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Supplementary data

of

Investigation of Intracellular trafficking Behavior of One-Dimensional Materials and Implications for Drug Delivery

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Experimental section

Materials

Aluminum (Al) foils with 0.30 mm thickness and >99.999% purity were purchased from Beijing Nonferrous Metal and Rare Earth Application Institute (Beijing, China). Sulfuric acid (H_2SO_4), acetic acid ($C_2H_4O_2$), phosphoric acid (H_3PO_4), copper(II) chloride ($CuCl_2$), ethanol (C_2H_5OH), hydrochloric acid (HCl), hydrogen peroxide (H_2O_2), perchloric acid ($HClO_4$),chromium trioxide (CrO_3), dimethyl sulphoxide (DMSO), acetone (C_3H_6O), $HgCl_2$ were purchased from Shenzhen Tianxiang Huabo Co., Ltd. (Shenzhen, China). Chloroquine (CQ), bovine serum albumin and 3-methyladenine (3-MA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 3-aminopropyltriethoxysilane (APTES) 3-aminopropyltriethoxysilane (APTES) were purchased from Alfa Aesar China. N-acryloxysuccinimide (NAS), acrylamide (AAm), N, N, N’, N’-tetramethylethylenediamine (TEMED), N, N-methylene bisacrylamide (BIS), ammonium persulphate (APS), and Fluorescein isothiocyanate (FITC) were obtained from Aladdin Industrial Co. LTD. (Shanghai, China). Antibodies against LC3, Arf-6, RhoA, Flotillin, Caveolin, Cdc42, P62, EEA1, Clathrin, were from Cell Signaling Technology. Lyso-Tracker Red and N-(3-Aminopropyl) methacrylamide hydrochloride were from Beyotime Biotechnology (Shanghai, China) and Polymer Science, Inc., respectively.

Plasmid and transfection

DsRed-Rab5 and DsRed-Rab7 were purchased from Addgene. The EGFP-LC3 and DsRed-LC3 plasmids were created in our own laboratory. And KSHV Flag-vBcl-2 plasmid was kindly provided by Professor Beth Levine from Department of Medicine, University of Texas. Rab family genes in the T Vector and sub-cloned into EGFP-C1 and DsRed-C1 were kindly provided by Professor Jiahuai Han’s Lab. All the plasmids were confirmed by automated DNA sequencing. And cells were transiently transfected with the plasmids by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Synthesis of AANTs

AANTs were synthesized using a modified pulse anodization approach under

galvanostatic mode.[1] Briefly, an aluminum foil with size of 7 cm^2 was firstly sonicated in EtOH for 15 min, washed with ultrapure water and then evaporated to dryness under nitrogen stream. Before anodization, aluminum foils were electropolished in a mixture of EtOH and $HClO_4$ 4:1 (v/v) at 20 V and 5 °C for 7 min. Then, to prepare the first NAA layer on the surface, the first step anodization was carried out in 0.3 M H_2SO_4 at 25 V under 6 °C for 23.5 h. The second step of pulse anodization, including mild anodization (MA) for 5 s with a current of 3 mA followed by hard anodization (HA) for 2 s with a current of 450 mA, was carried out under galvanostatic conditions at 1 °C. Area of the aluminum foils contacted to the electrolyte solution was 0.95 cm^2. The conditions were repeated 30 cycles. After that, the aluminum substrate residue was removed by wet chemical etching in a saturated $HgCl_2$ solution. Free-standing AANTs were obtained by immersing into 0.2 M $CuCl_2$ and 6.1 M HCl solution and gentle ultrasonication.

Functionalization of AANTs with fluorescein isothiocyanate (FITC)

AANTs were functionalized with FITC procedures similar to reference.[2] Briefly, free-standing AANTs were treated with boiled hydrogen peroxide (30 wt%) for 15 min to add more hydroxyl groups on its surface, followed by cooling down to room temperature. Then, the AANTs were washed with ultrapure water for three times. Next, the AANTs were reacted with 5 vol% APTES in absolute ethanol for 2 h under the stirring and then washed by acetone and ethanol repeatedly to remove unreacted APTES molecules. APTES-functionalized AANTs were immersed in 400 μL FITC/EtOH (2.5 mg ml^{-1}) for 1 h, and then washed repeatedly with EtOH. Finally the AANTs were stored in ultrapurewater till further use.

