope (CLSM)	Leica	TCS SP8 STED 3X
Transmission electron microscope (TEM)	FEI	Tecnai G2 Spirit BioTWIN
Smartchemi 910 Plus	Sagecreation	306006
Multimode microplate reader	TECAN SPARK	TECAN SPARK 10M
Cytomics FC 500 instrument	Beckman	N/A
Zetasizer Nano S	Malvern	N/A

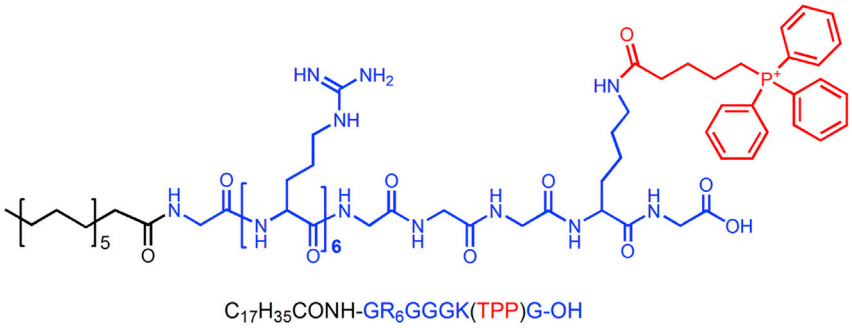

Figure 2. Chemical structure of the mitochondria-targeting peptide TACP

MATERIALS AND EQUIPMENT

Prepare all the solution and cell culture medium according to the following table and keep storage at 4°C until use.

Meo-PEG-*b*-PDPA polymer and TACP solution			
Reagent	Stock concentration	Final concentration	Amount
Meo-PEG-*b*-PDPA polymer	N/A	10 mg/mL in DMF	5 mL
TACP peptide	N/A	5 mg/mL in DMSO	2 mL

Note: The polymer and peptide would precipitate from the solution when storage at 4°C. Before use, place the solution into 37°C water bath for 2–3 min and manually shake the glass vial to ensure the dissolution of the polymer and peptide. The polymer and peptide solution could be stored at 4°C for up to 4 weeks.

Digestion solution			
Reagent	Stock concentration	Final concentration	Amount
DMEM high glucose	1×	1×	94 mL
Penicillin/streptomycin	100×	1% (v/v)	1 mL
Collagenase I	N/A	2 mg/mL	200 mg
Pronase	N/A	0.4 mg/mL	40 mg
Hyaluronidase	N/A	2 mg/mL	200 mg
FBS	N/A	5% (v/v)	5 mL
Total	N/A	N/A	100 mL

⚠ CRITICAL: Prepare the digestion solution inside a standard laboratory biosafety cabinet at the day of isolation of liver fibroblasts and avoid repeated freeze-thaws of the digestion solution. Before use, pre-warm the solution in 37°C water bath for ~8–10 min.

Cell culture medium			
Reagent	Stock concentration	Final concentration	Amount
DMEM high glucose	1×	1×	445 mL
Penicillin/streptomycin	100×	1% (v/v)	5 mL
FBS	N/A	10% (v/v)	50 mL
Total	N/A	N/A	500 mL

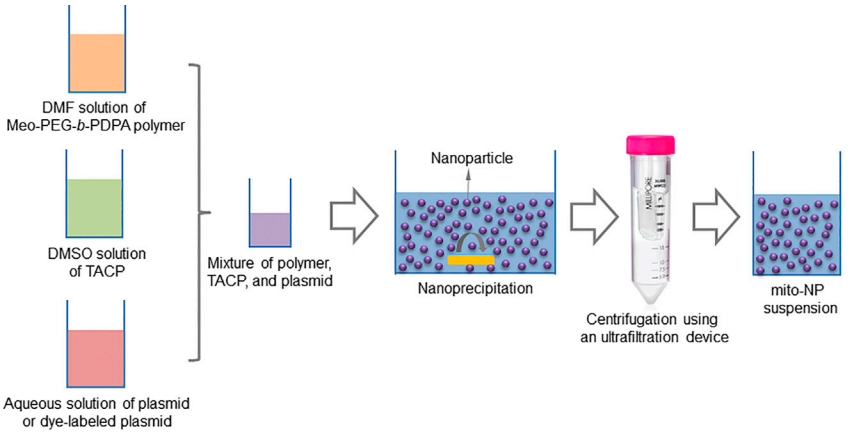

Figure 3. Schematic illustration of the preparation of the mitochondria-specific mito-NP

