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Centrifugation Free Magnetic Isolation of Functional Mitochondria Using Paramagnetic Iron Oxide Nanoparticles

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Abstract

Subcellular fractionation techniques are of tremendous importance in the field cellular biology, drug development, and many others. With the emergence of organelle targeted nanoparticle (NP) platforms, it is getting increasingly important to isolate target organelles in order to determine localization and activity of these agents. Mitochondria targeted NPs attracted attention of researchers around the globe since dysfunctions of this organelle can result in a wide range of diseases. Conventional mitochondria isolation methods involve high-speed centrifugation. The inherent problem associated with high speed centrifugation-based isolation of NP-loaded mitochondria using these conventional methods can arise from settling down of NPs along with the organelle irrespective of whether these NPs are truly associated with mitochondria or not. We report development of a mitochondria-targeted paramagnetic iron oxide nanoparticle, Mito-magneto, that enables isolation of mitochondria under the influence of a magnetic field. The mitochondria isolated using this centrifugation-free method are not only intact, pure and respiration active but also eliminates artifacts that are typically associated with isolation of NP-loaded mitochondria using centrifugation.

Keywords

Mitochondria isolation; Centrifugation-free method; Iron oxide nanoparticles; Magnetic resonance imaging

Introduction

Mitochondria are the key players for maintaining cellular life and death processes by regulating vital cellular functions. Thus, mitochondrial dysfunctions can result in a number of human diseases (1, 2). Mitochondrial dysfunctions can be linked to cancer, cardiovascular diseases, neurodegenerative diseases, and many more that are yet to be understood (3-6). There is increasing interest and urgency towards technology development to access mitochondrial targets for treating mitochondrial dysfunction related diseases (7).

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Nanoparticle (NP) which holds promise as carriers for therapeutics and contrast agents for medical use can be an excellent addition to otherwise difficult to access mitochondrial targets (8). As the field of NP-enabled mitochondria targeting is booming, there is an increasing necessity to provide tools and techniques to accurately determine/quantify the localization and mitochondrial association efficacy of these NP platforms.

We and several other research groups have developed several NP-based cargos to achieve mitochondria-targeted delivery of payloads (8-18). Development and optimization of such targeted NP platforms would benefit from quantitative determination of mitochondrial localization and assessment of functional nature of the organelle and its components. Simple mitochondrial colocalization experiments to assess NP's distribution may not provide a complete picture and are not always possible to carry out. Isolation of pure and functional mitochondria can prove to be a very handy tool in such a scenario. Traditional methods for mitochondria isolation involve gradient or differential centrifugation based protocols (19) which may not be suitable for NP containing mitochondria, as the Brownian motions of NPs under centrifugal force (20) can cause precipitation of NPs in the pellet along with the mitochondria. These conventional high centrifugal force based methods for NP-associated mitochondria may generate erroneous results that are difficult to reproduce.

Centrifugation-free microbead based methods are available for cell sorting (21) and isolation of cellular organelles such as nuclei (22), endosome (23), lysosome (24), Golgi vessels (25), and plasma membranes (26). Along this line, magnetic beads with anti-mitochondrial membrane translocase of outer membrane (TOM)22 antibody on the surface were used for isolation of mitochondria from cells (27) as well as from a wide range of tissue samples (28). This method, though centrifugation free, requires immune precipitation. We, in our attempt to develop a centrifugation and immunoprecipitation free method, recently reported a mitochondria-targeted magnetic NP, Mito-magneto, for efficient isolation of mitochondria (29) (Figure 1). Mito-magneto, a Fe_3O_4 magnetite based magnetic NP with triphenylphosphonium (TPP) cation as the surface functionality, was designed and constructed to achieve a centrifugation and immune precipitation free mitochondria isolation protocol. Due to the presence of delocalized TPP cations on the surface, Mito-magneto utilizes mitochondrial membrane potential (ψ_m) which exists across the double membrane to associate itself with healthy mitochondrion and allows isolation of Mito-magneto associated healthy mitochondria by the use of a magnet (Figure 1). Further, advantage of iron oxide NPs (IONPs) is the contrast enhancement for magnetic resonance imaging (MRI) and hence an opportunity to image the isolated mitochondrial fraction. Mito-magneto based technology not only serves as an efficient tool for mitochondria isolation, but also provides a platform for new developments in MRI of mitochondria in various systems. Here, we describe a detailed protocol for Mito-magneto assisted centrifugation free mitochondria isolation technique.

Basic Protocol

Synthesis of Mito-Magneto

Oleic acid functionalized iron oxide nanoparticles (NT-IONP) were synthesized following a literature procedure (30). This procedure yields IONP that are soluble in chloroform and

hexane. The coating of oleic acids was then stripped off from the NP surface by repeated precipitation using ethanol. Mito-magneto was synthesized by resuspending NT-IONPs in DMF solution of TPP-(CH₂)₅-COOH (Figure 2). Mito-magneto can be characterized by ³¹P NMR for the presence of –TPP surface functionality, dynamic light scattering (DLS) for diameter, surface charge, and polydispersity index; transmission electron microscopy (TEM) for diameter and morphology (Figure 3). Toxicity of Mito-magneto was assessed by using rat H9C2 cardiomyocyte by following a conventional colorimetric (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or MTT assay, preliminary immunogenicity of Mito-magneto can be determined in RAW macrophages by analyzing secretion of interleukin (IL)-6 and tumor necrosis factor alpha (TNF- α) using enzyme-linked immunosorbent assay (ELISA), and effects of Mito-magneto on the components of mitochondrial oxidative phosphorylation (OXPHOS) can be determined by MitoStress assay on a Seahorse analyzer.