Characterization of AANTs

The shape and structure of AANTs were characterized by a scanning electron microscope (ZEISS, SUPRA-55, SAPPHIRE). Zeta potential was measured with a Malvern particle sizer Nano-ZS.

Cell culture

The HeLa cells were incubate in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS).

Cellular uptake of AANTs

Non-transfected or DsRed-Rab family gens were cultured with 1 mg/mL FITC-label AANTs at 37 ℃ for 4 h. Cells were incubated with Lyso-Tracker Red for 0.5 h for lysosome detection. After washing with PBS for three times, the cells were fixed with 4% paraformaldehyde for 10 min, stained with DAPI for 5 min and washed with PBS for three times. The Confocal microscopy was performed with a FLUO-VIEW laser scanning confocal microscope (Olympus, FV1000, Olympus Optical, Japan) under sequential scanning mode using a 60~100×objective.[3]

Autophagy assays

Cells were transfected with EGFP-LC3 under the conditions described above. Confocal microscopic images were used to calculate the EGFP-LC3 translocation. An anti-LC3 antibody was quantified by Level of LC3II protein.[4]

Immunoblotting

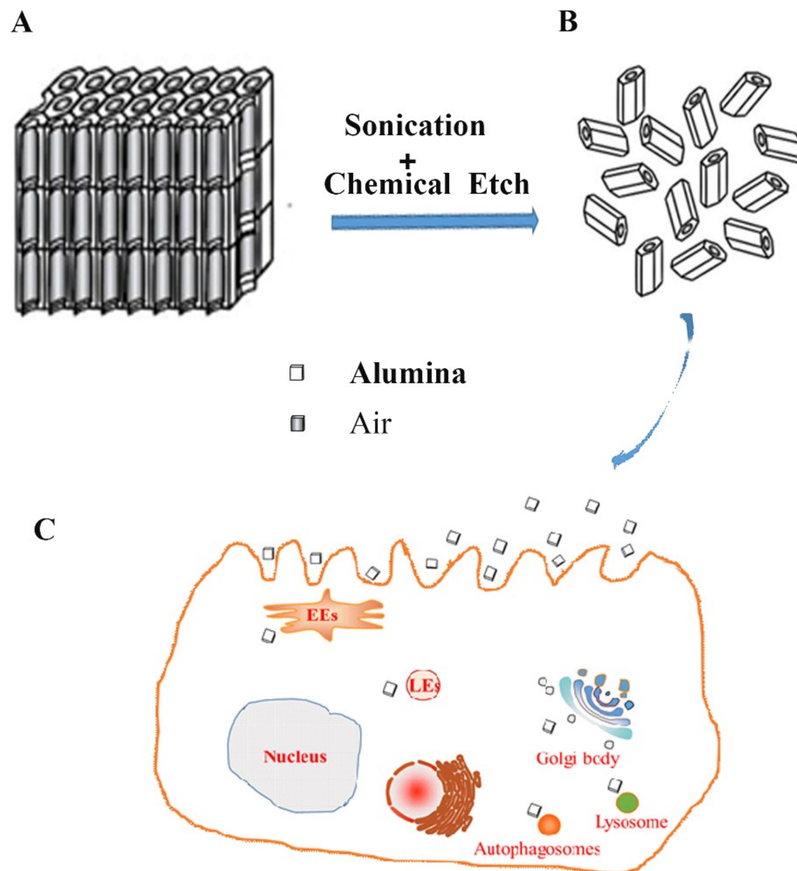
Immunoblotting analysis was similar to the reference.[5] In short, cell lysates were dissolved in 12% SDS-PAGE by immunoblotting with a LC3 antibody. An ECL detection system (Thermo Fisher Scientific, Schwerte, Germany) was used in this process.

Immunofluorescence assay

Primary antibodies, EEA1, P62, Caveolin, Cdc42, Clathrin, LC3, Flotillin, Arf-6, and RhoA were used to incubate the cells. the primary antibodies were detected by TRITC and FITC labeled secondary antibodies.