Note: Prepare the cell culture medium inside a standard laboratory biosafety cabinet, adjust the medium pH to 7.4, and store at 4°C for up to 2 weeks. Before use, pre-warm the medium in 37°C water bath for ~8–10 min.

Alternatives: This protocol uses Malvern Zetasizer Nano S and FEI TEM to examine the size and morphology of the mito-NP, respectively. The Zetasizer and TEM can be replaced by other instruments with similar functions. Regarding the Leica CLSM, TECAN Multimode microplate reader, Sagecreation Smartchemi 910 Plus, and Beckman Cytomics FC 500 instrument that are used to evaluate the *in vitro* and *in vivo* mitochondria-targeting ability of the mito-NP in this protocol, they can be also replaced by other instruments with similar functions.

STEP-BY-STEP METHOD DETAILS

Preparation and characterization of the mito-NP

Timing: ~2 days

The classic nanoprecipitation method is employed to prepare the mito-NP (Zhang et al., 2008). The detailed preparation is as follows (Figure 3).

1. Take 50 mg of Meo-PEG-*b*-PDPA polymer to 10 mL of glass vial and add 5 mL of DMF using a pipette (Metric, 100–1,000 μL).
2. Place the glass vial to a sonicator bath for 1–2 min sonication (50 W) to prepare homogenous polymer solution at a concentration of 10 mg/mL.
3. Similarly, take 10 mg of mitochondria-targeting peptide TACP and dissolve it in 2 mL of DMSO prepare homogenous peptide solution at a concentration of 5 mg/mL.
4. Take 200 μL of Meo-PEG-*b*-PDPA solution (step 2) and 50 μL of TACP solution (step 3) using a pipette (Metric, 20–200 μL) to 1 mL of glass vial and add 10 μg of circRNA-expressing vector (1 mg/mL aqueous solution).
5. Take 5 mL of commercially available HyPure water (molecular biology grade) to 20 mL of sterilized glass vial containing a stirring bar (15 mm in length and 5 mm in diameter) inside a standard laboratory biosafety cabinet.

Note: The pH value of the Hypure water should be a neutral pH that is higher than the pK_a (~6.35) of the Meo-PEG-*b*-PDPA polymer. If not, add small volume of NaOH aqueous solution (1 M) to adjust the solution pH to a neutral value using a pH meter.

6. Place the glass vial on a magnetic stirrer and use a pipette (Metric, 100–1,000 μL) to add the mixture (step 4) dropwise into the Hypure water under vigorously stirring at 400 × *g*.
7. Seal the glass vial and keep stirring on the magnetic stirrer at ~20°C–25°C for ~2–3 min followed by transferring the mito-NP suspension to an ultrafiltration device (15 mL, MWCO 100K).
8. Centrifuge at ~800–1,800 × *g* for 10 min to remove the organic solvent and free compounds.

Note: Although centrifugation at a low speed (<800 × *g*) could also remove the organic solvent and free compounds from the mito-NP suspension, longer experimental time is required, in which part of encapsulated circRNA-expressing vector may release from the mito-NP. Higher centrifugation speed (>1,800 × *g*) is not suggested because it usually induces the aggregation of the mito-NP. Our experience suggests that ~800–1,800 × *g* is an appropriate speed for the purification of mito-NP.

