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Biocompatible polydopamine platform for targeting delivery of nicotinamide mononucleotide and boosting NAD+ level in brain

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Materials and experimental section

Materials and reagents

Lactoferrin human (Lf), 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fluorescein isothiocyanate (FITC), 4',6-diamidino-2-phenylindole (DAPI), formaldehyde, nicotinamide mononucleotide (NMN) and Sulfo-Cy7 NHS ester reagents were purchased from Sigma Aldrich (USA). Dopamine hydrochloride, dimethyl sulfoxide (DMSO), ammonia and other chemicals were bought from Sinopharm Chemical Reagent Company (Shanghai, China). NAD/NADH kits were obtained from Abcam (Shanghai, China). Deionized water was 18 MΩ (Millipore, Billerica, Massachusetts). PC-12 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin pancreatin and phosphate buffer saline (PBS) were purchased from Gibco.

Instruments

Transmission electron microscopy (TEM) measurements were observed on a HITACHI H-7000FA (HITACHI, Tokyo, Japan). Scanning electron microscopy (SEM) images were taken with a JEOL JSM-6700F (JEOL, Tokyo, Japan). The UV-vis absorption spectra were measured with a Lambda 35 spectrometer (PerkinElmer, U.S.). Fourier transform infrared spectra were carried out with a Tensor 27 FTIR spectrophotometer. Confocal laser scanning microscope (CLSM) studies were observed using a Leica TCS SP8 microscope. The in vivo imaging of mice analysis was conducted using the PerkinElmer/IVIS Spectrum Small Animal Vivo 3D Optical Imaging System. Comprehensive mouse monitoring experiments were conducted using the

Complete Lab Animal Monitoring System (CLAMS, Columbus Instruments; Columbus, OH, USA).

SEM characterization

10 μL sample solution was held in a sample stage and kept at low temperatures for drying to avoid deformation. The sample was carefully loaded into the sample chamber of the SEM and the vacuum system was activated to remove the air. Finally, SEM images were taken with a JEOL JSM-6700F (JEOL, Tokyo, Japan).

TEM characterization

To prepare the TEM sample, a 10 μL sample was dropped onto a carbon-coated copper grid. After 10~15 min, filter paper was used to remove excess liquid. Finally, the sample was observed on a HITACHI H-7000FA (HITACHI, Tokyo, Japan) under the accelerating voltage of 200 kV.

FTIR characterization

The samples were oven-dried at 110 °C overnight and pulverized in an agate mortar before examination. Then a 0.20 mg aliquot of each powdered sample was mixed with 300 mg KBr in an agate mortar. FTIR spectra were carried out with a Tensor 27 FTIR spectrophotometer.

Preparation of polydopamine (PDA)

Ammonia (NH_4OH, 6 mL, 28-30%), ethanol (40 mL), and ultrapure water (90 mL) were mixed in a flask and stirred for 30 min at room temperature. Subsequently, dopamine hydrochloride (0.5 g) was completely dissolved in 10 mL of water, and this solution was added to the above mixture solution. The color of this solution transitioned from light brown to dark brown,

indicating the progress of the reaction. After 24 h, the final PDA samples were collected by dialyzing in ultrapure water for 12 h with a dialysis bag (molecular weight cut-off 1000 Da).

Preparation of FITC labeled PDA-Lf-NMN nanocomposite

Firstly, 2 mg of FITC was dissolved in 2 ml of DMSO. Then, 2 mL of PDA-Lf-NMN nanocomposite was added to the solution and stirred overnight under darkness. Finally, the FITC labeled PDA-Lf-NMN nanocomposite was obtained via centrifugation. FITC fluorescence signals were subsequently observed using CLSM (excitation at 488 nm).

Preparation of Sulfo-cy7-NHS ester labeled PDA-Lf-NMN nanocomposite (Cy7/PDA-Lf-NMN)

The Sulfo-cy7-NHS ester was dissolved in water to a concentration of 1 mg/mL. Then, 2 mL of PDA-Lf-NMN nanocomposite was added to the solution and stirred overnight under darkness. Finally, the Cy7/PDA-Lf-NMN nanocomposite was obtained via centrifugation.

Cell culture

PC-12 cells were incubated in a 5% CO_2 humidified incubator at 37°C. The culture medium was RPMI 1640 medium, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All reagents were dissolved in 1640 medium before their application.

Cellular uptake

PC-12 cells were seeded in 12-well plates at a density of 6×10^5 cells per well and allowed to incubate overnight. Then cells were incubated with FITC-labeled PDA-NMN-Lf (100 μg/mL) for 0.5 h, 1 h and 3 h, respectively. Besides, cells without any treatment were used as a control.

