Magnetic nanoparticles: An improved method for mitochondrial isolation

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Abstract. The ultrastructure, function, and physical and chemical properties of mitochondria have become important issues in scientific research. Current mitochondrial isolation methods mainly rely on the physical and chemical properties of mitochondria. Our team presents a fast and reliable new isolation method based on magnetic nanoparticles binding with monoamine oxidase-A (MAO-A) expressed in the mitochondria. MAO-A is expressed in the outer membrane of human mitochondria and is localized on the cytoplasmic side of the membrane, which makes it possible to isolate the mitochondria effectively, using a magnetic field. As shown in the present study, in comparison with differential centrifugation and density gradient centrifugation, the yield of mitochondria isolated by magnetic nanoparticle binding is higher, with greater mitochondrial purity and activity. The entire process, from cell harvesting to final isolation of the mitochondria, takes approximately 1 h. Magnetic nanoparticles provide a simple, practical approach for mitochondrial isolation.

Introduction

Magnetic particles are widely used in scientific research for biological applications, including drug delivery, biomedicine, magnetic resonance imaging (MRI) and bioseparation, due to their high liquid dispersion, chemical stability, surfacial modifiability and unique magnetic properties. Magnetic particles provide solid magnetic support for bioseparation. Many functional magnetic nanocomposites have been used for the specific isolation of phosphopeptides, glycopeptides, histidine-tagged proteins, bacteria and cells from complex biological samples in order to find biomarkers to study disease mechanisms and drug actions.

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The mitochondrion is an important organelle in a variety of eukaryotic cells. It participates in many important life processes, including energy generation, the production of oxygen free radicals, the maintenance of intracellular calcium homeostasis, signal transduction, apoptosis and so on (1-5). As many studies have suggested links between mitochondrial dysfunction and diseases such as obesity (6), cancer (7,8), aging (9), neuro-degeneration disease (10) and cardiac disease (11), the study of mitochondria has gained importance in many fields, including biomedicine, drug development and proteomics. Studies on mitochondria require the isolation of active mitochondria (12).

Mitochondria are commonly extracted from metabolically active cells, e.g., myocardial, liver and kidney cells. However, mitochondria are easily contaminated by other cellular components during extraction. Furthermore, the special bilayer structure and enzyme system of the mitochondria may be destroyed under certain physiological conditions. These difficulties in mitochondrial extraction have limited the research on mitochondria. Differential centrifugation has been the most commonly used method for purifying mitochondria. Density gradient centrifugation (13-16) can also isolate highly purified mitochondria, but it requires large amounts of time and energy; it also requires a high-speed centrifuge, which is a large piece of equipment. In this study, we introduce a faster and more convenient method for mitochondrial isolation, based on magnetic nanoparticle binding with a monoamine oxidase-A (MAO-A) monoclonal antibody. The results of this isolation method show high yields of mitochondria, improved mitochondrial activity and no contamination by other subcellular components. This method provides a more effective method for mitochondrial isolation and will thus facilitate future studies on mitochondrial function.

Materials and methods

Materials. Tetraethoxysilane (TEOS), ethylene glycol, 3-aminoproplyl triethoxysilan (99%), sodium cyanoborohydride ($NaCNBH_3$), sucrose, HEPES, Tris, SDS, TritonX-100, EDTA, EGTA, and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO, USA). Nycodenz was purchased from Axis-Shield Co. Flotillin-1, cytochrome *c*, and COX IV antibodies were purchased from Sigma. Lamin B, GAPDH and VDAC antibodies were purchased from Cell Signaling Co.

LAMP-1, catalase and MAO-A antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ammonium hydroxide and sodium acetate anhydrous were obtained from Tianjin Chemical Plant (Tianjin, China). Glutaraldehyde (GA, 25% w/v aqueous solution) was purchased from Beijing Chemical Reagent (Beijing, China).

