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Mitophagy induced by nanoparticle–peptide conjugates enabling an alternative intracellular trafficking route

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ABSTRACT

The intracellular behaviors of nanoparticles are fundamentally important for the evaluation of their biosafety and the designs of nano carrier-assisted drug delivery with high therapeutic efficacy. It is still in a great need to discover how functionalized nanoparticles are transported inside the cells, for instance, in a complicated fashion of translocation between different types of cell organelles. Here we report a new understanding of the interactions between nanoparticles and cells by the development of polyoxometalates nanoparticle–peptide conjugates and investigation of their intracellular trafficking behaviors. The as-prepared nanoparticles are featured with a unique combination of fluorescence and high contrast for synchrotron X-ray-based imaging. Functional surface modification with peptides facilitates effective delivery of the nanoparticles onto the target organelle (mitochondria) and subsequent intracellular trafficking in a dynamic mode. Interestingly, our experimental results have revealed that autophagy of mitochondria (mitophagy) can be induced by NP-peptide as a cellular response for recycling the damaged organelles, through molecular mediation associated with the change of mitochondrial membrane potential. The biological effects induced by NP-peptide reciprocally affect the distribution patterns and fates of nanoparticles in the cell metabolism by providing an alternative route of intracellular trafficking. The new understanding of the mutual activities between nanoparticles and cells will enrich our approaches in the development of nanobiotechnology and nano-medicine for disease treatments.
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1. Introduction

The development of multifunctional nanoparticles promises versatile approaches for enhanced bio-imaging [1–3], nano carrier-assisted drug delivery [4,5], and theranostic treatment [6–8] in clinical applications. It is fundamentally critical to investigate the delivery mechanism of the nanoparticles crossing various biological barriers including cell membranes, their behaviors of intracellular trafficking, and their final fates thereof [9–11]. It has been demonstrated that peptide-conjugated nanoparticles can effectively be delivered onto the cell membrane containing targeted receptor proteins [12,13], internalized by cells [14], or further transported into the cell organelles such as nuclei [15,16] and mitochondria [17,18]. The mediation by the peptides at the nano-bio interfaces is believed to play a key role in deciding the distribution or intracellular trafficking behaviors of nanoparticles [14,19]. A selection of peptides with certain sequences of amino acids has been screened out successfully for those various purposes [14]. Among them, quantum dots (QD) conjugated with TAT peptides have been applied for intracellular trafficking studies, revealing that those nanoparticles were tethered in the inner surface of vesicles after endocytosis. The QD-loaded vesicles can actively be transported by molecular machines along microtubule tracks to an asymmetric perinuclear region, or pinched off toward outside from filopodia [20]. Researchers have also demonstrated that both the particle size and bioconjugation of TAT peptide are important in assisting mesoporous silica nanoparticles into cell nuclei for the cell organelle-targeted drug delivery [16]. However, little is known regarding how the nanoparticles are translocated from one type of cell organelles to another different type after their cellular

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internalization. It remains a great challenge to understand the interactions between the biological effects induced by nanoparticles and their metabolism accompanying the host cell responses during their intracellular trafficking.

Herein we report our new efforts in addressing this challenge by the development of polyoxometalates (POM) nanoparticles conjugated with peptides and investigation of their intracellular behaviors. Although Lanthanide-containing POMs exhibit excellent photoluminescent properties including tunable narrow-banded emission, large Stokes shift, long lifetime of the excited states, examples of POM-based nanomaterials in cell imaging are quite scarce because of the undesired quenching effect of fluorescence in aqueous solutions [21,22]. Therefore we have developed a facile approach to prepare POM nanoparticles with the improved fluorescent property. This approach is robust and characteristic of encapsulating nano-scale assembly of Eu/W containing polyoxometalates with in situ-polymerized acrylic acids (PAA), that is, EuW_{10}@PAA. Encapsulation of PAA stabilizes POM nanoparticles in aqueous solutions and enhances their fluorescence by reducing the quenching effect of water molecules [21]. Simultaneously it promotes biocompatibility of POM-based nanomaterials and provides enriched carboxyl groups for subsequent bioconjugation with a mitochondria-targeting peptide. These NP–peptide conjugates are not only fluorescent, but also effective as a contrast reagent contributed by the Eu and W elements for synchrotron X-ray-based cell imaging. We have demonstrated that they can be delivered to mitochondria with high efficiency, and then investigated the distribution and dynamic translocation of NP-peptide between different types of cell organelles. Interestingly, our experiments have revealed that mitophagy is a cellular response for the exposure of mitochondria to the accumulation of NP-peptide. This autophagic degradation is likely to be mediated by Parkin, a protein recruited due to the changes of mitochondria membrane potential. Reciprocally, these processing steps hosted by the cells have made important changes in the distribution landscape of nanoparticles through alternative intracellular trafficking. Therefore, the results suggest a new understanding of the interactions between how nanoparticles could induce biological effects, and how a cellular response could affect the final fate of nanoparticles by providing a different route to transport nanoparticles between distinct types of cell organelles.