After the incubation, all cells were fixed with 4% formaldehyde for 10 min and stained with DAPI. FITC fluorescence signals were subsequently observed using CLSM.

Cell viability assay

PC-12 cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated for 24 h. The initial medium was discarded and replaced with fresh medium containing different concentrations of Lf, NMN and PDA-Lf-NMN (0, 1, 5, 20, 100, 200 μg/mL). The cells were further incubated at 37 °C for 24 h and treated with 20 μL of MTT (5 mg mL^{-1}). After 4 h incubation, 150 μL of DMSO was added. Finally, the optical density (OD) of each well was measured at 490 nm using a microplate reader. Cell viability was calculated according to the following formula:

$$\text{Cell viability} = \frac{(Ae - Ab)}{(Ac - Ab)} \times 100\%$$

Ae: Absorbance of the experimental group; Ab: Absorbance of the blank; Ac: Absorbance of the control group.

In vitro hemolysis assay

Blood samples stabilized by heparin sodium were obtained from C57BL/6 mice. Erythrocytes were isolated from the whole blood through centrifugation at 3000 rpm for 5 min and then washed with PBS. The erythrocyte suspension was diluted to a 1/5 concentration with PBS. The diluted erythrocytes (150 μL) were incubated with various concentrations of PDA-Lf-NMN (25, 50, 100, 200 and 300 μg/mL) for 4 h at 37°C. In parallel, a positive control was prepared by adding erythrocytes (150 μL) to H_2O, and a negative control was prepared by adding the same volume of erythrocytes to PBS. After incubation, all the mixtures were centrifuged at 8000 rpm

for 10 min to collect the supernatant. The absorbance of the supernatant at 570 nm was measured using UV-vis spectrophotometry. The Hemolysis percentage was calculated as follows:

Hemolysis percentage (%) = (sample absorbance - negative control absorbance) / (positive control absorbance - negative control absorbance) × 100%

Mice

Three-month-old C57BL/6 mice and Kun Ming (KM) mice were purchased from the Hubei Provincial Center for Disease Control and Prevention. The mice were housed under standard environmental conditions (temperature and humidity), with ad libitum access to food and water, and maintained on a 12-hour light/dark cycle. C57BL/6 mice had unrestricted access to high-fat chow or water and were allowed to age under these conditions until reached 21 months. All the animal experiments were approved by an ethical committee of Medical College, Wuhan University of Science and Technology (2023143). All ethical obligations were complied with.

In vivo imaging

For imaging and tissue distribution studies, the healthy KM mice and C57BL/6 mice were injected with labeled PDA-Lf-NMN via the tail vein. The non-injected mice served as the control. After anesthetizing using isoflurane by an animal anesthesia respiratory system (Shenzhen Reward Life Science Co., Ltd.), the signals in the mice's brains were monitored using an IVIS system (excitation/emission at 745/800 nm) and a positron emission tomography (PET) system.

Detection of NAD+ level

C57BL/6 mice (male, 3-month-old) received an injection of saline or PDA-Lf-NMN (4 mg/kg/day and 8 mg/kg/day) or NMN (400 mg/kg/day and 800 mg/kg/day) for 16 days. To

investigate the NAD+ level in the brain, all mice were anesthetized using isoflurane by an animal anesthesia respiratory system (Shenzhen Reward Life Science Co., Ltd.). Then their hippocampal tissues were collected for analysis by the NAD/NADH kit. Hippocampus samples were homogenized using a cryogenic homogenizer, and the resultant supernatant was collected post-centrifugation. All the procedures were carried out following the protocols provided by the NAD/NADH kit. To determine the effects of PDA-Lf-NMN and NMN supplementation on the NAD+ levels for old mice, HFD old mice (male, 21-month-old) were also treated with saline, PDA-Lf-NMN (8 mg/kg/day) or NMN (800 mg/kg/day) for 16 days under the same conditions. The mice were treated the same as above and subjected to detection of NAD+ level by NAD/NADH kits.

In vivo toxicity studies

C57BL/6 mice (male, 3-month-old) were administered an injection of saline, PDA-Lf-NMN (4 mg/kg/day and 8 mg/kg/day) or NMN (400 mg/kg/day and 800 mg/kg/day) for 16 days. Post-experiment, the mice were sacrificed and the blood was collected for analysis. Major organs (heart, liver, spleen, lung, and kidney) were harvested and stained with H&E to assess biosafety.

In vivo energy consumption studies

Experiments were conducted using a Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments; Columbus, OH, USA) under controlled temperature and light conditions (12 h light/dark cycle). Mice were individually housed in separate cages with unrestricted access to chow and water (n = 4). Rates of O_2 consumption (V_{O2}) and CO_2 production (V_{CO2}), recorded every 11 minutes for a total duration of 48 hours, were used to calculate the respiratory exchange

ratio (RER) values as $V_{CO2}/_{VO2}$. Activity levels were determined based on beam breaks, and food intake was measured gravimetrically.