Materials

Fe(acac)₃ or tris(acetylacetonato)iron(III)
1,2-hexadecanediol
Oleic acid
Oleyl amine
Benzyl ether
Sand bath (for heating reaction mixture to 200-300 °C)
Nitrogen gas cylinder
Triphenylphosphine
6-bromohexanoic acid
Acetonitrile
Diethyl ether
3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT)
Dimethylsulfoxide (DMSO)
Hexanes
Ethanol
Dimethyl formamide (DMF)
Coating buffer (*see recipes*)
Wash buffer (*see recipes*)
Assay diluent (*see recipes*)
Lipopolysaccharide (LPS)
Rotavap
Beckman Coulter Microfuge 22R centrifuge

Branson 2510 sonicator bath

1.5 mL Thick-walled centrifuge tubes

Additional reagents and equipment for Dynamic Light Scattering (DLS), Transmission Electron Microscopy (TEM), Nuclear Magnetic Resonance (NMR) and Inductively Coupled Plasma-Mass Spectrometry (ICP-OES) characterization of Mito-magneto: Malvern Zetasizer Nano ZS DLS system, ZEN1002 dip cell cuvettes, Philips/FEI Tecnai 20 microscope, CDCl_3 , DMSO-d_6 and 500 MHz Varian NMR spectrometer, Perkin Elmer Optima 8300 ICP-OES (ICP-OES) spectrometer.

Synthesize Oleic acid Capped Iron Oxide Nanoparticles (NT-IONP)

1. Dissolve 706 mg of $\text{Fe}(\text{acac})_3$ in 20 mL of benzyl ether and stir vigorously.
2. Add 1.89 mL of oleic acid to the reaction mixture.
3. Add 1.97 mL of oleyl amine to the reaction mixture.
4. Add 2.58 g of 1,2-hexadecanediol to the reaction mixture and heat the reaction mixture on a sand bath to 200 °C with constant stirring under N_2 atmosphere for 2 h.
5. After 2 h, raise the temperature of the sand bath to 300 °C and reflux the reaction mixture for 1 h.
6. Remove the sand bath and cool the reaction mixture to room temperature.
7. Add 40 mL of ethanol to the mixture.
8. Centrifuge the mixture at 6000 rpm at RT for 10 min to pellet down a black material.
9. Decant the supernatant gently and resuspend the black material in 10 mL of hexanes.
10. Add 50 μL each of oleic acid and oleyl amine to the solution and mix well.
11. Remove any undispersed residue from the solution by centrifugation at 6000 rpm at RT for 10 min.
12. Add 40 mL of ethanol to precipitate the NT-IONPs as black material.
13. Collect the NPs by centrifugation at 6000 rpm at RT for 10 min.
14. Redissolve the pellet in 5 mL of hexanes and store at 4 °C for further use.

Synthesize 6-carboxyhexyltriphenylphosphonium bromide [TPP-(CH_2)₅-COOH]Br

15. Dissolve 2 g of 6-bromohexanoic acid and 2.82 g of triphenylphosphine in 40 mL of acetonitrile.
16. Reflux the reaction mixture at 90 °C under nitrogen atmosphere for 24 h.
17. Cool the reaction mixture to room temperature.

- 18 Evaporate solvent from the mixture to dryness using a rotavap.
- 19 Triturate the resultant sticky solid with hexanes until it yields white powder.
- 20 Wash the white powder with diethyl ether (2×20 mL).
- 21 Dry the resultant white product under vacuum and store at 4 °C for further use.

Synthesize Mito-magneto

- 22 To 100 μL of NT-IONP solution (10 mg/mL) in hexanes, add 1 mL of ethanol and vortex the mixture for 1 min.
- 23 Collect the resultant precipitate by centrifugation at 6000 rpm for 10 min at 4 °C.
- 24 Remove the supernatant and resuspend the precipitate in 100 μL of hexanes.
- 25 Repeat the precipitation for two more times.
- 26 Add 1 mL DMF solution of TPP-(CH₂)₅-COOH (100 mg/mL) to NT-IONP precipitate.
- 27 Sonicate the resultant mixture for 1 h with occasional vortexing to ensure complete resuspension of the iron oxide nanoparticles into the solution.
- 28 Transfer the contents into a thick walled 1.5 mL centrifuge tube and centrifuge it for 1 h at 14000 rpm and 4 °C to get rid of any larger aggregates.
- 29 Collect the supernatant in a new thick walled 1.5 mL centrifuge tube and centrifuge the contents at 14000 rpm for 4 h at 4 °C.
- 30 Resuspend the Mito-magneto pellet thus obtained in 100 μL of DMF and store at 4 °C for future use.

Characterize Mito-magneto

- 31 Resuspend 20 μL of as synthesized Mito-magneto in 980 μL of DMF in a ZEN 1002 cuvette and measure size (diameter, nm), PDI, and surface charge (zeta potential, mV).
- 32 Dilute 20 μL of as synthesized Mito-magneto with 980 μL of water containing 1% DMF (or hexanes for NT-IONP) and drop 8 μL of the resultant solution on a copper grid and record TEM images after the grids are dried overnight.
- 33 Lyophilize 20 μL (30 mg/mL) of Mito-magneto or NT-IONPs and dissolve the resultant powder in 500 μL of CDCl₃ (for NT-IONP) or DMSO-d₆ (for Mito-magneto) to record ³¹P NMR spectra.
- 34 Quantify the concentration of iron in Mito-magneto solution by ICP-OES analysis of a 2500× diluted sample of Mito-magneto in water to a final volume of 1 mL.

