A novel nanoparticle system targeting damaged mitochondria for the treatment of Parkinson's disease

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Research

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Abstract

Background

Mitochondrial damage is one of the primary causes of neuronal cell death in Parkinson's disease (PD). In PD patients, the mitochondrial damage can be repaired or irreversible. Therefore, mitochondrial damage repair becomes a promising strategy for PD treatment.

Methods

We use hyaluronic acid nanoparticles (HA-NPs) of different molecular weights to protect the mitochondria and salvages the mild and limited damage in mitochondria. Our HA-NPs with 2,190 kDa HA can improve the mitochondrial function of SH-SY5Y cells and PTEN induced putative kinase 1 (PINK1) knockout mouse embryo fibroblast (MEF) cells. In cases of irreversible damage, we use NPs with ubiquitin specific peptidase 30 (USP30) siRNA to promote mitophagy. Meanwhile, by adding PINK1 antibodies, our NPs can selectively target the irreversibly damaged mitochondria, preventing the excessive clearance of healthy mitochondria.

Results

Our HA-NPs with 2,190 kDa HA can protect the mitochondria and salvage the mitochondrial function of the mild and limited damage conditions in both SH-SY5Y cells and PINK1 knockout MEF cells. NPs with USP30 siRNA and PINK1 antibodies can selectively target and promote the clearance of irreversibly damaged mitochondria both in vitro and in vivo.

Conclusions

We successfully designed and developed NPs for the treatment of PD that can target different stages of mitochondrial damage. This strategy is expected to overcome the problems associated with the excessive clearance of healthy mitochondria and has great potential for clinical applications. Our study provides a novel strategy for PD treatment.

Background

Parkinson's disease (PD) is a common neurodegenerative disease with an incidence of about 1.7%. More than 6 million individuals are diagnosed with PD worldwide and there is an increased tendency for occurrence among youth [1]. The pathological feature of PD is the self-selective loss of dopaminergic neurons, which are the main source of dopamine (DA) in the mammalian central nervous system; these neurons project to the striatum of the substantia nigra compacta (SNc).

One of the significant factors that contributes to the dopaminergic neuron death in PD is mitochondrial dysfunction [2–6]. In the dysfunctional mitochondria, the morphology undergoes destruction [7], the consumption of hydrogen peroxide and the clearance of reactive oxygen species (ROS) in the
mitochondria is decreased, and the oxidative stress levels are increased [8]. Moreover, depletions in mitochondrial DNA (mtDNA) accumulate with normal aging in the SN and occur 9% more often in PD patients than in age-matched controls [9, 10]. mtDNA expression at the transcription level is lower in the postmortem tissue of PD patients [11]. Besides, the mutations of mtDNA occurrence is increased [12] and dopaminergic neurons exhibit an inflammatory phenotype in the brains of PD model mice [13].

The mitochondrial dysfunction caused dopaminergic neurons damage is gradually becoming severe over aging. The mild and limited damage to the mitochondria can be repaired by regulating the mitochondrial dynamics, and the irreversibly damaged mitochondria can be cleaned by mitophagy. Therefore, treatment targeting the mitochondria constitutes an important strategy for treating PD [14].

Hyaluronic acid (HA) is a nonsulfated polysaccharide distributed throughout the extracellular matrix of mammals. It is a member of the glycosaminoglycan family and is synthesized at the inner leaflet of the plasma membrane as a large, unbranched polymer of repeating disaccharides of glucuronic acid and N-acetylglucosamine [15]. It has been verified that HA provides protection against oxidative stress-caused cellular damage through a mitochondrial-controlled pathway [16–20]. For example, HA can improve mitochondrial function by improving mtDNA integrity, and enhancing ATP production and the cell viability of chondrocytes [16]. Further, human placenta-derived mesenchymal stem cells (PDMSCs) cultured on HA-coated surfaces have a 58% higher average mitochondrial mass and increases in the mtDNA copy number compared to noncoated tissue culture surfaces, as well as a threefold increase in the gene expression of the mitochondrial biogenesis-related gene PGC-1α. This increase in mitochondrial biogenesis led to a hyaluronan dose-dependent increase in the mitochondrial membrane potential, ATP content, and oxygen consumption rate, with ROS levels shown to be at least three times lower compared to the control [21]. Therefore, in this study, we aimed to explore the effect that HA exerts on mitochondrial functions of neurons, and applied it to PD treatment.

USP30, a deubiquitinating enzyme (DUB) located in the mitochondria, can delay mitophagy by inhibiting the recruitment of Parkin. In addition, USP30 has also been proven to regulate basal pexophagy, independent of PINK1 and Parkin [22]. Inhibiting the expression of USP30 in cells with mitochondrial dysfunction caused by a Parkin mutation can alleviate the mitochondrial damage caused by Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and their accumulation. For example, in Drosophila, knocking down USP30 can protect dopaminergic neurons from paraquat-induced PD [23]. However, knocking out USP30 will also significantly enhance the mitophagy of healthy mitochondria [22]. Thus, the strategy of knocking out USP30 to treat PD has a possible risk, as the mitochondria could be over-cleared, leading to energy starvation. Thus, systems that can selectively clear irreversibly damaged mitochondria, but not the healthy mitochondria, may represent an alternative for treating PD.

