Mitochondria-Targeted Near-Infrared Fluorescent Carbon Dots for Assessing ATP Levels in Cardiomyocytes under Exercise-Simulated Metabolic Stress

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Experiment

Characterization and instrument

Fluorescence spectrometer (Hitachi, Japan) was measured by record the fluorescence changes of Mit-CDs. The microstructure and lattice information of the Mit-CDs are obtained by the a Talos F200X electron microscope (FEI, Czech Republic). The X-ray powder diffraction (XRD) pattern of the Mit-CDs was obtained by the Bruker D8 Advance X-ray diffractometer (Bruker, Germany). Fourier Transform infrared spectroscopy 5700 infrared spectrum analyzer (Bruker, Germany) was used to record Fourier transform infrared (FTIR) spectrum. X-ray photoelectron spectroscopy (XPS) was collected on a PHI 5000 Versa Probe III spectrometer (ULVAC-PHI, Japan). The fluorescence lifetime was measured by an Edinburgh FLS1000 fluorescence spectrophotometer (Edinburgh, UK).

Synthesis of Mit-CDs

In a typical synthesis, 0.35 g *o*-Aminophenol and 0.5g 4-Formylbenzeneboronic acid were dissolved in 5mL H_2O and 10 mL DMF; after constant stirring, the resultant solution was successively transferred into a 25 mL poly(tetrafluoroethylene) liner stainless steel autoclave. After being heated at 180 °C for 10 hours and then Cooled down to room temperature. Subsequently, the Mit-CDs crude product was centrifuged at 12000 rpm for 10 min to remove large particles. The solution dialyzed with a 8000 Da dialysis bag for 72 h. Finally, the purified solution freeze-dried to obtain a brown powder and store in refrigerator (4 °C), in addition, Mit-CDs were simultaneously prepared as a stock solution at 10 mg mL^{-1} or further use.

Detection of ATP

The detection of ATP was performed at room temperature in an aqueous solution of Mit-CDs at pH 7.4 using PBS excited at 480 nm. Stock standard solutions of 300 mM ATP were prepared by dissolving an appropriate amount of ATP in aqueous and calibrating the volume to 150 mL in a volumetric flask. Other metal ion solutions were prepared in the same way before use. The fluorescent intensity of the Mit-CDs was recorded after different concentrations of ATP aqueous solution was added to the same

amount of Mit-CDs (10 mg/L) respectively and the mixed solutions were reaction for 5 min. To evaluate the selectivity of Mit-CDs, their optical responses to various metal ions and activity molecule. The fluorescence spectra were collected subsequently from 560 nm to 720 nm and the slit widths of the excitation and emission were both 2.5 nm and voltage as 680 V.

Detection of ATP in real samples

Human serum samples were thawed and centrifuged at 12,000 rpm for 10 min to remove impurities. A 10 μL aliquot of the supernatant was diluted 20-fold with PBS to minimize matrix effects. The diluted serum (10 μL) was mixed with 190 μL of Mit-CDs solution and incubated at 37 °C for 30 min. The fluorescence intensity was then recorded subsequently from 560 nm to 720 nm and the slit widths of the excitation and emission were both 2.5 nm and voltage as 680 V. All experiments involving human serum samples were performed in compliance with the relevant institutional guidelines and the principles outlined in the Declaration of Helsinki, and were approved by the Ethics Committee of Xi'an Medical University (No. XYLS2025353). Informed consent was obtained from all participants. No identifiable personal information was collected or used, and no diagnostic or clinical decision-making analysis was involved.