9. Add 5 mL of Hypure water to resuspend the mito-NP inside a standard laboratory biosafety cabinet and then centrifuge at ~800–1,800 × *g* for 10 min.
10. Repeat step 9 and then disperse the mito-NP into 1 mL of Hypure water inside a standard laboratory biosafety cabinet for the next experiments.
11. Take 10 μL of the mito-NP suspension and add to a copper grid. After air-drying at ~20°C–25°C for around 12 h, observe the mito-NP morphology on a Tecnai G2 Spirit BioTWIN TEM.
12. Take 200 μL of the mito-NP suspension and then disperse in 1 mL of Hypure water. Subsequently, transfer the mito-NP suspension to a cuvette and then measure the particle size using Malvern Zetasizer.
13. The NPs without mitochondria-targeting ability are prepared as control NPs. The Meo-PEG-*b*-PDPA polymer and an amphiphilic peptide without mitochondria-targeting TPP group ($C_{17}H_{35}$CONH-GR6GGGKG-OH, synthesized by GL Biochem Ltd) are used to prepare the control NPs. The preparation protocol is similar as the mito-NP by changing the peptide TACP with the above amphiphilic peptide without mitochondria-targeting ability.

Note: If the prepared mito-NP and control NPs are not immediately used for the following *in vitro* or *in vivo* experiments, frozen storage at −80°C is needed, and the encapsulated circRNA-expressing vector could maintain its biological function within 4–6 months.

⚠ CRITICAL: Since the Meo-PEG-*b*-PDPA is sharp pH-responsive polymer with a pK_a of ~6.35, the formed mito-NP could disassemble once the solution pH is lower than the pK_a. Therefore, the solution pH of Hypure water needs to be examined before the mito-NP preparation and the pH value must be higher than the pK_a. In addition, to determine the encapsulation efficiency of the circRNA-expressing vector and evaluate the mitochondria-targeting ability of the mito-NP, labeling the circRNA-expressing vector with fluorescent dye Cy5 using *Label* IT Tracker Intracellular Nucleic Acid Localization Kit and then encapsulating the resulting vector into the mito-NP. The vector encapsulation efficiency is determined by comparing the fluorescence intensity of Cy5 (E_x = 647 nm, E_m = 662 nm) of the mito-NP to the standard curve.

Isolation of primary liver fibroblasts

Timing: ~10 h

Liver is one of the important metabolic organs and the mitochondria of liver fibroblasts play vital roles in the development and progression of liver immunometabolic diseases such as non-alcoholic fatty liver disease (NAFLD) (Friedman et al., 2018). Therefore, the primary liver fibroblasts are isolated and used as an example to evaluate the mitochondria-specific gene delivery of the mito-NP.

The detailed isolation of primary liver fibroblasts is as follows (Figure 4).