Magnetic nanoparticles linked with a MAO-A antibody. Fe_3O_4 magnetic nanoparticles were synthesized according to a previously published procedure (17). The synthesized Fe_3O_4 magnetic nanoparticles (100 mg) were washed twice with ethanol, re-dispersed in ethanol (80 ml), and sonicated for 0.5 h. Ammonia (25%, 1.00 ml), water (20 ml) and TEOS (1.0 ml) were added to the obtained Fe_3O_4 suspension, and the resulting mixture was stirred vigorously at 40˚C for 6 h in an oil bath. The resultant magnetic Fe_3O_4/SiO_2 core-shell nanoparticles were rinsed three times with ethanol (100 ml) and were then dispersed in isopropanol (45 ml) with sonication for 10 min. APTES (1.0 ml) was added, and the mixture was stirred at 70˚C for 12 h under a nitrogen atmosphere. After cooling to an ambient temperature, the resultant nanoparticles were washed twice with isopropanol, ethanol and PBS buffer, sequentially, with the help of a magnet at each step. The amine of the produced material (500 μg) was activated under a vortex agitator for 2 h after adding 500 μl of 10% glutaraldehyde solution in PBS buffer. The magnetic particles were retained by a magnet and the solution was removed. The particles were then washed three times with PBS (500 μl) buffer. A MAO-A antibody (40 μg) solution was mixed with 50 μl of PBS buffer, and the obtained magnetic particles were incubated with the MAO-A antibody (40 μg) solution for 2 h under vortex. Finally, the resultant magnetic material was washed four times with PBS buffer. Magnetic nanoparticles were re-dispersed in 1 ml PBS buffer containing 0.02% (w/w) NaN_3 before they were ready for use.

Mitochondrial preparation. The isolation and purification of the mitochondria was performed as previously described, with some modifications (18). A total amount of $1x10^7$ HepG2 and HeLa cells were harvested. The pre-cooling united extraction buffer (0.2 mol/l mannitol, 50 mmol/l sucrose, 1 mmol/l EDTA, 1 mmol/l EGTA, 10 mmol/l HEPES, pH 7.4, 50 mmol/l DTT, 5 mmol/l protease inhibitor cocktail and 1 mmol/l PMSF) was added at a 5:1 (w/v) ratio. The buffer and the cells were transferred into a dounce homogenizer and homogenized for several strokes until up to 80% of the cells had ruptured. A total amount of 30 μl magnetic nanoparticles linked with the MAO-A antibody were added to the ruptured cells and incubated for 20 min at 4˚C. A magnet was then used to bring the magnetic nanoparticles to the tube wall. The tube contents were washed three times with potassium phosphate buffer. The precipitate was collected and centrifuged at 15,000 x g for 10 min. The resulting mitochondrial crude extract was dissolved in 12 ml 25% Nycodenz (Axis-Shield Co.) for full suspension. Density gradient centrifugation was performed as previously described (19); the total time was not more than 1 h. Finally, mitochondria were fully suspended in the isolation buffer.

Scanning electron microscopy. The mitochondrial suspension was centrifuged, and the supernatant was removed. The pellet was fixed overnight with 2.5% glutaraldehyde. After washing three times with PBS, the pellet was dehydrated for 10 min at each concentration of a graded ethanol series (50, 70, 80, 90, 95 and 100%). The pellet was immersed in pure tert-butyl alcohol and was then placed into a 4˚C refrigerator until the tert-butyl alcohol solidified. The frozen samples were dried by placing them into a vacuum bottle. Mitochondrial morphology was evaluated by scanning electron microscopy (SEM; Philips model XL30).

Determination of mitochondrial respiratory function. The mitochondrial respiratory control rate is an extremely sensitive index for evaluating the structural integrity and oxidative phosphorylation of mitochondria. Mitochondrial respiratory function was determined using the Estabrook oxygen electrode method (20). The reaction temperature was 29˚C; the total volume was 2.0 ml (0.25 M sucrose, 5 mM $MgCl_2$, 15 mM KH_2PO_4, 50 mM Tris HCl and pH 7.2). Mitochondrial suspension and substrate (50 μl) were added. A dissolved oxygen meter was used to detect the oxygen consumption of states 3 and 4, from which the respiratory control ratio (RCR) was calculated.

Analysis of mitochondrial membrane potential. The density of mitochondria in the suspension was adjusted to $1x10^5$/ml, and then 10 μg/ml JC-1 solution was added. The solution was fully mixed and incubated in a 5% CO_2 dark incubator for 30 min. The excess unbound dye was then washed twice with 1X assay buffer, and the precipitate was resuspended in PBS. The cells were analyzed by flow cytometry (BD Co., Franklin Lakes, NJ, USA) with an emission wavelength of 488 nm and an excitation wavelength of 488 nm. JC-1 monomers and aggregates were visualized on the FL1 and FL2 detectors, respectively. Because a dual wavelength was emitted by JC-1, electronic compensation was applied to modify the overlap of the green (monomer) and red fluorescence (polymer). FL1-H and FL2-H represented the fluorescence intensity of red and green, respectively. Quantitative analysis was carried out using CellQuest analysis software.