Novel object recognition test

Firstly, the HFD old mice (male, 21-month-old) were treated with PDA-Lf-NMN (8 mg/kg/day) for 16 days to compare the effects of PDA-Lf-NMN supplementation on old mice by a novel object recognition test. Then, the HFD old mice (male, 21-month-old) were divided into three groups: the control group and two sample groups. The mice were administered with saline, PDA-Lf-NMN (8 mg/kg/day) and NMN (800 mg/kg/day) for 16 days, respectively. After the treatment, all mice were subjected to the novel object recognition test. The test was divided into a training phase and a trial phase, conducted in a square box measuring 25 × 25 cm at the base and 40 cm in height. The walls of the box were uniformly gray. During the training phase, two identical cubes, A and B, were placed on the floor of the box. Each mouse was introduced into the box with its back toward the two objects and allowed to explore the field for 10 min. After an hour, cube B was replaced with a cylindrical object C. The mice were then permitted to explore the enclosure for an additional 5 minutes. Throughout the experiment, the time spent exploring each object was recorded. Exploration was defined as the mouse aligning its nose with an object within a distance of ≤2 cm and/or touching the object with its nose. Sitting or climbing on the objects was not considered exploratory behavior. All objects used in the study were constructed from washable, odorless plastic. Data were collected and analyzed using VisuTrack Animal Behavior Analysis Software (model: XR-VT, Shinsoft, Shanghai). The percentage of time spent exploring the new object relative to the total time spent exploring both objects was used as a measure of new object recognition. The recognition index (RI) served as the main metric, calculated according to the following formula:

$$RI = \frac{\text{time with novel object C}}{\text{time with familiar object A} + \text{time with novel object C}}$$

Live subject statement

All experiments strictly complied with international and national laws and regulations on animal experiments to ensure the ethicality and legality of all in vivo experiments. The experimental protocol was approved by the Ethical Review Committee of Wuhan University of Science and Technology, China (Approval No. 2023143) prior to implementation, and conformed to the standards of the International Guide for the Care and Use of Laboratory Animals. All animal experiments followed the 3R principle (Replace, Reduce, Optimize) to minimize animal use and suffering. We are committed to conducting all experimental operations under professional supervision to ensure that the welfare and rights of laboratory animals are maximally respected and protected.

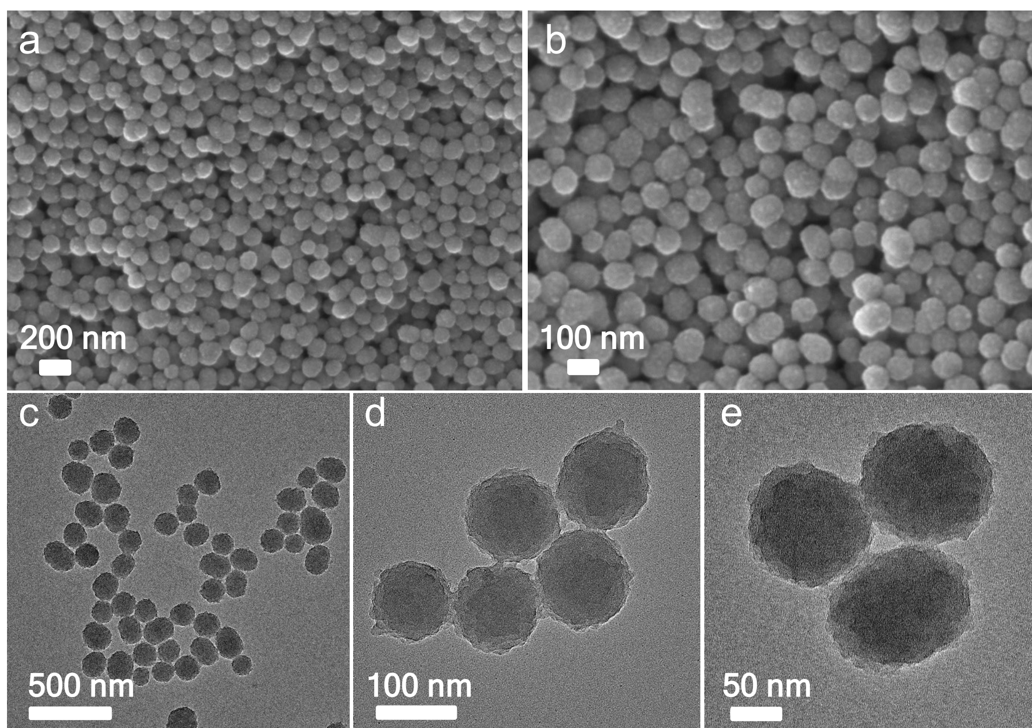

Figure S1. (a-b) SEM images of PDA. (c-e) TEM images of PDA.