PINK1 protein is transported into the mitochondria and degraded normally [24]. However, the permeability of the transition pores (PTP) of the mitochondria are blocked under stress. Thus, PINK1 cannot be successfully transported into the mitochondria. As a result, PINK1 aggregates on the outer mitochondrial membrane (OMM) surface. It interacts with the translocase of the outer membrane (TOM) protein to form
macromolecular complexes that are anchored on the OMM. Therefore, PINK1 protein accumulation could be used as an intracellular target of damaged mitochondria [25].

In this study, we invented different nanoparticles to either reverse the mitochondrial damage or selectively clear the irreversibly damaged mitochondria during the process of PD, without affecting healthy mitochondria. We showed that our HA-NPs could protect the mitochondria from damage and rescue mitochondrial function. We also showed that NPs grafted with USP30 siRNA could clear the irreversibly damaged mitochondria. In addition, to achieve the selective clearance of irreversibly damaged mitochondria, we added PINK1 antibodies to the NPs grafted with USP30 siRNA; thus, the clearance of healthy mitochondria was avoided. This is because PINK1 is only expressed at the damaged mitochondria.

Methods

Materials and animals

The C57BL/6 mice were bought from Harbin Medical University. All animal experiments were approved and performed in accordance with the guidelines set forth by the Harbin Institute of Technology Committee on Animal Resources.

SH-SY5Y cells was bought from the National Collection of Authenticated Cell Cultures (Shanghai, China). MEF+/+ and MEF PINK1−/− cells were bought from the America Tissue Culture Collection (ATCC; Manassas, VA, USA). Minimum essential medium (MEM), F12, Gluta-max, sodium pyruvate, and non-essential amino acids (NEAA) were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Dulbecco’s Modified Eagle Medium (DMEM) was purchased from HyClone (Thermo Fisher Scientific). Fetal bovine serum (FBS) was purchased from Roche (Basel, Switzerland). Phosphate buffered saline (PBS) was purchased from Gibco (Thermo Fisher Scientific). Cell counting kit-8 (CCK-8), penicillin–streptomycin solution, total superoxide dismutase (SOD) assay kit, MTT cell proliferation and cytotoxicity assay kit were purchased from Beyotime Biotechnology (Jiangsu, China). PINK1 antibody was purchased from Abcam plc (Cambridge, UK). Anti-CD44/FITC was purchased from eBioscience (San Diego, CA, USA). Goat anti-Mouse IgG/FITC was purchased from ZSGB-BIO (Beijing, China). 4’,6-diamidino-2-phenylindole (DAPI), sodium triplyphosphate (TPP), polyformaldehyde, pyruvate, L-Glutamine, and Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Chitosan was purchased from Mengjiu (Shanghai, China). Hyaluronic acid (HA) was purchased from Freda (Shandong, China).

Polymer synthesis

Synthesis of B6 peptide-HA

The prepared 1.25 mg/mL HA solution underwent a pH adjustment to about 5 with MES (21.3 mg/mL) and NaCl (29.2 mg/mL). NHS (5.8 mg/mL) and EDC (19.4 mg/mL) were added under stirring at room
temperature to activate the carboxyl group of the HA. After 10 minutes, B6 peptide solution was added (1 mg/mL; 14 µL per 1 mL HA). The mixture was placed for 24 hours and then dialyzed for 24 hours. Lastly, the mixture was lyophilized.

**Synthesis of B6 peptide-HA-CS-TPP**

In all, 50 µL of TPP solution (0.5 mg/mL) per 1 mL of B6 peptide-HA solution was mixed, and a 1 mL CS solution (0.6125 mg/mL) was added dropwise. The mixture was stirred for 30 minutes and then incubated for 20 minutes to form a stable nanoparticle. The mixture was centrifuged (13,000 g, 10 minutes) and lyophilized, and the nanoparticles were collected.

**Synthesis of B6 peptide–HA-PINK1 antibody–CS-TPP**

The PINK1 antibody was added to 5 mL of sodium periodate solution (10 mg/mL), shaken in the dark for 30 minutes, and then 1 mL was added to the B6 peptide–HA-CS-TPP NP solution (50 mg/mL); the mixture was then shaken in the dark for 4 hours. Overall, 1 mL NaBH₄ solution (1%) was added dropwise, and the mixture was reacted for 20 minutes. The mixture was then centrifuged (13,000 g, 10 minutes) and lyophilized, and the nanoparticles were collected.