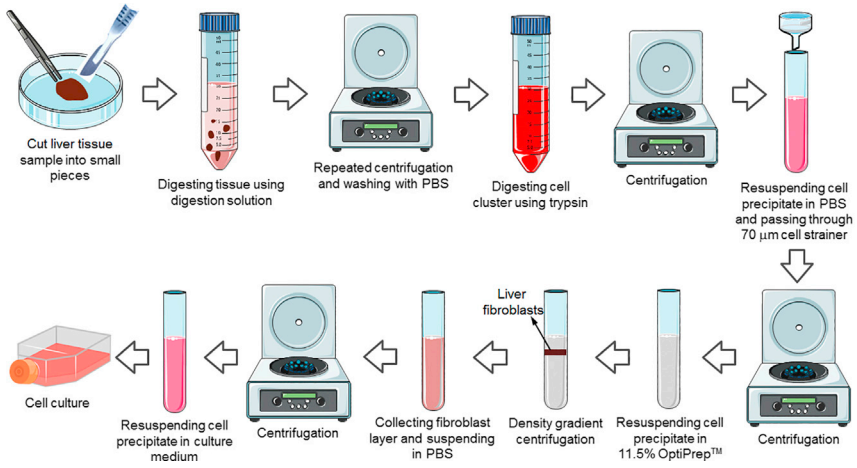

Figure 4. Schematic illustration of the isolation of primary liver fibroblasts

14. Collect surgically removed liver tissue during hepatic hemangioma resection or liver transplantation (Friedman et al., 1992; Zhai et al., 2019).
15. Place the liver tissue (~20 g) into a 50 mL of sterilized glass vial on ice and add 20 mL of DMEM containing 10% FBS and 1% penicillin-streptomycin.
16. Remove DMEM and then rinse the tissue with 10 mL of cold PBS solution containing 2% FBS and 1% penicillin-streptomycin thrice inside a standard laboratory biosafety cabinet.
17. Inside a standard laboratory biosafety cabinet, place the liver tissue on a 15 mm glass bottom cell culture dish and remove fat, necrotic tissue, and other miscellaneous tissue with sterilized forceps.
18. Inside a standard laboratory biosafety cabinet, cut the tissue into tiny pieces (~1–2 mm in diameter) with autoclaved surgical instruments on ice.
19. Inside a standard laboratory biosafety cabinet, transfer the tiny tissue pieces (~10 g) into a 50 mL of centrifuge tube and then add 10 mL of digestion solution (DMEM containing 5% FBS, 1% penicillin-streptomycin, 2 mg/mL collagenase I, 0.4 mg/mL pronase, and 2 mg/mL hyaluronidase).
20. Seal the centrifuge tube and then shake at 37°C with a speed of 150 rpm until the tissue pieces are digested.
21. Open the centrifuge tube inside a standard laboratory biosafety cabinet and add 40 mL of cold PBS solution followed by centrifugation at 450 × *g* for 10 min.
22. Remove the supernatant inside a standard laboratory biosafety cabinet and wash the pellet with 10 mL of cold PBS solution followed by centrifugation at 450 × *g* for 10 min.
23. After repeating step 22 thrice, add 10 mL of trypsin (0.05%) inside a standard laboratory biosafety cabinet and incubate the tube at 37°C for 20 min.
24. Resuspend the cell cluster using a pipette (Metric, 1–10 mL) inside a standard laboratory biosafety cabinet, add 1 mL of FBS to stop the digestion, and centrifuge at 450 × *g* for 10 min.
25. Remove the supernatant, resuspend the precipitate with 10 mL of PBS solution, and filter the cell suspension through a 70 μm cell strainer inside a standard laboratory biosafety cabinet.
26. Collect the filtered cell suspension and centrifuge at 20 × *g* for 5 min to deposit hepatocytes.
27. Transfer the supernatant to a new tube inside a standard laboratory biosafety cabinet and centrifuge for at 450 × *g* for 10 min.
28. Resuspend the precipitate with 11.5% OptiPrep inside a standard laboratory biosafety cabinet followed by density gradient centrifugation (1,400 × *g*, 20 min).
29. Gently collect the second layer (containing primary liver fibroblasts) from the top and transfer to a new tube inside a standard laboratory biosafety cabinet. After adding 40 mL of PBS solution, centrifuge the tube at 450 × *g* for 10 min.

30. Resuspend the cell precipitate with 10 mL of pre-warmed cell culture medium (DMEM containing 10% FBS and 1% penicillin-streptomycin) and culture the obtained primary fibroblasts in a 75 cm^2 cell culture flask at 37°C in a humidified atmosphere containing 5% CO_2.
31. When the primary liver fibroblasts attach to the cell culture flask and the cell proliferation reaches 70%–80% confluence, use the cells for the following experiments.

⚠ CRITICAL: In order to obtain more primary liver fibroblasts, the collected liver tissues should be dissected as tiny as possible before digestion. Occasionally, little amount of red blood cells (RBCs) may mix with the extracted primary liver fibroblasts, which can be removed via repeated change of the culture medium. If residual RBCs still exist, RBC lysis buffer is recommended. In addition, aseptic operation is recommended to avoid contamination and penicillin-streptomycin could be used during cell culture.