Determine Cellular Toxicity of Mito-magneto by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay

- 35 Plate 4000 H9C2 cells per well in a 96 well plate and allow them to adhere to the plate bottom for 24 h.
- 36 Add Mito-magneto (in 1% DMSO containing media)/NT-IONP (in 5% DMSO containing media) to the cells.
- 37 Incubate cells for 12 h.
- 38 Replenish cells with fresh media and incubate for another 60 h.
- 39 Add 25 μL of MTT (4 mg/mL in $1\times$ PBS) to each of the wells.
- 40 Incubate cells for 5 h.
- 41 Remove media and lyse cells with 100 μL of DMSO.
- 42 Read absorbance of the plate at 550 nm.
- 43 Set control values at 0% cytotoxicity or 100% cell viability for the untreated cells.
- 44 Express cytotoxicity as the mean percentage decrease of cell viability relative to untreated control cells $\pm$ standard deviation.

Determine Immunogenicity of Mito-magneto in RAW 264.7 Cells by ELISA

- 45 Plate 10,000 RAW 264.7 cells/well in a 96 well plate and allow them to grow for 24 h.
- 46 Treat cells with 50 $\mu\text{g}/\text{mL}$ Mito-magneto in 1% DMSO containing DMEM or lipopolysaccharide (LPS, 100 ng/mL) as positive control.
- 47 Incubate cells for 24 h.
- 48 Pre-coat 96 well ELISA plates with 100 μL of capture antibody (1:250) in coating buffer.
- 49 Wash ELISA plates three times with 300 μL of wash buffer per well.
- 50 Block plates with 100 μL of assay diluent per well for 1 h.
- 51 Wash ELISA plates three times with 300 μL of wash buffer per well.
- 52 Add 100 μL of supernatant media from the cell plates or standard per well and incubate at room temperature for 2 h.
- 53 Wash ELISA plates five times with 300 μL of wash buffer per well.
- 54 Incubate with 100 μL of detection antibody (diluted in assay diluent as per manufacturer's instructions) per well for 2 h.
- 55 Wash ELISA plates five times with 300 μL of wash buffer per well.
- 56 Incubate with 100 μL of streptavidin-HRP conjugate (diluted in assay diluent as per manufacturer's instructions) per well for 30 min.

- 57 Wash ELISA plates five times with 300 μL of wash buffer per well.
- 58 Incubate plates with 100 μL of substrate solution for 30 min in dark.
- 59 Read absorbance at 450 nm.
- 60 Plot absorbance of standard solutions against their respective concentrations to obtain a straight line standard curve.
- 61 Determine concentration of cytokines for the respective sample wells from the standard curve.

Determine Effect of Mito-magneto on Mitochondrial Bioenergetics of Cells by Seahorse Analyzer

- 62 Plate 40,000 cells/well in a XF24 cell culture microplate and allow them to grow for 24 h.
- 63 Add various concentrations of Mito-magneto (20, 40, 60 and 100 $\mu\text{g/mL}$) to cells (in quadruplets).
- 64 Incubate the cells for 24 h.
- 65 Pre-warm XF stress test optimization medium supplemented with sodium pyruvate and D-glucose to 37 °C.
- 66 Remove all but 50 μL of the culture medium from each of the wells.
- 67 Wash cells thrice with 450 μL of the pre-warm XF stress test optimization medium.
- 68 Add 450 μL of the XF stress test optimization medium to each of the wells and incubate cells at 37 °C without CO_2 .
- 69 Load oligomycin (2.0 μM) in port A and FCCP (2.0 μM) in port B, and a mixture of antimycin-A (1.0 μM) and rotenone (1.0 μM) in port C of the XF24 cartridge.
- 70 Measure oxygen consumption rates (OCR) for 16 min to establish baseline and subsequently after the addition of each of the additives using XF24 seahorse analyzer. Determine the various mitochondrial respiration parameters by subtracting the average respiration rates before and after addition of the additives.

Isolation of Mitochondria from Cultured Cells

Mitochondria was isolated from cultured cells using reagent-based and Mito-magneto based magnetic isolation methods (Figure 1) and comparison was made among the mitochondria isolated using the different methods as a proof of concept. MRI characterization of the cellular fractions proves the association of Mito-magneto with the mitochondrial fractions (Figure 4). Size, zeta potential, and PDI of the mitochondria were measured using DLS (Figure 4).

Reagent Based Isolation of Mitochondria

Materials—150 cm² cell culture flasks containing confluent monolayer (1-5×10⁷ cells) of H9C2 cardiomyocytes, J3TBG glioma, or A2780 ovarian cancer cells

Respective cell culture media: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% L-glutamine, 1% sodium pyruvate, 1% penicillin/streptomycin, and 10% FBS for H9C2 and J3TBG cells; Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin and 2 mM L-glutamine for A2780 cells

Phosphate buffered saline (1× PBS) of pH 7.4

0.25% Trypsin-EDTA solution

Reagents A and C (ice cold) from a mitochondria isolation kit (Cat. # 89874) procured from Thermo Fisher Scientific

Misonix Probe sonicator (Newtown, CT, USA)

1× Mitochondria Assay solution (*see recipes*)

1.5 mL Thick-walled centrifuge tubes

15 mL centrifuge tubes

Pierce® Bicinchoninic acid (BCA) protein assay kit to quantify amount of protein in the mitochondrial fractions

Malvern Zetasizer Nano ZS DLS system

Agilent (7 Tesla, 200 mm) horizontal bore magnet based MRI instrument

Treatment of Cells

1. Add 15 mL of (20 µg/mL) Mito-magneto solution in 1% DMSO containing media per flask of cells and incubate for 12 h.
2. Wash cells three times with 15 mL of 1× PBS per flask.