**Cell culture and treatment**

MEF cells were maintained in DMEM with 10% (v/v) FBS. SH-SY5Y cells were maintained in 43.5% (v/v) MEM with 43.5% (v/v) F12, 10% (v/v) FBS, 1% (v/v) Gluta-max, 1% (v/v) sodium pyruvate, and 1% (v/v) NEAA. The cell culture condition was set as 5% CO₂ and 37°C in a cell incubator with a humidified atmosphere.

For the CCCP treatment, the cells were seeded in 12-well plates at 5×10⁵ cells per well. The CCCP was diluted to 25 µM with fresh culture medium and cultured in 5% CO₂ at 37°C in an incubator.

**Cell viability assay**

SH-SY5Y cells, MEF+/+ , and MEF PINK1−/− cells were respectively seeded into 96-well plates at 5×10³ cells per well, and cultured in 5% CO₂ at 37°C in an incubator. After the cells were adhered, 100 µL of culture medium containing B6 peptide–HA-PINK1 antibody–CS-TPP nanoparticles (50 µg/mL) was added per well, and the control group was added with culture medium only. Each group was replaced with fresh culture medium after 4 hours; 10 µL of CCK-8 solution was then added to each well, mixed, and incubated for 4 hours in the incubator. The optical density of the viable cells was detected at a wavelength of 450 nm.

**Extracellular acidification and oxygen consumption rate assay**

To test the effect of different molecular weights of HA-NPs on neurons, SH-SY5Y cells were seeded in Seahorse testing plates. The HA-NPs were added with 35 kDa, 117 kDa, and 2,190 kDa HA (0.1%, w/v), respectively, when the cell density reached 95%. To test the repairing effect of different molecular weights
of HA-NPs on damaged neuron models, 100 µM H$_2$O$_2$ were added per well for 2 hours before adding the HA-NPs with 35 kDa, 117 kDa, 1,280 kDa, and 2,190 kDa HA (0.1%, w/v).

To test the effect of different molecular weights of HA-NPs on PINK1-deficient cells, MEF$^{+/+}$ and MEF PINK1$^{-/-}$ cells were respectively seeded in Seahorse testing plates. The HA-NPs were added with 117 kDa, 1,280 kDa, and 2,190 kDa HA (0.1%, w/v), respectively, to both MEF$^{+/+}$ and MEF PINK1$^{-/-}$ cells when the cell density reached 95%. To test the repairing effect of different molecular weights of HA-NPs on the damaged PINK1-deficient cell model, 100 µM of H$_2$O$_2$ was added per well for 2 hours before adding the HA-NPs with 35 kDa, 117 kDa, 1,280 kDa, and 2,190 kDa HA (0.1%, w/v).

After 12 hours, the extracellular acidification rates (ECAR) and cellular oxygen consumption rates (OCR) were determined in real time using a Seahorse XFe24 Analyzer (Seahorse Bioscience, North Billerica, MA, USA), and the experiments were conducted according to the manufacturer's protocol.

**SOD assay**

SH-SY5Y cells were seeded in 96-well plates at 5×10$^3$ cells per well and cultured in 5% CO$_2$ at 37°C in an incubator for 12 hours. Then, 100 µM of H$_2$O$_2$ was added per well. After 2 hours, the HA-NPs were added with 35 kDa, 117 kDa, 1,280 kDa, and 2,190 kDa HA (0.1%, w/v) for 2–3 days, and the SOD level was detected according to the manufacturer's protocol.

**MTT assay**

MEF$^{+/+}$ and MEF PINK1$^{-/-}$ cells were respectively seeded in 96-well plates at 5×10$^3$ cells per well and cultured in 5% CO$_2$ at 37°C in an incubator for 12 hours. Then, 100 µM of H$_2$O$_2$ was added per well. After 24 hours, the HA-NPs were added with 35 kDa, 117 kDa, 1,280 kDa, and 2,190 kDa HA (0.1%, w/v) for 24 hours. MTT (5 mg/mL, pH 7.4) was added for 4 hours, and then the optical density of viable cells was detected at a wavelength of 570 nm according to the manufacturer's protocol.

**Animal model and treatment**

C57BL/6 mice (male, 8 weeks; n=12) were injected with MPTP (30 mg/kg) intraperitoneally for 5 days to establish the PD model. The mice were divided into four groups featuring three mice each. An intravenous injection was administered to the tail every other day with 200 µg of PBS, B6 peptide–HA-PINK1 antibody–CS-TPP, B6 peptide-HA–CS-TPP-USP30 siRNA, and B6 peptide–HA-PINK1 antibody–CS-TPP-USP30 siRNA, respectively. The following tests were performed at the 7th day after the first injection.