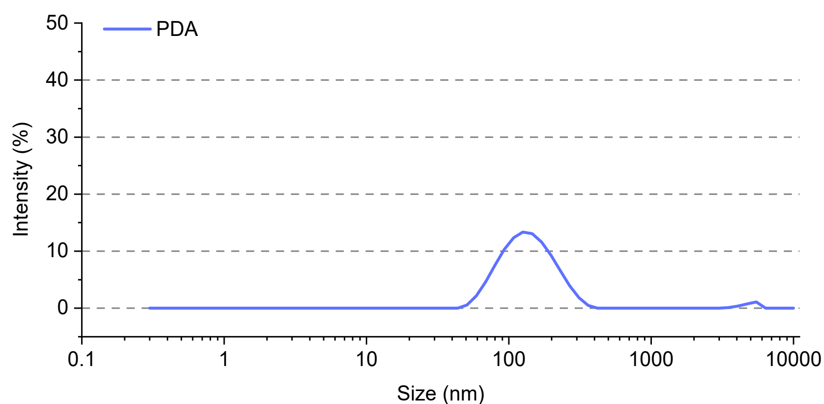

Figure S2. Dynamic light scattering (DLS) analysis of PDA.

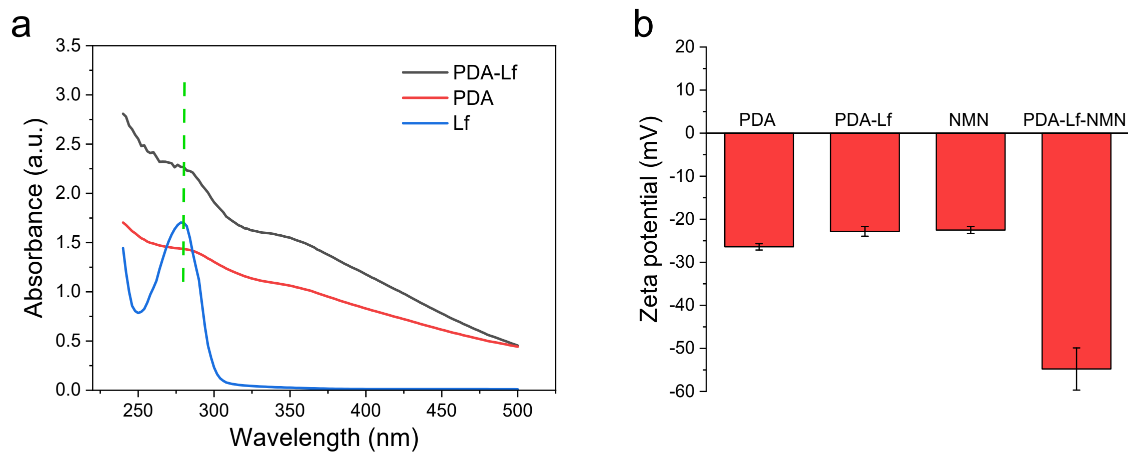

Figure S3. (a) UV-vis absorbance spectra of PDA, Lf and PDA-Lf. (b) The changes of zeta potential values of PDA, PDA-Lf, NMN, and PDA-Lf-NMN.

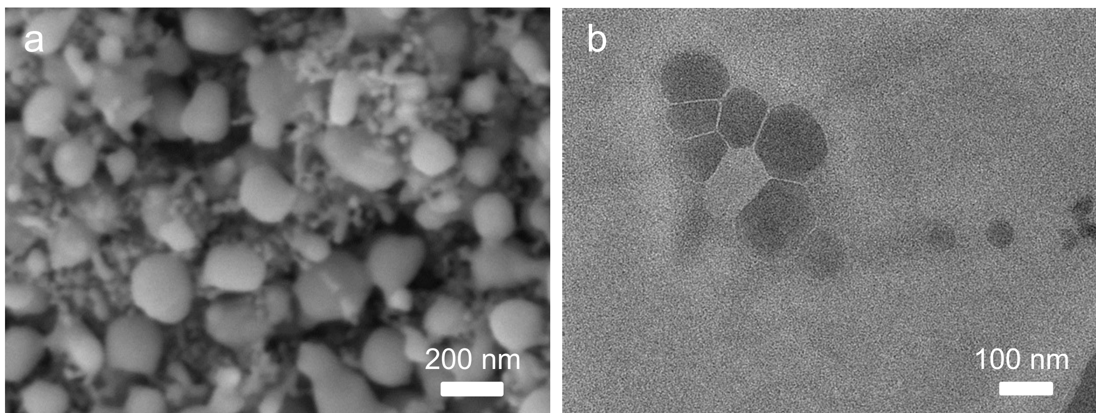

Figure S4. (a) SEM images and (b) TEM images of PDA-Lf-NMN nanocomposites.