Prepare Cells

- 3 Wash cells two times with 15 mL of 1× PBS per flask.
- 4 Add 3 mL of 0.25% trypsin-EDTA solution in each flask and incubate for 2-3 min.
- 5 Add 10 mL of the respective culture media.
- 6 Mix the media gently to ensure detachment of the cells from flask bottom.
- 7 Transfer the contents of the flask into 15 mL centrifuge tubes.
- 8 Centrifuge the tubes at 1800 rpm for 3 min at 4 °C to obtain the cell pellet.
- 9 Remove the supernatant media.

Isolate Mitochondria

- 10 Add 800 μL of ice cold reagent A to the cell pellet and mix well by pipetting up and down (five times).
- 11 Incubate the mixture on ice for 2 min.
- 12 Lyse the cells using 2 pulses of amplitude with 1 and 4 sec delay time using a probe sonicator.
- 13 Add 800 μL of ice cold reagent C to the mixture.
- 14 Remove nuclei, unlysed cells, and cell debris by centrifuging at $1300\times g$ for 5 min at 4°C .
- 15 Collect the supernatant in a separate centrifuge tube.
- 16 Centrifuge the supernatant at $12000\times g$ for 15 min at 4°C .
- 17 Remove the supernatant containing the cytosolic fraction.
- 18 Resuspend the pellet, comprised of the mitochondrial fraction, in 200 μL of $1\times$ mitochondria assay solution (MAS) and store at -20°C .

Characterize Isolated Mitochondria

- 19 Determine protein concentration in the mitochondrial fraction using BCA assay by adding 200 μL BCA working solution (1:50 of Reagents A:B) to 25 μL of diluted samples ($10\times$) or standard solution (various concentrations for the standard curve) and reading the absorbance at 562 nm.
- 20 Resuspend 20 μL of isolated mitochondria solution in 980 μL of water and measure size (diameter, nm), PDI, and surface charge (zeta potential, mV) using DLS instrument.
- 21 Embed various cellular fractions contain 20 $\mu\text{g}/\text{mL}$ of protein in 0.5% agarose gel blocks and embed these sample blocks in a bed of agarose gel and image using a 7T MRI instrument to confirm presence of iron in the mitochondrial fraction. Set the experimental parameters as follows: Slice width = 1 mm, Repetition Time (T_R) = 2500 ms and Echo Time (T_E) = 10.69 ms. Calculate transverse relaxivity (r_2) as per the equation $r_2[\text{Fe}] = 1/T_2 - 1/T_2^0$, where $1/T_2$ is the relaxation rate in presence of a certain concentration of IONPs, $1/T_2^0$ is the relaxation rate of pure water and $[\text{Fe}]$ is that particular concentration of Fe.

Magnetic Isolation of Mitochondria

This method utilizes Mito-magneto for the isolation of mitochondria from cultured cells. This is a magnetic isolation process and doesn't involve any high-speed centrifugation steps.

Additional Materials (also see reagent based isolation of mitochondria)

Mito-magneto solution (20 $\mu\text{g}/\text{mL}$) in 1% DMSO containing media
EasySepTM magnet

BD falcon conical tubes

Treatment of Cells

1. Add 15 mL of (20 $\mu\text{g}/\text{mL}$) Mito-magneto solution in 1% DMSO containing media per flask of cells and incubate for 12 h.
2. Wash cells three times with 15 mL of 1 $\times$ PBS per flask.

Magnetic Isolation of Mitochondria

- 3 Harvest cells and remove nuclei, unlysed cells and debris post cell lysis (*follow reagent based isolation of mitochondria steps 3-14*).
- 4 Collect the supernatant in a BD falcon conical tube and place it inside EasySep™ magnet.
- 5 Let the magnet stand on ice for 15 min.
- 6 Gently decant the supernatant without removing the magnet from around the tube.
- 7 Remove the tube from the magnet and rinse it with 200 μL of 1 $\times$ MAS to obtain the mitochondrial fraction and store at -20 °C.
- 8 Characterize the mitochondrial fraction by BCA assay, DLS, and MRI (*see reagent-based isolation protocol*).

Support Protocol 1

Citrate Synthase (CS) Activity Assay

CS catalyzes the condensation of acetyl coenzyme A and oxaloacetate (OAA) to produce citrate for the citric acid cycle. It is one of the key enzymes for mitochondrial functions. Thus, normal functioning of this enzyme can be assessed as a marker for healthy and functional mitochondria (Figure 5A).

Materials

Isolated mitochondria solution in 1 $\times$ MAS

Citrate synthase activity assay kit that contains citrate synthase assay buffer, citrate synthase substrate mix, and citrate synthase developer and glutathione (GSH) standard solution

96-well plates

Bio-Tek Synergy HT microplate reader

CS Activity Assay

1. Add 10 μL of mitochondria (containing 10 μg of protein) or GSH standard solutions per well of a 96-well plate in triplicates.

2. Add 50 μL of reaction mix containing CS assay buffer, CS substrate mix, and CS developer was added to the samples or standards.
3. Read Absorbance of the wells at 412 nm immediately after addition of the reaction mix in a kinetic mode from 0-40 min at 1 min intervals.
4. Plot GSH concentration vs. absorbance to obtain a straight line standard curve.
5. Determine the rate of change of thiol concentration for the samples over a linear range of absorbance change by comparing values with the standard curve.
6. Calculate citrate synthase activity from the formula:

$$\text{CS activity} = B / (T \times V) \times D \text{ (U/mL)}$$

Where: B is nmoles of thiols from standard curve

T is the duration of reaction (min)

V is the volume of sample added to each well

D is the dilution factor

Support protocol 2

Cytochrome C Oxidase (COX) Activity Assay

Cytochrome C oxidase (COX) is the complex IV of the electron transport chain (ETC). COX activity of the isolated mitochondria was determined to predict and assess the functional nature of mitochondrial ETC (Figure 5B).