In vitro evaluation of mitochondria-targeting ability of mito-NP

Timing: ~5 days

To evaluate the mitochondria-targeting ability, fluorescent dye (e.g., Cy5 and Cy7) labeled vector or fluorescence-expressing vector (e.g., pXCX-CMV-EGFP) (Glover et al., 2002) needs to be encapsulated into the mito-NP, which could facilitate to real-time track the vector distribution in the liver fibroblasts. Herein, GFP-expressing vector is used an example and encapsulated into the mito-NP, which can be used to evaluate the *in vitro* mitochondria-targeting ability and examine the transfection efficacy of the mito-NP.

32. Remove the medium from the cell culture flask and wash the primary liver fibroblasts with 10 mL of PBS solution thrice inside a standard laboratory biosafety cabinet.
33. Add 3 mL of 0.25% trypsin to digest the cells for 3–5 min and then stop digestion using 10 mL of DMEM containing 10% FBS.
34. Transfer the cell suspension into a 15 mL of centrifuge tube inside a standard laboratory biosafety cabinet and centrifuge the tube at 450 × *g* for 10 min.
35. Remove the medium and add 1 mL of cell culture medium to resuspend the cells using a pipette (Metric, 100–1,000 μL) inside a standard laboratory biosafety cabinet. Count the cell number using a cell counter and finally adjust the cell concentration to a value of 1×10^6 cell/mL.
36. Add 50 μL of the cell suspension into 15 mm of cell culture dish (5×10^4 cell/well) inside a standard laboratory biosafety cabinet and culture the cells in 2 mL of cell culture medium.
37. After 24 h incubation, replace the medium with 2 mL of fresh cell culture medium and add the mito-NP loading GFP-expressing vector at a plasmid concentration of 200 ng/mL. As a control, add the mixture of Lipofectamine 3000 and GFP-expressing vector to the liver fibroblasts at the same plasmid concentration.

Note: The pH of the medium used in this step should be a neutral pH. If the pH value is lower than the pK_a (~6.35) of the Meo-PEG-*b*-PDPA, add small volume of NaOH aqueous solution (1 M) to adjust the solution pH using a pH meter to a neutral value.

38. After 24 h incubation, remove the medium, wash the cells with 2 mL of PBS solution thrice, and add 2 mL of fresh medium for another 48 h incubation.
39. Remove culture medium and add 200 nM of MitoTracker Red diluted in 2 mL of HBSS buffer solution.
40. After 30 min incubation, remove the medium, wash the cells with 2 mL of PBS solution thrice, and stain the nuclei with 1.5 mL of Hoechst 33342 (diluted with PBS at 1:1000 ratio) for 10 min.
41. Remove the medium, add 2 mL of fresh PBS solution, and use confocal laser-scanning microscope to observe the intracellular GFP expression and colocalization of GFP with mitochondria.

⚠ CRITICAL: Since the encapsulated vector could gradually be released from the mito-NP, stronger mitochondria-targeting ability and better gene transfection could be achieved when using freshly prepared mito-NP. If large amount of NPs are needed, frozen storage at −80°C is recommended. In addition, in order to observe well-structured mitochondria, the live fibroblasts should be viewed under confocal laser-scanning microscope once the nucleus staining is finished.

In vivo evaluation of mitochondria-targeting ability

Timing: ~3 days

To evaluate the *in vivo* mitochondria-targeting ability, fluorescent dye such as Cy5 is needed to label the circRNA-expressing vector (Zhao et al., 2020), which is then encapsulated into the mito-NP and injected into healthy male C57BL/6J mice (5 weeks old) intravenously.

42. Label circRNA-expressing vector with Cy5 using Label IT Tracker Intracellular Nucleic Acid Localization Kit according to the manufacturer's instruction.
43. Repeat steps 1–9 to prepare the mito-NP loading Cy5-labeled circRNA-expressing vector.
44. After washing the NPs with 5 mL of PBS solution thrice, collect the NPs and resuspend the NPs in PBS solution at a vector concentration of 50 μg/mL.

Note: If the Cy5-labeled NPs are not immediately used for the following *in vivo* experiments, frozen storage at −80°C is needed.