**Biodistribution study of NPs in vivo**

C57BL/6 mice (female, 8 weeks of age; n=5) received an intravenous injection to the tail featuring 200 µg of HA–PINK1 antibody–CS-TPP, B6 peptide–HA-CS-TPP, and B6 peptide–HA-PINK1 antibody–CS-TPP, respectively. The mice were sacrificed at 3 hours after the injection of NPs, and the organs were collected. Images were captured with a camera and the results were analyzed using the Living Imaging System for Small Animals (IVIS Lumina; PerkinElmer, Waltham, MA, USA) according to the manufacturer's protocol.
Quantitative real-time PCR

Total RNA was extracted with the TRIzol reagent. The complementary DNA was synthesized using oligo (dT) primer and reverse transcriptase (FSQ-301; TOYOBO, Osaka, Japan), according to the manufacturer’s protocol. Reverse transcription polymerase chain reaction (RT-PCR) was performed in a 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific) with the Fast Start Universal SYBR Green Master [Rox] (Hoffman-La Roche Ltd, Basel, Switzerland) with the following cycling conditions: 2 minutes at 50°C; 10 minutes at 95°C; 45 cycles of 15 seconds at 95°C; and 1 minute at 60°C. RNAs were reverse transcribed using the PrimeScript kit (Takara, Dalian), and subjected to qRT PCR. The primer sequences are listed below: USP30-F: 5’-TCTTCCGCGTTGATGCCAGTT-3’; USP30-R: 3’-CAGCCATGGCTGCTGGTG-5’; GAPDH-F: 5’-CATGGCCTTCCGTGTTCTCTA-3’; GAPDH-R: 3’-CCTGCTTCACCACCTTCTTGA-5’. Relative changes were analyzed using the $2^{-\Delta\Delta CT}$ method.

Confocal immunofluorescence

Samples were fixed in 4% paraformaldehyde (pH 7.4, in PBS) for 15 minutes at room temperature. The samples were washed with PBS (pH 7.4) three times before being permeabilized via incubation in a solution of 0.2% Triton X-100 (Thermo Fisher Scientific) in PBS for 5 minutes at room temperature. Samples were washed three times with PBS (pH 7.4) and then blocked with 1% bovine serum albumin (BSA) (Thermo Fisher Scientific) in PBS (pH 7.4) for 1 hour at room temperature. For phalloidin–DAPI staining, rhodamine-conjugated phalloidin (0.8 U/mL in PBS), which stains F-actin, was then added to the samples and incubated for 1 hour. The samples were washed three times with PBS. For PINK1 antibody staining, the samples were stained by PINK1 antibody (1:200), goat anti-mouse immunoglobulin (Ig)G/FITC (1:200), and DAPI (1:1,000). The samples were washed with PBS after each staining step. All stained samples were visualized with ZeissLSM510 confocal microscopy (Carl Zeiss Microscopy GmbH, Jena, Germany).

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5.0 software. The results were presented as the means ± standard deviation. One-way analysis of variance (ANOVA) was used to compare multiple groups. ‘∗’, ‘∗∗’, ‘∗∗∗’, and ‘∗∗∗∗’ indicates a $P$-value of <0.05, 0.01, 0.0005, and 0.0001, respectively.

Results

Preparation and characterization of B6 peptide-HA-PINK1 antibody-CS-TPP NPs

As shown in Fig. 1a, we blended HA to the B6 peptide, which can help HA pass through the blood–brain barrier. We then added TPP, USP30 siRNA, and CS to form the basic NPs. The PINK1 antibodies were grafted in the last step. We used EDC/NHS to activate the carboxyl groups of HA, which react with the amino group of the polypeptide to form an amide bond, achieving B6 peptide-HA conjugates. We used
We characterized the charge of CS-TPP nanoparticles and also examined the particle size of the nanoparticles. The results showed that the average particle size of CS-TPP NPs was 150.8±5 nm and the zeta potential was −9.42±0.13 mV (Fig. 1c–1d). The dispersion of nanoparticles is relatively stable, and there is almost no sedimentation after standing for many days. Subsequently, the size, morphology, and distribution of CS-TPP nanoparticles were further characterized by scanning electron microscope (SEM). The results showed that the nanoparticles with uniform size and good dispersion had a CS-TPP NP particle size of about 160 nm (Fig. 1e). This is consistent with the SEM results. We obtained CS-TPP nanoparticles with good dispersion and stability.

The B6 peptide-HA, CS-TPP, and PINK1 antibodies were grafted, and the prepared nanoparticles were incubated with goat-anti-rabbit fluorescent secondary antibody, and red fluorescence (nanoparticles) and green fluorescence (PINK1 antibodies) were observed coincident (Fig. 1f), indicating that the PINK1 antibody was successfully grafted onto the surface of the nanoparticles. The prepared nanoparticles had a particle size of 117.2±49.6 nm and a dispersion coefficient of 0.595. The nanoparticles were incubated with MEF cells for 4 hours and immunofluorescence staining was observed (Fig. 1g). The R6G-labeled nanoparticles appeared at the same position as the mitochondria, indicating that the prepared nanoparticles could be taken up by the cells. The particle size analyzer and confocal experiments showed that the B6 peptide-HA-PINK1 antibody-CS-TPP nanoparticles were successfully prepared, and the size was suitable and uniform, which could be used for further experiments.