Materials

Isolated mitochondria solution in $1 \times \text{MAS}$

COX activity kit that contains cytochrome C solution, positive control, and assay buffer

96-well plate

Bio-Tek Synergy HT microplate reader

COX activity assay

1. Add 10 μL of mitochondria (containing 10 μg of protein), positive control, or assay buffer (blank) per well of a 96-well plate in triplicates.
2. Add 120 μL of cytochrome C solution to each of the wells containing the analytes.
3. Measure absorbance of these solutions at 550 nm quickly after addition of the reagent in a kinetic mode for 45-90 min at intervals of 1 min.
4. Calculate COX activity from the rate of depreciation of cytochrome C concentration over a linear range of absorbance change using the following formula:

$$\text{COX activity (Units/mg)} = (\text{OD} / t) / [\epsilon \times \text{protein (mg)}]$$

Where: OD is the change in absorbance at time t_1 and time t_2

$$t = t_2 - t_1$$

$\epsilon = 7.04 \text{ mM}^{-1} \text{ cm}^{-1}$ (molar extinction coefficient of reduced Cytochrome C at 550 nm)

Support Protocol 3

Complex V Activity Assay

Complex V is the terminal component of the mitochondrial ETC. It is also known as ATP synthase that participates in the production of ATP. Active ATP synthase is an indicator of functional nature of the mitochondrial ETC and thus ATP synthase activity of the isolated mitochondria can be determined (Figure 5C).

Materials

Isolated mitochondria solution in $1\times$ MAS

MitoTox™ Complex V OXPHOS Activity microplate assay kit consisting of complex V activity buffer containing ATP, pyruvate kinase (PK), lactate dehydrogenase (LDH), phosphoenolpyruvate, and NADH

Digitonin (5.5 mg/mL) solution

Oligomycin (2 mM) solution

$1\times$ MAS (*see recipes*)

Black bottom 96-well plate

Bio-Tek Synergy HT microplate reader

1.5 mL centrifuge tubes

Complex V Activity Assay

1. Add 40 μL of digitonin and mix with 360 μL isolated mitochondria.
2. Incubate the mixture on ice for 30 min.
3. Centrifuge at $12,000\times g$ for 20 min at 4°C .
4. Collect the supernatant containing exposed ATP synthase in a separate centrifuge tube and dilute this solution with $1\times$ MAS to a final protein concentration of 1 mg/mL.
5. Add 10 μL of exposed ATP synthase per well in a 96-well plate in triplicates.
6. Add 200 nM (final concentration) oligomycin to one set of ATP synthase.
7. Treat each of these wells with 200 μL of complex V activity buffer containing ATP, PK, LDH, phosphoenolpyruvate, and NADH.

8. Measure rate of depletion of NADH by recording the absorbance at 340 nm in a kinetic mode from 5-50 min at intervals of 1 min.
9. Calculate complex V activity from the rate of change of absorbance over a linear range using the formula:

$$\text{Rate (OD/min)} = (\text{Absorbance at } t_1 - \text{Absorbance at } t_2) / (t_2 - t_1)$$

Support Protocol 4

ATP Production Assay

Production of ATP by isolated mitochondria upon supply of adenosine diphosphate (ADP) as the substrate was quantified by luciferase based bioluminescent ATP quantification assay (Figure 5D).

Materials

Isolated mitochondria solution in 1× MAS
CellTiter-Glo[®] Luminescent cell viability assay kit
96-well plate
ADP solution (1 mM)
Bio-Tek Synergy HT microplate reader

ATP quantification

1. Add 10 μL of isolated mitochondria (10 μg with respect to total protein) per well in a 96-well plate in triplicates.
2. Add 1mM ADP solution to one set of triplicates.
3. Adjust volume of the solution to 20 μL using 1× MAS.
4. Add 100 μL of CellTiter-Glo reagent to each of the wells.
5. Incubate the plate at 37 °C for 30 min.
6. Record bioluminescence of each of the wells with a delay time of 10 min.
7. Express luminescence intensity as a measure of the amount of ATP produced.

Support Protocol 5

Determination of Purity of Mitochondrial Fraction by Western Blotting

To determine purity of the mitochondrial fraction, western blotting can be performed to detect contaminations of cytosolic, endoplasmic reticular, or nuclear marker proteins in the mitochondrial fraction. In our studies, anti-calnexin was used as an endoplasmic reticulum (ER) marker, anti-lamin A antibody as a nuclear marker, anti-mitochondrial transcription factor A (TFAM) antibody and anti-voltage dependent anion channels (VDAC1)/porin as mitochondrial markers.

Materials

Cellular fractions (nucleus and debris, cytosolic, and mitochondrial) suspended in 1× MAS

10 % sodiumdodecyl sulfate-polyacrylamide (SDS-PAGE) gel

nitrocellulose membrane

Tris-buffered saline with Tween 20 (TBST) buffer (*see recipes*)

Skimmed milk

Primary antibodies against TFAM, VDAC1/porin, lamin-A, and calnexin

Secondary goat anti-rabbit IgG H&L (HRP) preadsorbed and goat anti-mouse IgG H&L (HRP) preadsorbed antibodies

Clarity™ western ECL substrate

FluorChem HD2 system from Alpha Innotech or other similar imager

Orbital Shaker

Western Blotting

1. Load 20 μ g of protein from various cell fractions on a 10 % SDS-PAGE gel.
2. Resolve the proteins by running the gel at 150 mV for 60-90 min.
3. Transfer proteins onto nitrocellulose membrane at 100 mA for 1 h.
4. Block the membranes in 5% milk in TBST for 6 h.
5. Incubate the membranes with primary antibodies in 1% milk in TBST for overnight at 4 °C.
6. Wash membranes (3×, 10 min) with TBST buffer.
7. Incubate the membranes with secondary antibodies in 1% milk in TBST for 2 h at room temperature.
8. Wash membranes (3×, 10 min) with TBST buffer.
9. Treat the membranes with 1 mL of Clarity™ western ECL substrate for 1 min.
10. Acquire chemiluminescent images using FluorChem HD2 system in movie mode for 4 min.