45. Randomly divide six healthy male C57BL/6J mice into 2 groups (n = 3) and intravenously inject 200 μL of mito-NP (50 μg/mL vector-equivalent concentration in PBS solution) or naked Cy5-labeled vector (50 μg/mL in PBS solution).
46. Twenty-four hours post injection, sacrifice the mice, harvest the major organs (heart, liver, spleen, lung, and kidney) and image the major organs by Maestro 2 In-Vivo Imaging System to observe the accumulation of Cy5-labeled vector in each organ.
47. Cut the mouse liver tissue into tiny pieces and repeat steps 17–29 to extract the primary liver fibroblasts.
48. Use flow cytometry to analyze and compare the fluorescence intensity of Cy5 in the liver fibroblasts of mice treated with mito-NP or naked Cy5-labeled vector.
49. Extract the mitochondria from the liver fibroblasts using the commercial kit according to the manufacturer's instruction and use flow cytometry to analyze and compare the fluorescence intensity of Cy5 in the mitochondria of the liver fibroblasts of mice treated with mito-NP or naked Cy5-labeled vector.

⚠ CRITICAL: To eliminate the interference of blood autofluorescence, the harvested organs need to be washed repeatedly with PBS solution. Near-infrared (NIR)-emitting fluorescent dyes (e.g., Cy5, Cy5.5, and Cy7) are recommended to label the cirRNA-expressing vector.

EXPECTED OUTCOMES

At the end of the process, the obtained mito-NP should show a spherical morphology (Figure 5A) with an average size of ~100 nm (Figure 5B) and neutral surface charge (zeta potential, ± 5 mV). In addition, the NPs should be well-dispersed in aqueous solution and show good stability (Figure 5C).

Regarding the *in vitro* mitochondria-specific gene delivery, after incubation with the liver fibroblasts, the mito-NP should show stronger mitochondria-targeting ability and better gene transfection compared to the naked vector (Figures 5D and 5E).

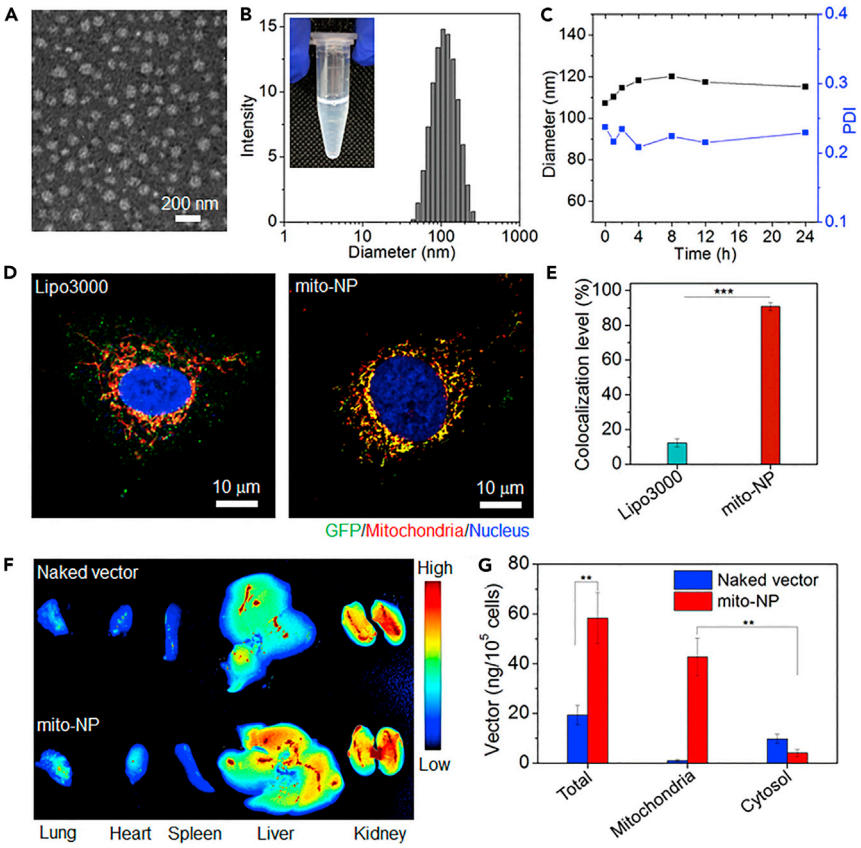

Figure 5. Characterizations of the mito-NP

(A) TEM image of the mito-NP.