We examined the biocompatibility of the B6 peptide–HA-PINK1 antibody–CS-TPP nanoparticles in MEF+/+, MEF PINK1−/−, SH-SY5Y cells, and the results showed that the addition of nanoparticles did not significantly change the cell viability (Fig. 1h). Our nanoparticles had good biocompatibility and could be used for further in vivo experiments.

**HA-NPs improve the function of reversibly damaged mitochondria**

In order to verify the repairing effect of our HA-NPs on reversibly damaged mitochondria in neurons using the Seahorse XFe24 sub-board program, we added a 0.1% HA-NP solution to SH-SY5Y cells treated with H₂O₂. We then used HA-NPs with 35 kDa, 117 kDa, 1,280 kDa, and 2,190 kDa HA to test the reparative effect of different molecular weights of HA. Oxidative phosphorylation of respiratory metabolism increased after the treatment of HA-NPs with 117 kDa and 2,190 kDa molecular weight HA on SH-SY5Y cells (Fig. 2a). After the HA-NPs with 117 kDa and 2,190 kDa HA was applied to the SH-SY5Y cells, the basic respiratory rate, maximum respiratory rate, H⁺ proton leakage, ATP production, and SOD activity of the cells were significantly higher than in the control group (Fig. 2b–2f). This showed that our HA-NPs can enhance the respiration rate of neurons, increase their metabolic intensity, enhance the potential
energy conversion during cell respiration, increase the production of ATP in the process of neural respiration, and enhance the ability of neurons to resist oxidative damage.

The PINK1/Parkin pathway exerts a protective function by activating mitophagy-related signaling pathways that can promote the clearance of abnormal mitochondria [26]. The PINK1 protein is transported into the mitochondria and degraded in a normal state [24]. Under stress, the accumulation of PINK1 recruits and activates Parkin, a phosphorylated Ub (pUb)-mediated E3 ligase. Eventually, a large amount of ubiquitin accumulates on the OMM, causing the mitophagy to degrade the damaged mitochondria, thereby maintaining the health of the mitochondria within the cell [27–30]. In PD patients, mitophagy dysfunction is considered to be a cause of neuron death [31–33]. The mutation of genes encoding components of the mitophagy pathway increases the risk of PD.

We used PINK1-deficient cells to further verify the repairing effect of different molecular weights of HA on the damaged mitochondria of PD. We respectively treated MEF+/+ and MEF PINK1−/− cells with HA-NPs and studied the oxidative phosphorylation of cell energy metabolism. The results showed that the HA-NPs with 117 kDa, 1,280 kDa, and 2,190 kDa HA had reparative and protective effects on both MEF+/+ and MEF PINK1−/− cells (Fig. 3a).

We found that the HA-NPs with 1,280 kDa and 2,190 kDa HA resulted in a significant increase in the basic respiratory rate, maximum respiration rate, $H^+$ proton leakage, and ATP production of MEF PINK1−/− cells. Meanwhile, the basic respiratory rate, maximum respiratory rate, $H^+$ proton leakage and ATP production in MEF+/+ cells were significantly greater than those in MEF PINK1−/− cells (Fig. 3b–3e). These results indicate that HA can enhance the basic respiratory rate of PINK1 knockout cells, increase metabolic intensity, and enhance potential energy conversion and mitochondrial metabolic activity during cellular respiration. We also found that the proliferation rate of PINK1 knockout cells was lower than that of wildtype cells. Our HA-NPs could significantly promote the proliferation of PINK1 knockout cells compared to the control group (Fig. 3f, 3g). Fluorescence staining of RH123 showed that the mitochondrial morphology of HA-NP-treated PINK1 knockout cells had more integrity (Fig. 3h). Studies have shown that HA enhances cell proliferation, metastasis, and adhesion by binding to the cell surface receptor CD44. We found CD44 expression was significantly upregulated in both MEF+/+ and MEF PINK1−/− cells following treatment with HA-NPs (Fig. 3i, 3j).

The NPs selectively target the irreversibly damaged mitochondria

Firstly, we identified two human USP30 shRNA sequences (Fig. 4a) and found the corresponding mouse gene sequence fragments. Through comparison, we chose the shRNA #2 gene sequence. To verify the interference efficiency of siRNA, we transfected MEF+/+ cells with siRNA by nanoparticles. The results showed that the expression of USP30 was significantly downregulated after siRNA transfection (Fig. 4b), indicating that siRNA has a certain interference and can be used in subsequent experiments. In order to
find a way to efficiently graft siRNA onto nanoparticles, we designed three sets of experiments: the control group was directly mixed with chitosan solution. In method 1, siRNA was directly mixed with nanoparticles. In method 2, siRNA was mixed with HA-TPP solution first, then mixed with the CS solution. The electrophoresis results showed that the nanoparticles could successfully encapsulate the siRNA under both methods, but the grafting efficiency of method 1 was minimal (Fig. 4c). Therefore, the subsequent experiments used method 2 to graft siRNA.