Western blot analysis. The prepared mitochondria were solubilized in lysis buffer (7.0 M urea, 2 M thiourea, 4% (v/v) CHAPS, 5 mM EDTA, 5 mM EGTA, 50 mM DTT, pH 7.4, and protease inhibitors and phosphatase inhibitor mixture), incubated on ice for 30 min and sonicated for 5 min. The supernatant was collected at 2500 x g, 4˚C for 30 min. Protein concentration was determined using the BCA method (BCA kit; Pierce, Rockford, IL, USA). The samples were separated by SDS-PAGE and transferred onto a PVDF membrane using the semi-dry method, then blocked with 5% non-fat milk overnight. The proteins were probed with their primary antibodies (diluted from 1:200 to 1:1000) (Bio-Rad, Hercules, CA, USA; Cell Signaling, CA, USA; Sigma; Santa Cruz Biotechnology) at 37˚C for 1 h, washed three times in TBST and then incubated with secondary antibodies (1:5000) at 37˚C for 1 h. After washing again, the resulting bands were detected using enhanced chemiluminescence. The band intensities were quantified using Quantity One software.

Statistical analysis. All data were statistically analyzed using SPSS 15.0. Mean values ± SD for various groups were compared

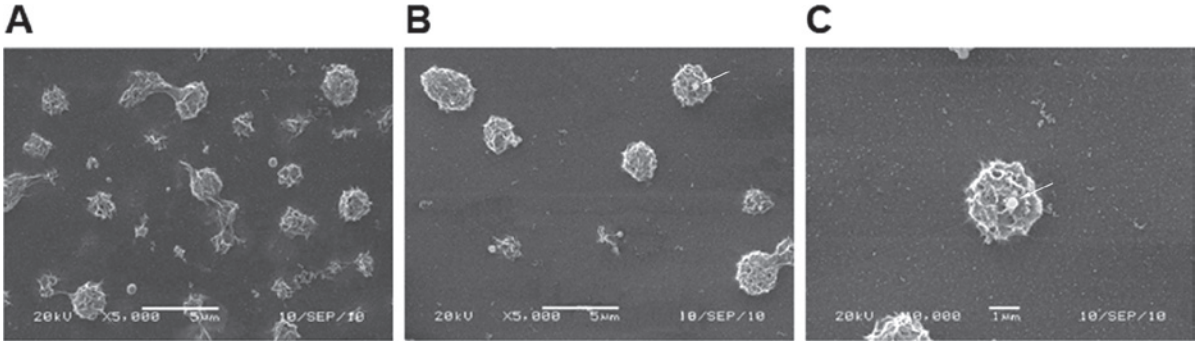

Figure 1. Morphological observation of mitochondria with scanning electron microscopy. (A) Mitochondria isolated with differential centrifugation. Mitochondria isolated by this method were contaminated with other subcellular components. (B) Mitochondria isolated with magnetic nanoparticles. Magnetic nanoparticles combined with mitochondria closely, with uniform distribution. Mitochondrial structure was intact. (C) x10,000 magnification of (B). The arrow indicates the binding site of a mitochondrion and a magnetic nanoparticle.

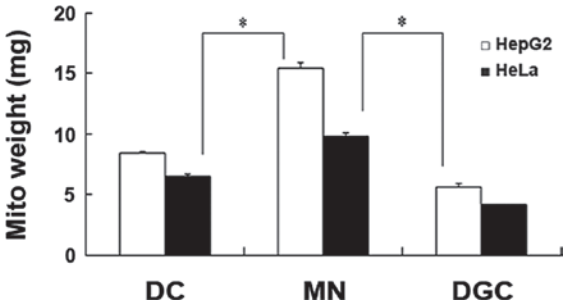

Figure 2. Yield of isolated mitochondria. Mitochondria were isolated from HepG2 and HeLa cells. The yield of the magnetic nanoparticle (MN) method was higher compared to that of differential centrifugation (DC) and density gradient centrifugation (DGC). Data represent the means ± SD of at least three separate experiments. *p<0.01. Mito, mitochondrial.

using the two-way ANOVA and LSD t-tests, as appropriate. Prior to the study, $p<0.05$ was selected as the criterion for significance of differences between groups.