Cytotoxicity measurements

AC-16 cells were seeded in 96-well plates at a density of 5×10^2 cells/mL, with 200 μL of culture medium per well. After 24 hours of incubation, the cells were treated with various concentrations of Mit-CDs (0, 5, 10, 20, 30, 60, 90, 180 μg/mL) for an additional 24 hours. Following treatment, the culture medium was carefully aspirated, and 200 μL of MTT solution (500 μg/mL) was added to each well. The plates were then incubated for 4 hours at 37 °C. After incubation, the MTT solution was removed, and 150 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The plates were gently shaken at 80 rpm for 10 min, and the absorbance was measured at 490 nm using a microplate reader. Cell viability = (OD value in dosing group /OD value in control group) ×100% The absorbance of every well at 490 nm was measured with a microplate reader. AC16 human cardiomyocytes were obtained from Xiamen Immocell Biotechnology Co., Ltd.

ATP imaging

Fluorescent imaging in AC-16 Cells were performed in the similar procedures, AC-16 Cells were inoculated in a confocal dish. After the cells adhered, 60 μg/mL Mit-CDs were added, and incubated in a 37℃ for 20 min at 5% CO_2. After the incubation, the cells were carefully washed 3 times with PBS. In the ATP detection positive group, Then the cells were supplemented with 50 uM ATP in the growth medium for 30 min at 37°C and imaged. In the negative control group, metformin (100 μM) was pre-incubated for 2 hours as a ATP inhibitor prior to incubation with the carbon dot solution.

Co-localization imaging

AC-16 cells were co-stained with 40 μM Mit-CDs and an organelle dye (100 nM Mito-Tracker Green (Beyotime, C1048), 50 nM Lyso-Tracker Green (Beyotime) for 15min (37℃). After washing with PBS, fresh DMEM was added, and the cells were imaged with a confocal laser scanning microscope (CLSM). For fluorescence imaging of Mito-Tracker Green (MTG), Lyso-Tracker Green (LTG), λex/λem = 488/500−530 nm; Mit-CDs: λex/λem = 514/580−650 nm.

Statistical methods

All experiments were conducted in triplicate, and data are presented as mean ± standard deviation (SD).