Next, to validate the targeting of nanoparticles to the dysfunctional mitochondria of the abnormality in the PINK1/Parkin pathway, we treated MEF+/+, MEF PINK1−/− cells for 24 hours with CCCP to induce mitochondrial damage. Then, nanoparticles were added and subsequently incubated for 4 hours. The results showed that the nanoparticles and mitochondria appeared at the same position in MEF+/+ cells. The fluorescence intensity of nanoparticles in mitochondria-damaged cells was significantly higher than in the control group. However, in MEF PINK1−/− cells, the aggregation of nanoparticles in the mitochondria was observed. There was no significant difference in MEF PINK1−/− cells either treated with CCCP or not (Fig. 5a–5b). Strikingly, in mitochondria-damaged MEC+/+ cells, a large amount of PINK1 protein accumulated in the outer membrane of the mitochondria, which resulted in the accurate identification and binding of nanoparticles grafted with the PIKN1 antibody. However, the lack of PINK1 protein in MEF PINK1−/− cells resulted in a lack of nanoparticle target sites and failed to target damaged mitochondria. Thus, we conclude that the B6 peptide–HA-PINK1 antibody-CS-TPP nanoparticle can target damaged mitochondria.

We next injected three nanoparticles of HA-PINK1 antibody–CS-TPP, B6 peptide–HA-CS-TPP, and B6 peptide-HA-PINK1 antibody–CS-TPP into C57BL/C mice (200 µg, respectively). We studied the nanoparticle distribution via in vivo imaging and immunohistochemistry. We showed that the fluorescence intensity of the B6 peptide–HA-CS-TPP and B6 peptide–HA-PINK1 antibody–CS-TPP nanoparticles groups was significantly higher than that of the HA-PINK1 antibody–CS-TPP nanoparticle group. The first two types of nanoparticles aggregated in the brain; the B6 peptide guided this aggregation. At the same time, fluorescence was detected in the liver and kidneys in all groups, but not in the heart and spleen (Fig. 5c). The immunofluorescence results showed that the number of nanoparticles in the brain cells of mice injected with nanoparticles coated with B6 peptide was significantly higher than those without B6 peptide. The colocalization of nanoparticles and PINK1 protein showed that the grafting of the PINK1 antibody onto nanoparticles significantly enhanced the accumulation of nanoparticles in damaged mitochondria (Fig. 5d).

**NPs promote the mitophagy of irreversibly damaged mitochondria and rescue the mitochondrial function of neurons**

We studied whether the nanoparticles we obtained could repair cell damage in vivo and in vitro. We treated MEF+/+ cells with phosphate buffered saline (PBS), B6 peptide-HA-PINK1 antibody-CS-TPP
nanoparticles, and B6 peptide-HA-PINK1 antibody-CS-TPP-USP30 siRNA nanoparticles after CCCP treatment. The results showed that the intensity of RH123 was significantly reduced after CCCP treatment. After adding B6 peptide-HA-PINK1 antibody-CS-TPP nanoparticles, the intensity was still low and there was no significant difference compared to the control group. In the B6 peptide-HA-PINK1 antibody-CS-TPP-USP30 siRNA nanoparticle group, the intensity did not reach normal cell levels, but increased significantly compared to the other two groups (Fig. 6a). In addition, when compared with the non-CCCP group, the expression levels of USP30 were significantly downregulated in the other groups. Compared with the CCCP-inducing group, the expression of USP30 was significantly inhibited by NPs, in particular, the NPs grafted with USP30 siRNA (Fig. 6b).

Next, we established a Parkinson mouse model by intraperitoneally injecting MPTP. We tested the amount of USP30 expression in the brain tissue of PD model mice following the injection of PBS, B6 peptide-HA-PINK1 antibody-CS-TPP, B6 peptide-HA-CS-TPP-USP30 siRNA, and B6 peptide-HA-PINK1 antibody-CS-TPP-USP30 siRNA nanoparticles, respectively. We found that the USP30 expression in the brain of mice injected with the B6 peptide-HA-PINK1 antibody-CS-TPP nanoparticle was increased compared to the PBS group. In contrast, the USP30 expression levels in the brain tissues of the other two groups were significantly downregulated (Fig. 6c).

In our study, the expression of USP30 in the cells treated with B6 peptide-HA-CS-TPP-USP30 siRNA nanoparticles and the PINK1 antibody is greater than in cells without the grafted PINK1 antibody. This suggests that our NPs can selectively promote the progress of mitophagy.

In order to verify the repair of damaged mitochondria by NPs in neurons, we use SH-SY5Y cells treated with H$_2$O$_2$. Oxidative phosphorylation of respiratory metabolism increased after the treatment of 117 kDa and 1,280 kDa molecular weight HA NPs on SH-SY5Y cells (Fig. 7a). Furthermore, after the NPs were applied to SH-SY5Y cells, the basic respiratory rate, maximum respiratory rate, H$^+$ proton leakage, ATP production, and SOD activity of the cells were significantly higher than in the control group (Fig. 7b–7d). Thus, the findings show that our NPs can enhance the respiration rate of neurons, increase the metabolic intensity, enhance the potential energy conversion during cell respiration, increase the production of ATP in the process of neural respiration, and enhance the ability of neurons to resist oxidative damage.