Results

Synthesized magnetic nanoparticles modified with a MAO-A antibody with well-defined magnetic core/silica shell (Fe_3O_4/SiO_2) structures. Magnetic microspheres were obtained from a solvothermal reaction and coated with a layer of silica shell by a sol-gel reaction. Compared with magnetic microspheres coated with a polymer shell (such as polystyrene), magnetic microspheres encapsulated with a silica shell had a greater magnetic response and more diverse surfacial modification. Transmission electron microscopy (TEM) observation showed that the Fe_3O_4 magnetic particles were almost spherical in shape and had a mean diameter of 240 nm (Fig. 1A). Remarkably, every magnetic microsphere was composed of many magnetic nanoparticles (diameter of approximately 15 nm). A TEM image of Fe_3O_4/SiO_2 core-shell microspheres showed that the dark magnetite particles were individually coated with a uniform grey silica shell with a thickness of approximately 40 nm. The amine group was further functionalized on the silica shell. Glutaraldehyde, a commonly used biofunctional reagent, was employed to lengthen the bond between the antibody and the magnetic solid support. Sodium cyanoborohydride was applied to reduce C=N to C-N to covalently bind the antibody to the support, enhancing the stability of the antibody and its resistance to changes in the microenvironment such as pH value and organic solvents. The Fe_3O_4/SiO_2 microspheres modified with monoamine oxidase antibody exhibited superparamagnetic properties as well as high magnetization with a saturation value of approximately 75.77 emu x g^{-1} (Fig. 2).

Fe_3O_4/SiO_2 microspheres have three major benefits for mitochondrial isolation. First, the thick silica shell of the microspheres not only protects the Fe_3O_4 magnetic cores from leaching in an acidic environment in the medium but also provides easier and more effective functional modification. Moreover, the Fe_3O_4/SiO_2 core-shell microspheres are well dispersed in aqueous solutions and have good biocompatibility. Second, a suitable space arm is formed to improve the flexibility of the antibody, thereby improving its ability to capture mitochondria. Third, the material's superparamagnetic properties enable the quick isolation of material from sample matrices using a magnet, facilitating the manipulation of materials in enrichment procedures without centrifugation. All of these benefits of the Fe_3O_4/SiO_2 microspheres modified with a MAO-A antibody make them suitable for fast and specific mitochondrial isolation from biological samples.

Mitochondrial isolation using magnetic nanoparticles. To determine whether magnetic nanoparticles are capable of successfully isolating mitochondria from different cells, we used scanning electron microscopy. The results showed that after the mitochondria were incubated with magnetic nanoparticles at 4˚C for 20 min, the majority of the mitochondria were combined with the nanoparticles. The structure of the isolated mitochondria was round or oval with the magnetic nanoparticles firmly bound and uniformly distributed. The mitochondrial structure was intact. The purity of the isolated mitochondria was over 90%, demonstrating that magnetic nanoparticles linked with a MAO-A antibody combine with mitochondria specifically, making the mitochondrial isolation more effective (Fig. 1).

Yield of isolated mitochondria. We isolated mitochondria from an equal number of HepG2 and HeLa cells using various methods. We found diverse mitochondrial yields with each different method. In HepG2 cells, the nanoparticle method