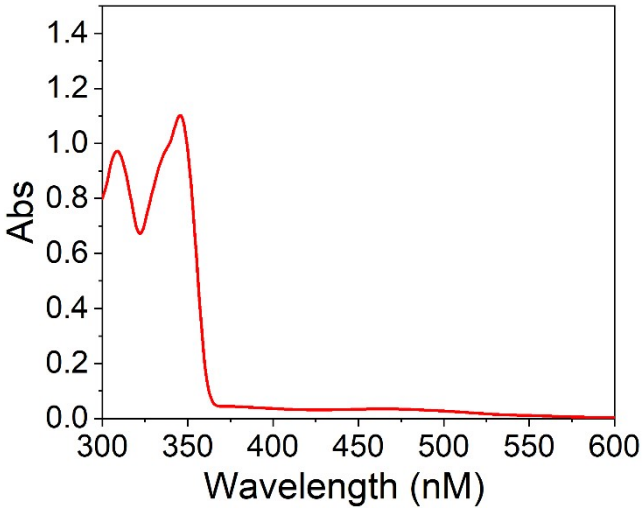

Figure S1 Uv-Vis spectrum of Mit-CDs

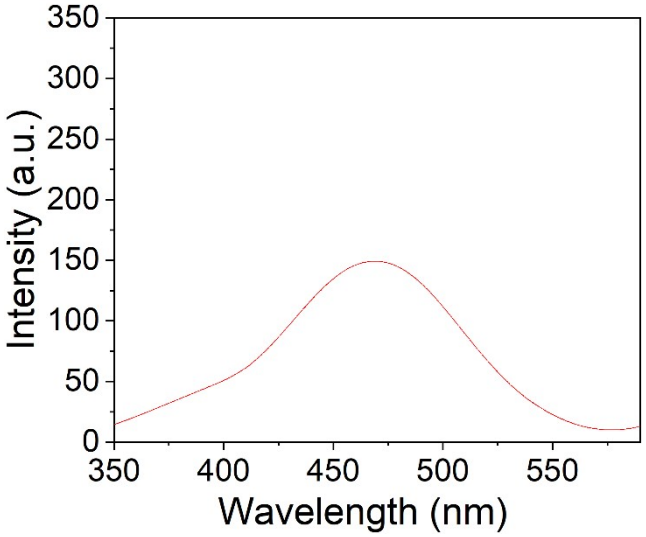

Figure S2 Emission excited spectrum of Mit-CDs

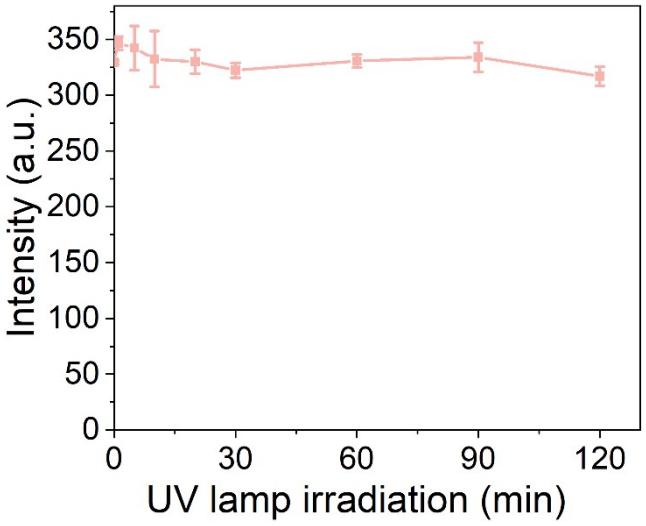

Figure S3 Fluorescent stability of Mit-CDs with different UV irradiation time.

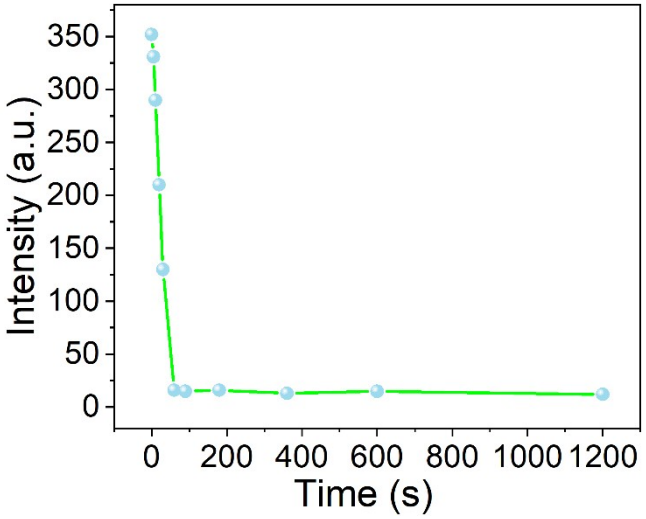

Figure S4 The reaction time as the best conditions for recognizing ATP.

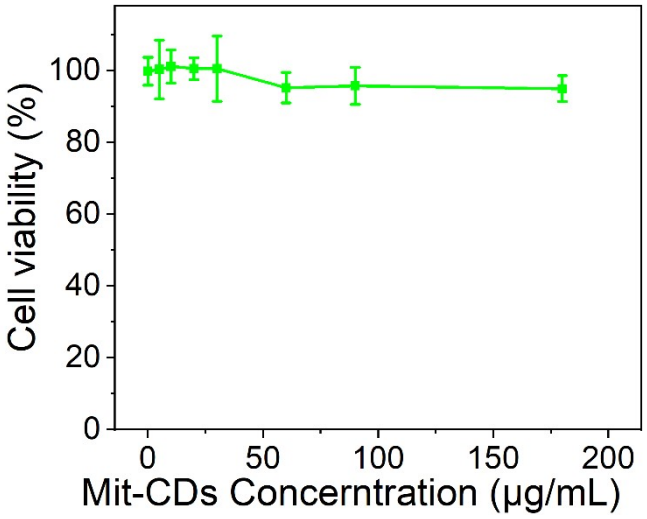

Figure S5 The MTT Curve of Mit-CDs.