2. Experimental section

2.1. Chemicals and reagents

All chemicals used in our experiment were analytical grade without further purification. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-Hydroxysuccinimide (NHS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich. The mitochondria-targeting peptide (purity 95%, Dmt-D-Arg-Phe-Lys-NH2; Dmt = 2′,6′-dimethyltyrosine) was purchased from GL Biochem Ltd. (Shanghai, China). Calcein-AM, PI, Mito Red, and Hoechst 33342 were purchased from Dojindo Laboratories (Kumamoto, Japan). LysoTracker® Deep Red was purchased from Life Technologies. Mitochondrial Membrane Potential Assay Kit containing JC-1 dye was purchased from Fanbo Biochemicals Co. Ltd. (Beijing, China). Glutaraldehyde and acrylic acid were purchased from Sinopharm Chemical Reagent Co, Ltd. (Shanghai, China). Cell culture medium (RPMI 1640) was obtained from Hyclone. Fetal bovine serum (FBS) was purchased from GIBCO. ATG5, LC3, and GAPDH antibodies were purchased from Cell Signaling Technology. Parkin antibody and P62 antibody were purchased from Abcam. FUNDC1 was purchased from Santa Cruz Biotechnology, Inc.

2.2. Preparation of EuW_{10}@PAA nanoparticles and NP–peptide conjugates

$Na_9EuW_{10}O_{36}\cdot 32H_2O$ was prepared as described by Yamase [Yamase et al., *Bull. Chem. Soc. Jpn.* 1993]. The synthesis of EuW_{10}@PAA nanoparticles was complete in three steps performed in one pot. First, EuW_{10} containing Polyoxometalates ($Na_9EuW_{10}O_{36}\cdot 32H_2O$, 100 mg) was dispersed into 4 mL acrylic acid with 5 mg ammonium persulfate (APS) as the initiator. Then deionized water was dropped into the mixture with vigorous stirring for 6 h at room temperature. Finally, the mixture was centrifuged (12000 rpm for 5 min) and washed with deionized water for three times to remove the residual acrylic acid and APS in the supernatant. The precipitate was further dialyzed using 4K dialysis membrane for one day to obtain purified EuW_{10}@PAA nanoparticles. Mitochondria targeting peptide was covalently conjugated onto carboxyl-functionalized EuW_{10}@PAA nanoparticles through the well-established EDC and NHS coupling strategy. Briefly, 0.1 mmol EDC and 0.25 mmol NHS were dissolved in 2 mL phosphate buffered saline (PBS) solution (pH 7.4) containing 2 mg EuW_{10}@PAA. The mixture was stirred at room temperature for 30 min to activate the carboxylic group of EuW_{10}@PAA. Subsequently, 0.01 mmol peptide was added to the above solution. The mixture was stirred for 24 h at room temperature. The EuW_{10} @PAA nanoparticles–peptide conjugates (NP-peptide) were collected by centrifuge at 14800 rpm for 5 min at 4 °C and washed with deionized water for 3 times. They were stored at 4 °C for future use.

2.3. Characterization of the nanoparticles and NP-peptide

The transmission electron microscopy (TEM) images were obtained using a Philips CM300 transmission electron microscope (acceleration voltage 200 kV). The size distribution and zeta potentials of nanoparticles were obtained by dynamic light scattering (DLS) with Zetasizer Nano ZS (ZEN3690, Malvern). The fluorescence spectra were obtained with the FluoroMax 4 luminescence spectrometer (HORIBA Jobin Yvon). NP and NP-peptide were deposited on the MALDI sample plate and then mixed with the matrix solution of CHCA (40% v/v, 1 μL). MALDI-TOF mass spectrometry was performed in positive reflection mode on a 5800 Proteomic Analyzer (Applied Biosystems, Framingham, MA, USA) with a Nd:YAG laser at 355 nm, a repetition rate of 200 Hz, and an acceleration voltage of 20 kV. External mass calibration was performed by using standard peptides from myoglobin digests.

2.4. Synchrotron-based X-ray microfluorescence (m-XRF)

The silicon nitride (Si_3N_4) window (1 × 1 mm^2, 200 nm thick) was used to support the cell for synchrotron-based X-ray fluorescence experiments. Then MCF-7 cells were incubated with NP-peptide at 37 °C in 5% CO_2 for 24 h and then washed by PBS for three times. After fixed by 2.5% glutaraldehyde, ethanol (50%–100% concentration gradient) was used for the dehydration series, and then dehydrated in vacuum overnight. Imaging of the elemental distribution of Eu and W inside the cells was carried out using Synchrotron-based X-ray microfluorescence at Beamline 15 U (BL15U), Shanghai Synchrotron Radiation Facility (SSRF), China.

2.5. Cell culture and microscopy imaging

MCF-7 cells from American Type Culture Collection (ATCC) were cultured in RPMI-1640 cell medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were grown at 37 °C and 5% CO_2. MCF-7 cells were plated in 35 mm glass-bottomed culture dishes and grown up till 60% confluency. Then the

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