**Discussion**

The late-onset characteristic and severity of PD require early diagnosis and targeting therapy. Many PD risk loci have been shown to be related to mitochondrial dysfunction caused by impaired mitochondrial homeostasis. For example, PD cases related to PRKN, PINK1 and LRRK2 show significant interference in mitochondrial-related pathways [2, 34–36]. The dysfunction caused by mitochondrial damage is a significant factor that contributes to the dopaminergic neuron death in PD [2–6].

Mitochondrial dysfunction is gradually accumulated and becoming severe over aging [14]. Therefore, we designed and developed NPs for the treatment of PD that can target different stages of mitochondrial
damage. For the mild and limited damaged mitochondria, our HA-NPs could protect the mitochondria from damage and rescue mitochondrial function by regulating the mitochondrial dynamics. The mechanism of our HA-NPs repairing mitochondria may be due to the improvement of mtDNA integrity and the promotion of mitochondrial biogenesis. Whereas there are evidences show that HA can improve mitochondrial function by improving mtDNA integrity in chondrocytes [16], and increase the gene expression of the mitochondrial biogenesis-related gene PGC-1α in PDMSCs [21]. HA may also enhance the biological occurrence and function of mitochondria by activating the P38 MAPK pathway [37], or perhaps it could enhance the mitochondrial network function by activating SIRT1 [38].

As for the irreversible damaged mitochondria, they can be cleaned by mitophagy driven by PINK1/Parkin pathway under normal conditions [39]. However, Parkin gene mutation is one of the most common causes of autosomal recessive PD [40]. It can lead to autosomal recessive inherited early-onset PD (AR-JP) [40], and about 50% of AR-JP cases are related to Parkin gene [41]. When the Parkin gene is mutated, the Parkin protein loses the function of ubiquitin ligase and cannot activate the autophagy function of the damaged mitochondria, resulting in excessive accumulation of the damaged mitochondria and leading to programmed cell death. Therefore, promoting mitophagy of the irreversible damaged mitochondria has become an important direction for PD's drug development. Although studies have shown that knocking down USP30 gene expression can promote mitophagy in PD models [23], this unselective clearance will also significantly enhance the mitophagy of healthy mitochondria with a high risk of energy starvation [22]. Thus, according to the feature that PINK1 protein accumulated on the surface of damaged mitochondria [42], we added PINK1 antibodies on our USP30 siRNA-based NPs for targeting the damaged mitochondria exclusively. And the results showed that our NPs with USP30 siRNA and PINK1 antibodies can selectively target and promote the clearance of damaged mitochondria both in vitro and in vivo.

Our results also showed that with the exception of neurons, our nanoparticles aggregated in other kinds of cells of the SNc, which indicates that the nanoparticles do not exhibit neuronal selection, but rather target the damaged mitochondria in all kinds of cells. However, neurons are integrated with supporting cells, such as astrocytes, in continuous material exchange and information exchange. Studies have shown that neuronal cells release damaged mitochondria into the astrocytes. During circulation, astrocytes also release healthy mitochondria to supply neuronal cells and maintain normal neuronal function [43].

Conclusions

We successfully designed and developed NPs for the treatment of PD that can target different stages of mitochondrial damage. Our HA-NPs with 2,190 kDa HA can protect the mitochondria and salvage the mitochondrial function of the mild and limited damage conditions in both SH-SY5Y cells and PINK1 knockout MEF cells. NPs with USP30 siRNA and PINK1 antibodies can selectively target and promote the clearance of irreversibly damaged mitochondria both in vitro and in vivo (Fig. 8). This strategy is expected
to overcome the problems associated with the excessive clearance of healthy mitochondria and has great potential for clinical applications. Our study provides a novel strategy for PD treatment.

**Abbreviations**

PD
Parkinson's disease

HA-NPs
Hyaluronic acid nanoparticles

PINK1
PTEN induced putative kinase 1

MEF
Mouse embryo fibroblast

USP30
Ubiquitin specific peptidase 30

DA
Dopamine

SNc
Substantia nigra compacta

mtDNA
Mitochondrial DNA

CCCP
Carbonyl cyanide 3-chlorophenylhydrazone

CS
Chitosan

TPP
Sodium tripolyphosphate

**Declarations**

**Ethics approval and consent to participate**

All animal experiments were approved and performed in accordance with the guidelines set forth by the Harbin Institute of Technology Committee on Animal Resources.

**Consent for publication**

Not applicable.

**Availability of data and materials**

Not applicable.
Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

W.T designed the project; Y.C was the major contributor in analyzing the data and writing the manuscript; L.Y and J.Z synthetized the NPs and performed the cell examination; L.Yao and J.Z established the mice PD model and performed the animal tests; H.Y helped writing the manuscript. All authors read and approved the final manuscript.