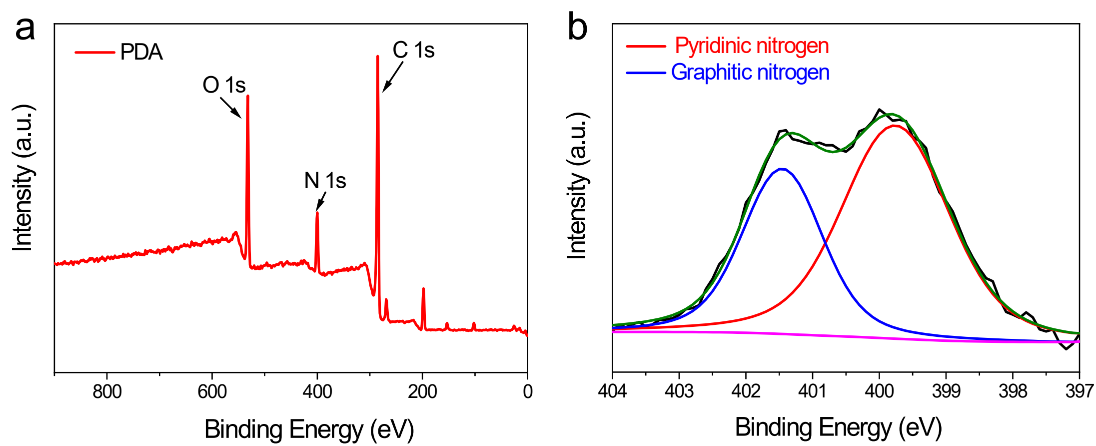

Figure S5. (a) XPS spectrum of PDA, (b) XPS spectrum of N 1s.

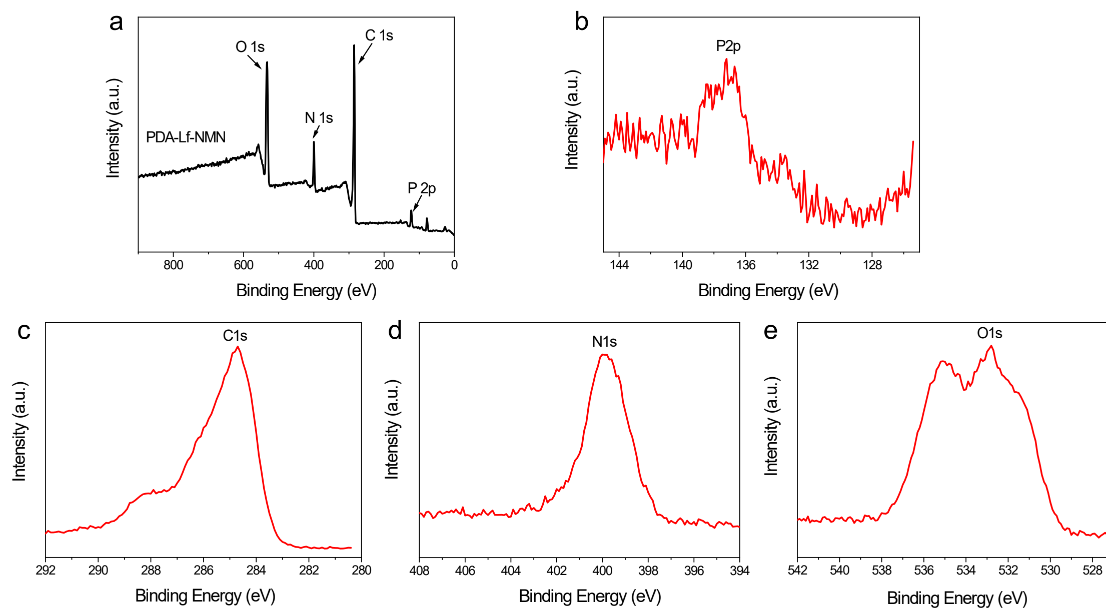

Figure S6. XPS spectra of PDA-Lf-NMN. (a) full survey spectra, (b) P 2p peak, (c) C 1s peak, (d) N 1s peak, (e) O 1s peak.