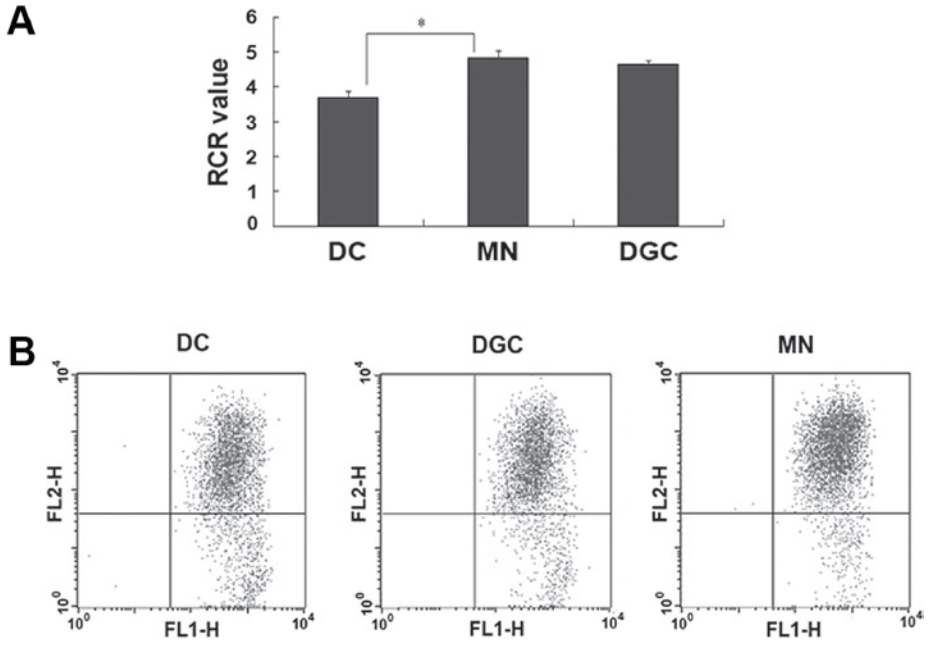

Figure 3. Mitochondrial respiratory control rate and membrane potential. (A) Three methods were used to test the mitochondrial respiratory rate. (B) Flow cytometry was used to analyze mitochondrial membrane potential. Data represent the means ± SD of at least three separate experiments. *p<0.01.

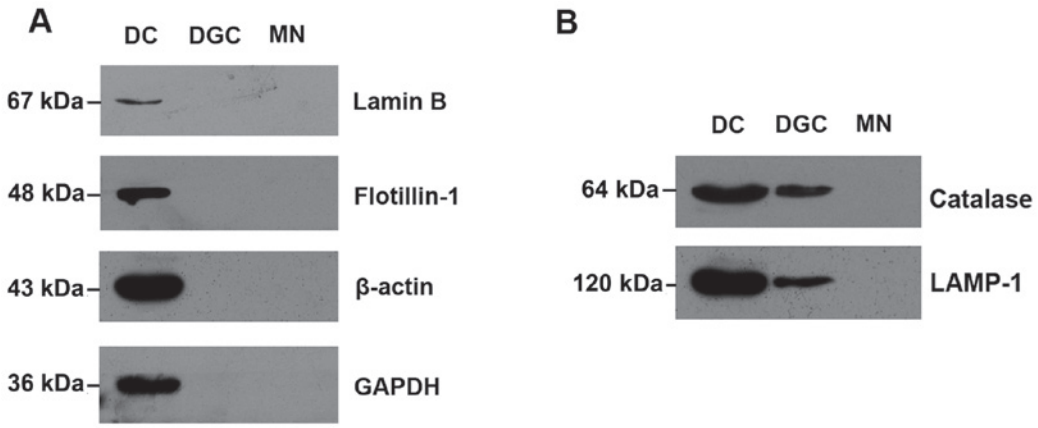

Figure 4. Purity of the mitochondria. (A) Western blot analysis of lamin B, flotillin-1, β-actin and GAPDH expression in mitochondria obtained using the differential centrifugation (DC), magnetic nanoparticle (MN) and density gradient centrifugation (DGC) methods. (B) Western blot analysis of catalase, LAMP-1 in mitochondria obtained using the DC, MN and DGC methods. Each group had the same sample volume; each independent experiment was repeated three times.

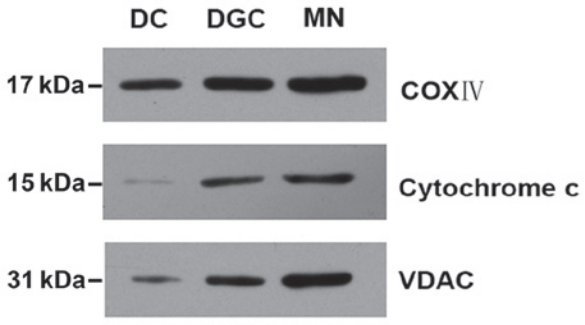

Figure 5. Western blot analysis of COXIV, cytochrome *c* and VDAC expression in mitochondria obtained using the differential centrifugation (DC), magnetic nanoparticle (MN) and density gradient centrifugation (DGC) methods. Each group had the same sample volume; each independent experiment was repeated three times.

gave a yield two-fold higher than differential centrifugation and three-fold higher than density gradient centrifugation. These results indicated that the highest mitochondrial yield was obtained using magnetic nanoparticles (Fig. 2).