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**Figures**
Figure 1

Characterization of the B6 peptide–HA-PINK1 antibody–CS-TPP NPs. a) Schematic of the NP formation. b) Fourier transform infrared spectrometer detection for B6 peptide–HA grafting. c) The average particle size of the CS-TPP NPs. d) Zeta potential of CS-TPP NPs. e) Scanning electron microscopy characterization of the CS-TPP NPs. f) Preparation and characterization of B6 peptide–HA-PINK1 antibody–CS-TPP NPs. Left: PINK1 antibody grafting. Right: Nanoparticle size and distribution. g) Uptake
of nanoparticles by MEF cells. h) Survival rate of different cells following incubation with the nanoparticles.

Figure 2

HA repairs damaged mitochondria in neurons. a) Changes in the oxidative phosphorylation of respiration in SH-SY5Y cells treated with different molecular weights of HA. b) Basic respiratory rate of SH-SY5Y cells treated with different molecular weights of HA. c) Maximum respiration rate of SH-SY5Y cells treated with different molecular weights of HA. d) H+ proton leakage of SH-SY5Y cells with different molecular weights of HA. e) ATP production of SH-SY5Y cells treated with different molecular weights of HA. f) The ability of HA to scavenge oxygen radicals (n=3; *P<0.05; **P<0.01; ***P<0.001)
Figure 3

HA-NPs repair PINK1 knockout cells. a) Effect of HA-NPs with different molecular weights of HA on MEF+/+ and MEF PINK1-/- cell respiration oxidative phosphorylation. (Left: 117 kDa HA. Right: 1,280 kDa and 2,190 kDa HA). b) Basic respiratory rate of MEF+/+ and MEF PINK1-/- cells treated with HA-NPs. c) Maximum respiration rate of MEF+/+ and MEF PINK1-/- cells treated with HA-NPs. d) H+ proton leakage of MEF+/+ and MEF PINK1-/- cells with HA-NPs. e) ATP production of MEF+/+ and MEF PINK1-/- cells
treated with HA-NPs. f) Effect of HA-NPs with different molecular weights of HA on MEF+/+ cell proliferation. g) Effect of HA-NPs with different molecular weights of HA on MEF PINK1-/- cell proliferation. h) Potential of the mitochondrial membrane. i) CD44 expression of MEF+/+ cells. j) CD44 expression of MEF PINK1-/- (n=3; *P<0.05; **P<0.01; ***P<0.001).

Figure 4

Grafting and identification of USP30 siRNA. a) USP30 siRNA sequence. b) Grafting of siRNA under different methods. c) USP30 siRNA interference efficiency (n=3; **P<0.01).
Figure 5

Targeting of the B6 peptide–HA-PINK1 antibody–CS-TPP nanoparticles. a) Left: Localization of nanoparticles in MEF+/+ cells. Right: Relative fluorescence intensity of nanoparticles in MEF+/+ cells under two conditions (n=3; **P<0.01). b) Left: Localization of nanoparticles in MEF PINK1-/- cells. Right: Relative fluorescence intensity of nanoparticles in two MEF PINK1-/- cells under different conditions (n=3). c) Distribution of nanoparticles in different mouse tissues. d) Left: Injection of mouse brain slices
with different nanoparticles. Middle: Fluorescence intensity analysis of NPs in mouse brain slices injected with different nanoparticles (n=5; **P<0.01). Right: Colocalization analysis of different nanoparticles and PINK1 protein (n=5; *P<0.05).

Figure 6

Nanoparticles repair damaged cells both in vivo and in vitro. a) Left: Repair of MEF+/+ cell damage induced by CCCP by nanoparticles. Right: Quantitative analysis of the fluorescence intensity of RH123
staining under different treatment conditions (n=3; *P<0.05; **P<0.01; ***P<0.001). b) USP30 expression of MEF+/+ cells under different treatment conditions (n=3; *P<0.05; **P<0.01; ****P<0.0001). c) USP30 expression in the brain tissues of PD mice with different nanoparticles (n=3; *P<0.05; **P<0.01; ***P<0.001).

Figure 7

NPs repair damaged mitochondria in neurons. a) Changes in the oxidative phosphorylation of respiration in SH-SY5Y cells treated with different molecular weights of HA. b) Basic respiratory rate of SH-SY5Y cells treated with different molecular weights of HA. c) Maximum respiration rate of SH-SY5Y cells treated with different molecular weights of HA. d) H+ proton leakage of SH-SY5Y cells with different molecular weights of HA. e) ATP production of SH-SY5Y cells with different molecular weights of HA.
molecular weights of HA. e) ATP production of SH-SY5Y cells treated with different molecular weights of HA. (n=3; *P<0.05; **P<0.01; ***P<0.001).

Figure 8

Schematic of our nanoparticle system strategy for PD treatment.