Supprot Protocol 6

Tem Imaging of Isolated Mitochondria

To study the integrity and intact morphology of the isolated mitochondria, TEM images of mitochondria can be obtained.

Materials

1× MAS (*see recipes*)

Isolated mitochondria solution in 1× MAS

2% glutaraldehyde in 1× MAS

1% OsO₄ in 1× MAS

Deionized water

Ethanol (30, 50, 75, 95, and 100%) in deionized water

Propylene oxide

Spurr

Araldi

JEOL JEM 1011 Transmission Electron Microscope or similar microscope

TEM Imaging

1. Collect mitochondrial pellet by centrifugation at 12000×g for 15 min at 4 °C.
2. Treat mitochondria with 2% glutaraldehyde in 1× MAS for overnight.
3. Recover the mitochondria by centrifugation at 12000×g for 15 min at 4 °C.
4. Rinse fixed mitochondria with deionized water (3 times) and recover the pellet by centrifugation at 12000×g for 15 min at 4 °C.
5. Embed the mitochondrial pellet in 2% low melting agarose gel and allow it to solidify at 4 °C.
6. Treat the mitochondria-embedded agarose block with 1% OsO₄ solution for 1 h.
7. Rinse the block three times with deionized water.
8. Dehydrate the sample sequentially in ethanol (30, 50, 75, 95, and 100%) for 15 min in each step.
9. Repeat the dehydration step with 100% ethanol two times.
10. Rinse the samples twice for 15 min in 100% propylene oxide.
11. Infiltrate the sample sequentially with 50% and 75% spurr in propylene oxide for 120 min each.
12. Treat the agarose embedded sample twice with 1:1 Araldi spurr mixture for 30 min each.
13. Embed the agarose block in 1:1 Araldi spurr mixture and allow it to polymerize at 70-80 °C for overnight.
14. Slice the sample into 20 nm thick slices.
15. Mount the slices on copper grids and image using TEM instrument.

Supprot Protocol 7

Isolation of Mitochondria from Cultured Cells Using Tom22 Based Magnetic Beads

To determine the extent to which the mitochondria isolated by the protocol developed by us compares with a standard centrifugation-free mitochondria isolation method, we performed mitochondria isolation using TOM22 based magnetic beads. We then performed all the tests for functional nature of the isolated mitochondria and found that quality of mitochondria isolated using both the methods compare reasonably well with one another (Figure 5).

Additional Materials (also see reagent-based and magnetic mitochondria isolation protocols)—TOM22 magnetic beads based Mitochondria MidiMACS starting kit (Cat. # 130-094-872) containing lysis buffer, anti TOM22 microbeads, LS column and magnet, Orbital Shaker.

Mitochondria Isolation from Cultured Cells using anti-TOM22 microbeads

1. Harvest cells by trypsinization following method mentioned in reagent-based or magnetic isolation protocols.
2. Suspend cells in 1 mL of ice-cold lysis buffer.
3. Isolate nuclei, debris and unlysed cells following method mentioned in reagent-based or magnetic isolation protocols.
4. Add 50 μ L of anti-TOM22 microbeads to the supernatant and incubate for 1 h under gentle shaking on an orbital shaker at 4 °C.
5. Pour the mixture in a LS column fitted to the magnet provided with the kit.
6. Collect the cytosol fraction at the bottom of the LS column.
7. Remove the LS column from the magnet and collect the mitochondrial fraction by forceful purging of 1 mL of separation buffer through the LS column using the plunger.
8. Store the mitochondrial fraction at -20 °C for future use.

Supprot Protocol 8

Isolation of Mitochondria from Cells Pretreated with Mitochondria-Targeted and Non-Targeted Polymeric Nanoparticles

Mitochondria isolation from NP treated cells may often give an impression that the NPs are associated with the mitochondria. This may be a mere artifact that arises due to the sedimentation of the NPs along with the mitochondria under conditions of high centrifugal speeds. We have developed a targeted polylactide-*co*-glycolide (PLGA)-block (*b*)-polyethylene glycol (PEG) polymer appended with TPP cation, PLGA-*b*-PEG-TPP which upon self-assembling forms NPs which demonstrate high mitochondrial association property. To demonstrate that magnetic isolation can predict better results in understanding mitochondrial association property of such targeted NPs, we pretreated cells with quantum dot (QD) loaded targeted NPs before isolating mitochondria using reagent based or magnetic

isolation methods (Figure 6). As control, we also used non-targeted NPs composed of PLGA-*b*-PEG-OH.

Additional Materials (also see reagent-based and magnetic mitochondria isolation protocols)—Synthesis of PLGA-PEG-QD, QD loaded NPs from PLGA-*b*-PEG-TPP (mitochondria targeted), and PLGA-*b*-PEG-OH (non-targeted) NPs were as described in previously reported procedures (12, 14). PLGA-COOH of inherent viscosity of 0.18 dL/g was purchased from Lactel. HO-PEG-OH (MW 3350) purchased from Sigma-Aldrich. Qdot 705 ITK or amino PEG quantum dot was purchased from Invitrogen. Amicon centrifugal filters with a MW cutoff of 100 kDa were procured from Millipore. Beckman Coulter Allegra X 15R centrifuge was used for amicon filtration of nanoparticles. VG PlasmaQuad 3 ICP mass spectrometer.