Quality of isolated mitochondria. The goal of any isolation method is to obtain mitochondria with a high biological activity. In order to clarify the impact of different isolation methods on mitochondrial biological activity, we examined the mitochondrial respiratory function as well as the alteration of mitochondrial membrane potential. Our results showed that the mitochondria obtained from all three methods had good activity, but the mitochondria isolated by magnetic nanoparticles had the largest activity, and their membrane potential tended to be the most stable (Fig. 3).

Purity of mitochondria. Another goal of any isolation method is to obtain highly-purified mitochondria. Contamination by other subcellular components may interfere with the results of any study performed using the isolated mitochondria. Previous studies have demonstrated that isolated mitochondria may easily be contaminated by cell nuclei, cell membranes, cytoplasm, lysosomes and peroxisomes, among other components. Contamination of isolated mitochondria by lysosomes and peroxisomes is particularly frequent. Using western blot analysis, we found that the mitochondria isolated by differential centrifugation exhibited lamin B, flotillin-1, β-actin and GAPDH, representing cell nuclei, cell membranes, cytoskeletons and cytoplasm, respectively. The levels of these proteins were higher in the mitochondria isolated by differential centrifugation than by the magnetic nanoparticle method or by density gradient centrifugation. Furthermore, LAMP-1 and catalase, which mark peroxisomes and lysosomes, had almost no expression in the mitochondria isolated by the magnetic nanoparticle method. This suggests that the mitochondria isolated using magnetic nanoparticles have higher purity than those isolated using other methods (Fig. 4).

Analysis of mitochondrial membrane protein. We measured the content of the mitochondrial inner membrane protein, COXIV, the mitochondrial membrane space protein, cytochrome *c*, and the mitochondrial outer membrane protein, VDAC, to investigate the biological activity of the mitochondrial membranes. The results suggest that COXIV, cytochrome *c* and VDAC were expressed at higher levels in the mitochondria isolated by the magnetic nanoparticle method than those isolated by differential centrifugation or density gradient centrifugation. These results demonstrate that mitochondria isolated by magnetic nanoparticles have greater levels of biological activity (Fig. 5).

Discussion

Mitochondria, which serve as the center of energy production, metabolism and apoptosis, play crucial roles in all living organisms and appear to be closely involved in the development of various diseases (21). Therefore, the study of mitochondrial structure and function has become increasingly important. Mitochondrial isolation is an indispensible technology for this research.

There are many ways to isolate mitochondria. Laboratories adopt different isolation methods depending on several factors (22-24). The choice of an isolation method depends on whether the method is capable of reducing contamination from other cellular components, whether mitochondria obtained using the method have good structure and activity, and whether the isolation method is convenient, among other factors. At present, differential centrifugation or density gradient centrifugation are the two methods most commonly used to isolate mitochondria. Mitochondria isolated using these methods were suitable for some kinds of mitochondrial function research, such as the detection of oxidative phosphorylation activity. However, these isolation methods are not able to provide the highly-purified mitochondria required to study the relationship between mitochondria and disease development. We designed a simpler, faster mitochondrial isolation method based on a combination of magnetic nanoparticles and a monoclonal MAO-A antibody. Using $1x10^7$ cells for each method, we found that the magnetic nanoparticle method produced a higher mitochondrial yield than either differential centrifugation or density gradient centrifugation. Moreover, the mitochondria isolated using the magnetic nanoparticle method contained no contamination from other subcellular components. The magnetic nanoparticle method for mitochondrial isolation has many advantages: high yield, good reproducibility, greater mitochondrial activity and higher purity. Furthermore, this method could be applied to various cell lines, such as HepG2 and HeLa. This method, using magnetic nanoparticles as separation media, obtained high-purity, intact mitochondria with good activity and shortened the time required for mitochondrial isolation. This study has demonstrates an efficient, practical method for mitochondrial isolation.

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