Statistical methodology

All results in the present study are reported as the mean ± S.D of three independent experiments. (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).



Scheme1. Schematic illustration showing fabrication of AANTs and the pathways of the AANTs in the cells. (A) Resulting nanoporous anodic alumina structure obtained after pulse anodization of aluminum foil. (B) Free-standing AANTs obtained after wet chemical etching and sonication. (C) Schematic representation of the degradation pathway of AANTs in cells.

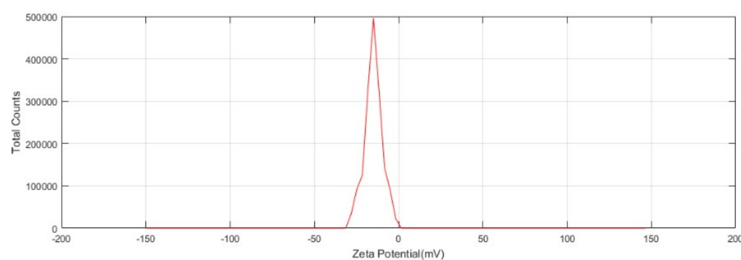


Figure S1. Zeta potential of AANTs

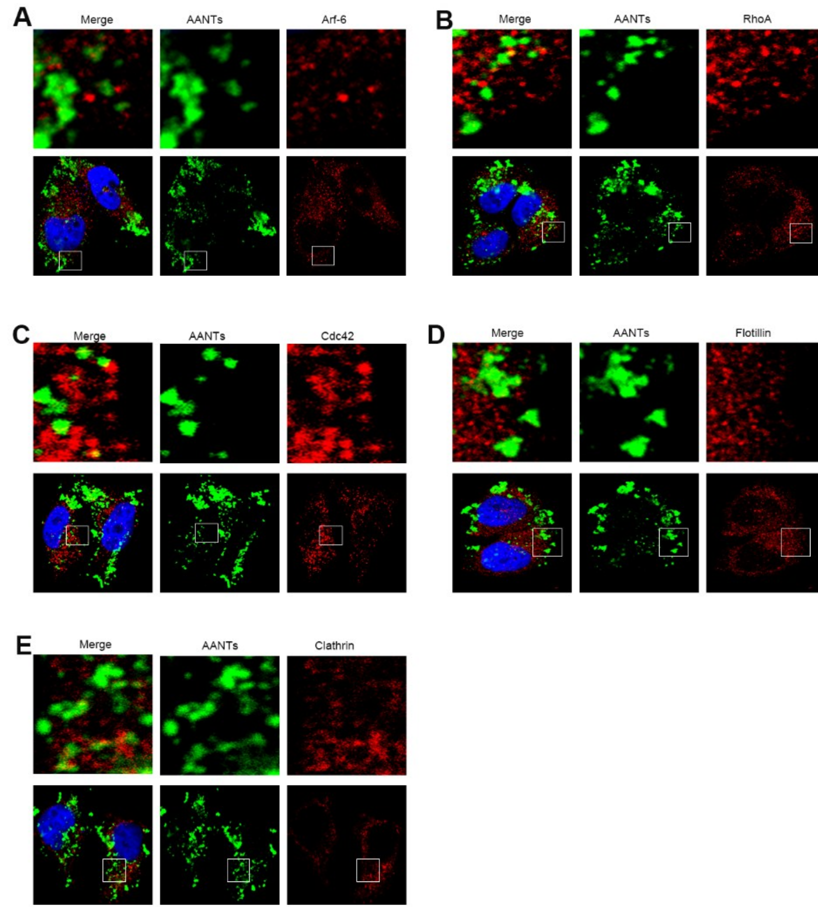


Figure S2. AANTs cannot enter cells through Arf-6, Clathrin, Flotillin, Cdc42, or RhoA endocytosis. Confocal images of HeLa cells, which were treated with 1 mg/mL FITC-labeled AANTs for 4 h. Arf-6, Clathrin, Flotillin, Cdc42, and RhoA were detected with respective specific primary antibodies. Scale bars: 10 μm.

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