Mitochondria Isolation from T/NT-QD-NP Pretreated Cells

1. Treat ($1-5 \times 10^7$ cells) of H9C2 cardiomyocytes, J3TBG glioma, or A2780 ovarian cancer cells with 50 $\mu\text{g/mL}$ T/NT-QD-NP in fresh media.
2. Add Mito-magneto (20 $\mu\text{g/mL}$) along with T/NT-QD-NP to one set each of the treated cells.
3. Incubate the cells for 12 h.
4. Isolate mitochondria, nucleus, and cytosol fractions from the Mito-magneto treated cells following magnetic isolation protocol and from the Mito-magneto untreated cells following the reagent based isolation protocol.
5. Quantify amount of cadmium (Cd) present in the various fractions using ICP-MS and normalize the Cd content with respect to protein concentration of the cellular fractions based on BCA assay performed on the various cellular fractions.

Reagents and Solutions

Use Millipore water in all recipes and protocols unless mentioned otherwise

1× Mitochondria assay solution (MAS)

210 mM Mannitol
70 mM Sucrose
5 mM Tris-HCl (pH 7.5)
1 mM EDTA (pH 7.5)

10 × Tris-buffered saline with Tween 20 (TBST) buffer

20 mM Tris (pH 7.5)
150 mM NaCl
0.1% Tween 20

Coating Buffer

3.03 g Na_2CO_3
6.0.g NaHCO_3
1000 mL distilled water
pH adjusted to 9.6

Wash Buffer

1× PBS (pH 7.4) containing 0.05% Tween 20

Assay Diluent

10% Fetal Bovine Serum (FBS) in 1× PBS (pH 7.4)

Commentary**Background Information**

With the emergence of NP platforms that can target mitochondria for a variety of biologically relevant purposes, isolation of NP-associated mitochondria has become an absolute necessity to understand the targeting properties of these newly developed nanomaterials. Mitochondria of cells are dynamic organelles that undergo continuous fission/fusion processes. Furthermore, the mitochondrial membrane potential that exists across the double mitochondrial membranes also varies depending on the cell type and the diseases stage. Conventional high speed centrifugation based techniques are not suitable for isolation of NP-containing mitochondria as it may give erroneous distribution pattern of NPs among the cellular compartments.

The method devised here is a centrifugation or immunoprecipitation free method that enables magnetic isolation of mitochondria. This method was tested in three different cell lines with different mitochondrial membrane potential values. This method yields similar amounts of mitochondria as that obtained using conventional reagent based isolation. Mitochondria isolated by this technique have intact morphology as determined by TEM studies. The DLS data on isolated mitochondria suggest that these are negatively charged particles with diameter 0.3 to 0.5 microns that matches well with the reported literature values for size mammalian cell mitochondria (31). The mitochondria isolated using this technique are pure as determined by western blotting which shows strong enrichment of mitochondrial markers in the mitochondrial fraction. Multiple activity assays for various mitochondrial proteins and processes also confirm that the mitochondria are functional and respiration active.

The only limitation of this technique is that Mito-magneto cannot be separated from the mitochondrial fraction after isolation. It should be noted that we are using only 20 $\mu\text{g}/\text{mL}$ of Mito-magneto in this process and our analyses demonstrated that cellular and mitochondrial health is not affected by this concentration of Mito-magneto. This was confirmed by MTT assay and MitoStress test analysis. MTT assay suggested that mito-magneto are non-toxic to cells up to a concentration of 100 $\mu\text{g}/\text{mL}$. MitoStress test analyses on cells revealed that

Mito-magneto did not have any adverse effect on mitochondrial bioenergetics up to a concentration of 60 $\mu\text{g/mL}$. A comparison of Mito-magneto based mitochondria isolation with TOM22 magnetic beads based mitochondria MidiMACS kit indicate that TOM22 technology involves the use of expensive TOM22 antibody coated magnetic beads. Moreover, the MidiMACS kit requires immunoprecipitation technique whereas the technique described herein is immunoprecipitation and centrifugation free.

Critical Parameters and Troubleshooting

The protocol described herein, for magnetic isolation of mitochondria, is simple and easy to carry out. Nevertheless, there are certain crucial parameters that need more attention. First and most important is the quality of Mito-magneto that is synthesized. The particles should be small (<10 nm in diameter), monodisperse, and must bear a high positive surface charge (zeta potential > 20 mV). In the final step of Mito-magneto purification, minimum volume of DMF should be used to resuspend the NPs for use in mitochondria isolation.

All the tubes, magnet, buffers etc., which are used for mitochondria isolation should be pre-chilled on ice. Depending on the cell type, the probe sonicator sequence should be optimized for optimal disruption of cells and minimum harm to the mitochondria. Isolated mitochondria should be isolated in minimum possible amount of buffer and frothing of the mitochondria solution should be avoided. Frothing may lead to deactivation of mitochondrial enzymes. Freshly isolated mitochondria samples produce best results for functional assays and TEM imaging studies. The magnet should be on around the tube while decanting the cytosolic fraction into a separate tube. Purity of the mitochondrial fraction can be improved by repeating the magnetic isolation step 2-3 times but doing so may decrease the yield of mitochondrial fraction.

Anticipated Results

Mito-magneto based magnetic isolation protocol described here provides a centrifugation and immunoprecipitation free method which can produce pure, intact, and functional mitochondria from live cells for multiple purpose. Most importantly as learnt from our data and this protocol, Mito-magneto can be used as a powerful tool to investigate mitochondrial association properties of other nanomaterials since this mitochondria isolation technique does not require high-speed centrifugation and hence, possible errors arising from nanoparticle precipitation along with the mitochondrial component under the influence of centrifugation can be avoided. The method described here is capable of eliminating such artifacts associated with centrifugal isolation of nanoparticle-loaded mitochondria.