(B) Optical image and size distribution of the mito-NP dispersed in PBS solution.

(C) Size change of the mito-NP dispersed in PBS solution for 24 h.

(D) Representative fluorescent images of the liver fibroblasts transfected with the complexes of Lipo3000 and GFP-expressing vector or mito-NP loading GFP-expressing vector. The mitochondria and nuclei are stained with MitoTracker Red and Hoechst 33342, respectively.

(E) Statistic analysis of the colocalization of GFP with the mitochondria from (D).

(F) Biodistribution of Cy5-labeled cirRNA-expressing vector in the main organs of the mice received the intravenous injection of naked Cy5-labeled vector or mito-NP loading Cy5-labeled vector.

(G) Quantitative analysis of the amount of Cy5-labeled vector in the mitochondria of the liver fibroblasts extracted from the mouse liver in (F).

In regard to the *in vivo* mitochondria-specific gene delivery, after intravenous injection into the mice, the mito-NP should show higher accumulation in the liver tissues due to their large size (Figure 5F). More importantly, due to their good mitochondria-targeting ability, more mito-NP should accumulate in the mitochondria of the liver fibroblasts compared to the naked vector (Figure 5G).

LIMITATIONS

In this protocol, there is limitation at the storage of the mito-NP. As the vector, i.e., nucleic acid, is physically encapsulated into the NPs via electrostatic interaction, the vector could be gradually released as time goes by. Frozen storage of mito-NPs at −80°C could significantly inhibit the vector release, however, repeated freeze-thaw cycles would induce destroy of the nanostructure, leading to unsatisfactory experimental results. Our experiences suggest that the optimal experimental results could be achieved when using the freshly prepared mito-NP. If large scale of NPs is needed, frozen storage is necessary after NP preparation. Moreover, before the frozen storage, the NPs are recommended to be divided into different tubes according to the experiment arrangement;

and only one tube of NPs should be used for the each experiment to avoid the repeated freeze-thaw cycles.

TROUBLESHOOTING

Problem 1

The Meo-PEG-*b*-PDPA polymer and mitochondria-targeting peptide TACP are respectively dissolved in DMF and DMSO, and then used for the preparation of the mito-NP. Therefore, the residual organic solvents probably mix in the mito-NP aqueous solution, which may induce potential cytotoxicity.

Potential solution

Since the organic solvents used in this protocol are water miscible, repeated washing the obtained NPs with Hypure water or PBS solution could effectively remove the residual organic solvents.

Problem 2

The vector with relatively large size may restrict its encapsulation into the mito-NP, which ultimately induces low gene transfection efficacy.

Potential solution

Since the encapsulation of the vector is based on its electrostatic interaction with the mitochondria-targeting peptide TACP, increasing the nitrogen/phosphate (N/P) ratio by using more TACP could improve the encapsulation efficiency of the vector.

Problem 3

As the vector is physically encapsulated into the mito-NP via electrostatic interaction, the vector could be gradually released from the NPs as time goes by.

Potential solution

Frozen storage could significantly inhibit the vector release from the mito-NP. If large amount of NPs are needed, NPs could be divided into different tubes before the frozen storage. Then, only one tube of NPs should be used for the each experiment, which can avoid the destroy of the nanostructure induced by repeated freeze-thaw.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Xiaoding Xu (xuxiaod5@mail.sysu.edu.cn) and Sicheng Su (sushch@mail.sysu.edu.cn).

Materials availability

All unique/stable reagent generated in this study will be made available on request but we may require a payment and/or a completed Materials Transfer Agreement if there is potential for commercial application.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

S.S., J.L, S.C., and X.X. conceived and wrote the protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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