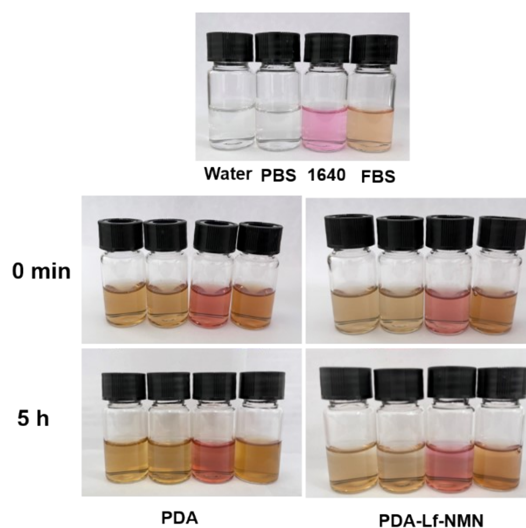

Figure S7. The colloidal stability of PDA and PDA-Lf-NMN in water, PBS, cell medium and fetal bovine serum (FBS).

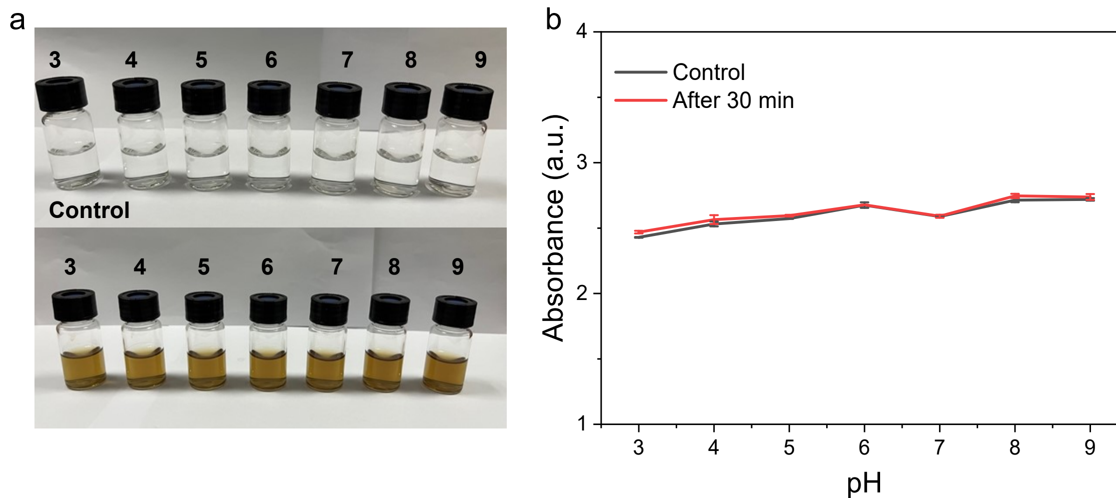

Figure S8. (a) The colloidal stability of PDA-Lf-NMN in different pH media. (b) UV-vis absorbance spectra of PDA-Lf-NMN at 260 nm in different pH media before and after 30 min.

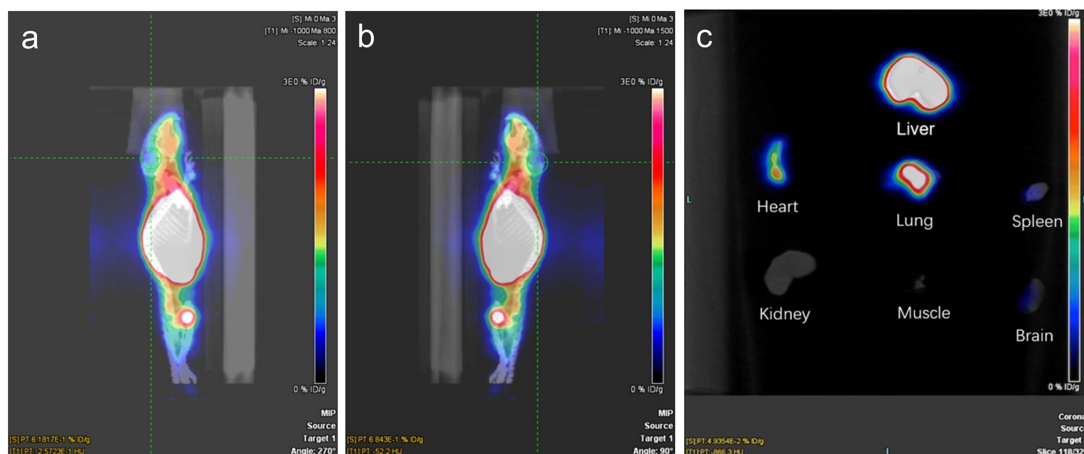

Figure S9. In vivo positron emission tomography (PET) imaging of (a, b) mice and (c) organs administrated with PDA-Lf-NMN.