Time Considerations

We have included anticipated time for each of the steps in the protocol. The total estimated time of the whole procedure starting from synthesis of the precursors, Mito-magneto, characterization of Mito-magneto, plating of cells for mitochondria isolation could be ~15 days. Synthesis and characterization of mitochondria targeting ligand and the non-targeted IONPs can take up to 7 days. Isolation of mitochondria using Mito-magneto will depend on the doubling time of the type of cells, as it is crucial for the flask to become confluent. An overview of the workflow of the magnetic mitochondria isolation process is as follows: Day

1: plating of cells; Day 3-4: treatment with Mito-magneto, harvesting of cells, isolation of mitochondria, functional activity assays, beginning of TEM sample preparation; Day 5-6: western blotting, continuation of TEM sample preparation; Day 7: TEM slice preparation and imaging of isolated mitochondria.

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Significance Statement

We report the development of a centrifugation free technique for magnetic isolation of pure, intact, respiration active and functional mitochondria using a newly developed mitochondria targeted iron oxide nanoparticles, Mito-magneto. The ease of synthesis of Mito-magneto and the ability to detect these nanoparticles in the subcellular fractions post-isolation add additional advantages in addition to a centrifugation free mitochondria isolation protocol. This technology was validated in different cell lines from multiple species with varied mitochondrial membrane potentials. This technique is found to be highly relevant since it is capable of eliminating possible artifacts associated with centrifugal isolation of nanoparticle containing mitochondria.

Figure 1. Schematic illustration of mitochondria isolation procedures employing magnetic and reagent-based protocols. This figure is reproduced with slight modifications based on our previous publication: *Nanoscale* 8(47):19581-19591.

Figure 2. Schematic showing the steps involved during synthesis of Mito-magneto. This figure is reproduced with slight modifications based on our previous publication: *Nanoscale* 8(47): 19581-19591.

Figure 3.

(A) ^{31}P NMR of Mito-magneto nanoparticles, DLS plots for hydrodynamic diameter (B) and zeta potential (C) of Mito-magneto. (D) TEM image of Mito-magneto. This figure is reproduced with slight modifications based on our previous publication: *Nanoscale* 8(47): 19581-19591.

(B)

Characterization of Isolated Mitochondria by DLS					
Cell Line	Isolation Method	Mito-magneto	Diameter (nm)	PDI	Zeta potential (mV)
A2780	Reagent	-	421.4 $\pm$ 33.5	0.744 $\pm$ 0.198	-44.1 $\pm$ 2.5
A2780	Reagent	+	402.6 $\pm$ 14.7	0.656 $\pm$ 0.047	-43.1 $\pm$ 2.1
A2780	Magnetic	+	424.6 $\pm$ 22.6	0.557 $\pm$ 0.064	-45.1 $\pm$ 4.3
J3TBG	Reagent	-	365.6 $\pm$ 7.7	0.450 $\pm$ 0.029	-34.2 $\pm$ 0.7
J3TBG	Reagent	+	371 $\pm$ 8.8	0.482 $\pm$ 0.046	-32.5 $\pm$ 0.4
J3TBG	Magnetic	+	377.9 $\pm$ 3.6	0.457 $\pm$ 0.014	-33.4 $\pm$ 0.4
H9C2	Reagent	-	381.9 $\pm$ 2.6	0.647 $\pm$ 0.111	-34.5 $\pm$ 1.5
H9C2	Reagent	+	354.7 $\pm$ 7.6	0.447 $\pm$ 0.030	-30.3 $\pm$ 6.0
H9C2	Magnetic	+	272.3 $\pm$ 6.3	0.438 $\pm$ 0.050	-31.9 $\pm$ 0.6

Figure 4.

(A) T_2 -weighted MRI ($T_R = 2500$ ms, $T_E = 10.69$ ms) imaging of cellular sub-fractions isolated using reagent-based or magnetic methods to confirm association of Mito-magneto NPs with the mitochondrial fraction. Cells treated with 20 $\mu\text{g/mL}$ of mito-magneto were subjected to sub-fractionation after 12 h of incubation. Cyto: cytosolic fraction; Mito: mitochondrial fraction. (B) Size, PDI and Zeta potential of mitochondria isolated from all three cell types. This figure is reproduced with slight modifications based on our previous publication: *Nanoscale* 8(47):19581-19591.

Figure 5.

Functional nature of mitochondria isolated from J3TBG cells as demonstrated by determination of (A) citrate synthase activity as Krebs cycle marker, (B) COX activity as ETC marker, (C) ATP synthase activity as a ATP synthesis marker, and (D) production of ATP by the isolated mitochondria upon supply of ADP substrate. This figure is reproduced with slight modifications based on our previous publication: *Nanoscale* 8(47):19581-19591.

Figure 6.

(A) Mitochondria targeted and non targeted NPs which were used to investigate the validity and superiority of Mito-magneto based mitochondria isolation technique for determination of mitochondria targeted NP distribution. (B) A schematic of Mito-magneto based mitochondria isolation for other nanomaterial cellular distribution. (C) Determination of Cd using ICP-MS analyses for the quantification of a mitochondria-targeted NP (T-QD-NP) and non targeted NP (NT-QD-NP) in various sub-cellular fractions isolated by conventional reagent based method and Mito-magneto based magnetic separation from J3TBG cells. This figure is reproduced with modifications based on our previous publication: *Nanoscale* 8(47): 19581-19591.