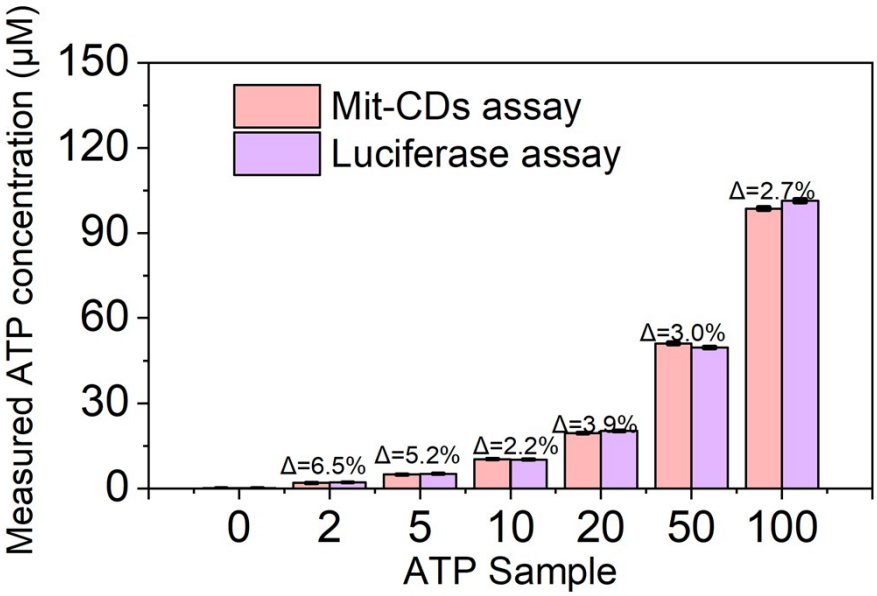

Figure S6 Validation of ATP detection by Mit-CDs using a luciferase-based ATP assay.

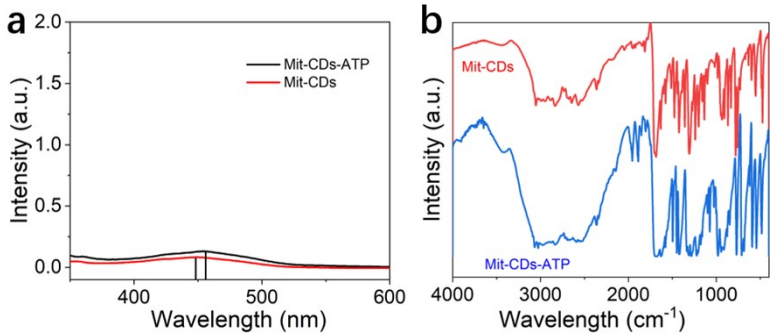

Figure S7 (a) UV-Vis measurements of Mit-CDs before and after ATP addition, (b) FT-IR of Mit-CDs in the absence and presence of ATP.

Table S1. Comparison of the analytical performance of Mit-CDs with other reported ATP probes

Probe	LOD	Specificity	Stability	Ref.
NIR-P	21 nM	Excellent	-	[1]
Fluorescein	0.5 μM	Good	-	[2]
CDs	-	Good	Excellent	[3]
Rh6G-ACFPN	4.72 mM	Moderate	Good	[4]
ROMP	27 nM	Excellent	Excellent	[5]
AD-3	0.01 mM	Moderate	Good	[6]
Mito-A	0.43 μM	Good	Good	[7]
DNA-M	142.6 pM	Excellent	Good	[8]
Zn-1/FBγCyD	18.9 nM	Moderate	Good	[9]
RhE-AuNC	74.9 μM	Moderate	Moderate	[10]
P1	20.80 nM	Good	Moderate	[11]
This article	**21.3 nM**	**Excellent**	**Excellent**	

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