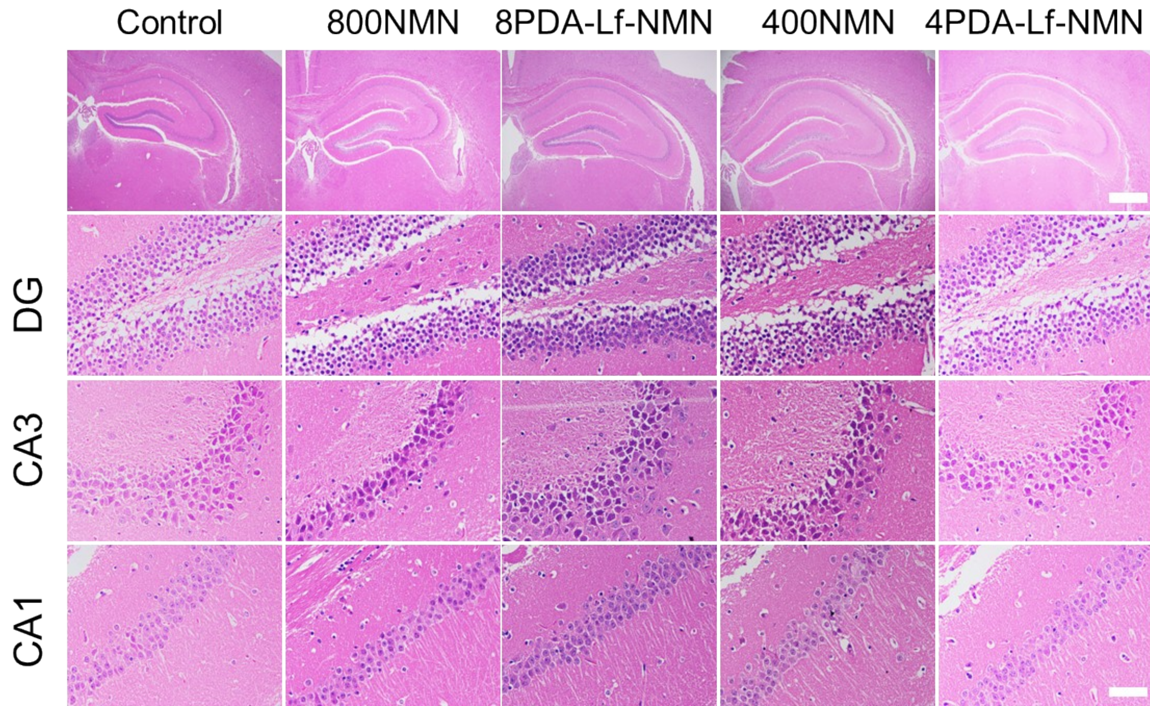

Figure S10. Histology H&E staining of the hippocampus for young mice (3 months) (scale = 50 μm, 500 μm).

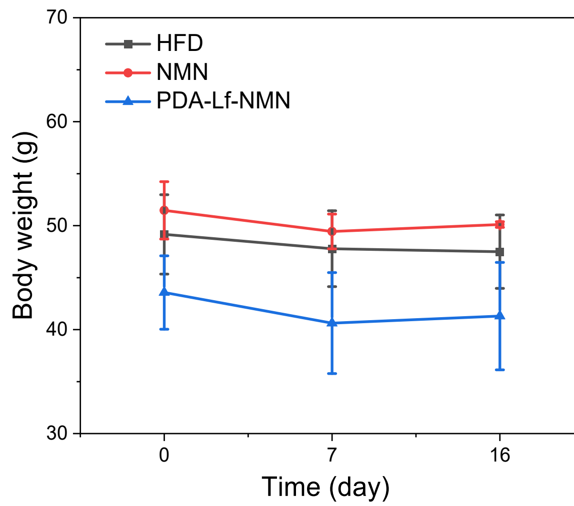

Figure S11. Effects of in vivo treatment with PDA-Lf-NMN and NMN on body weight.

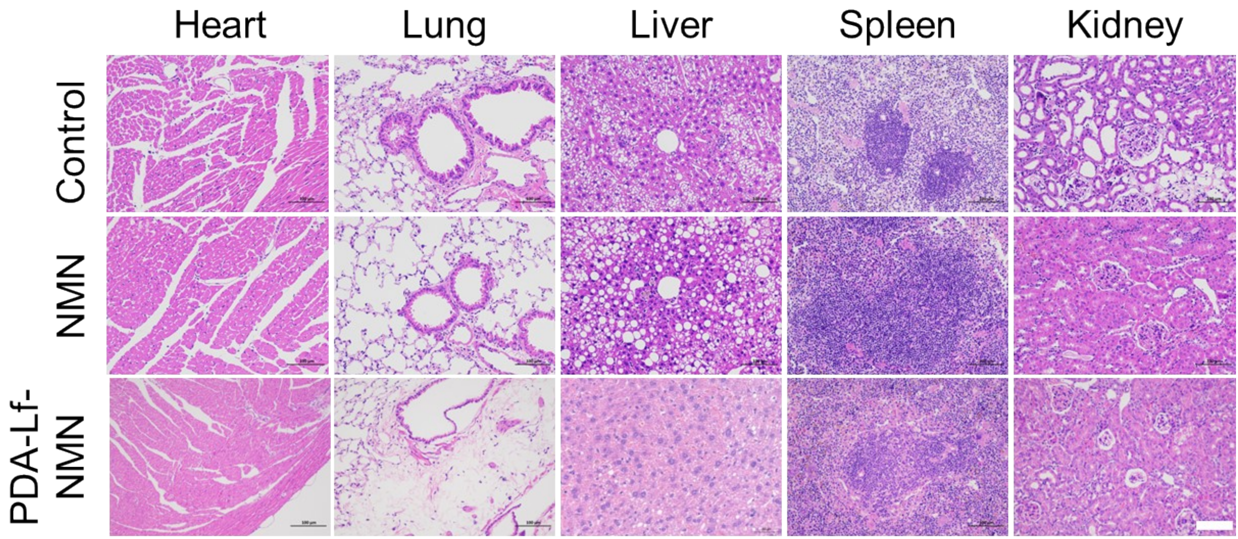

Figure S12. Histology H&E staining of heart, lung, liver, spleen, and kidney for HFD old mice (21 months) (scale = 100 μm).

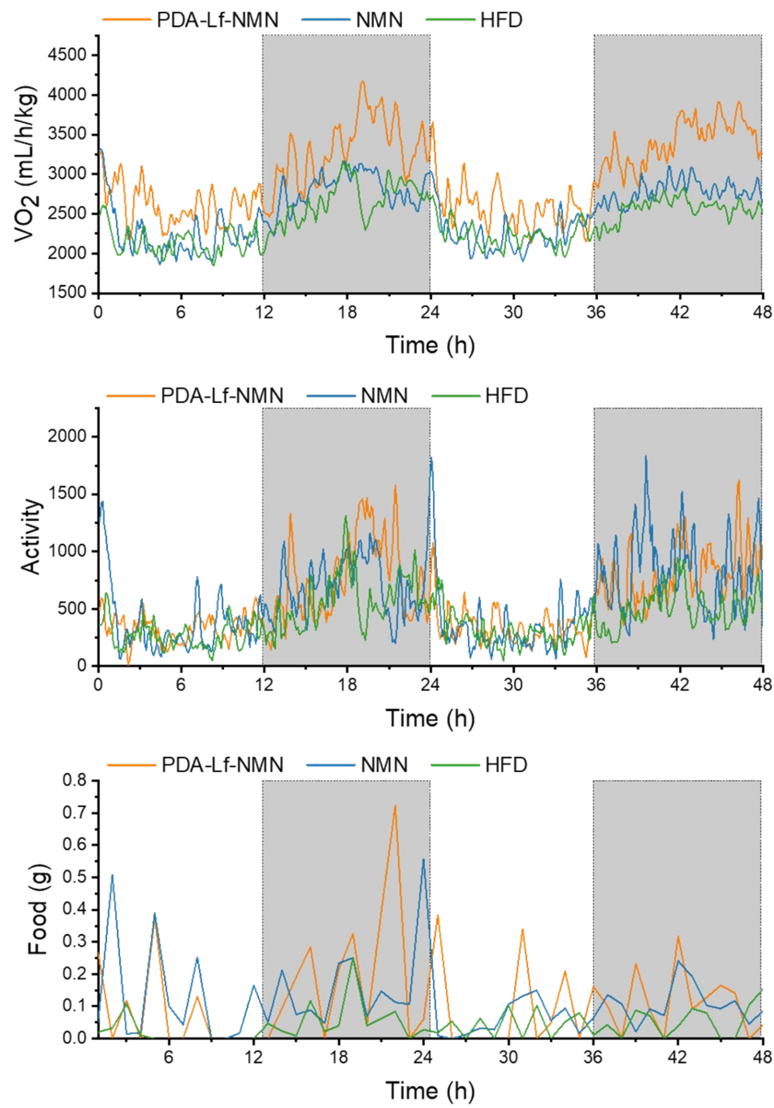

Figure S13. (a) Corresponding VO_2 (normalized to body weight), (b) activity and (c) food consumption from metabolic cages (n = 4, 3 days recording).

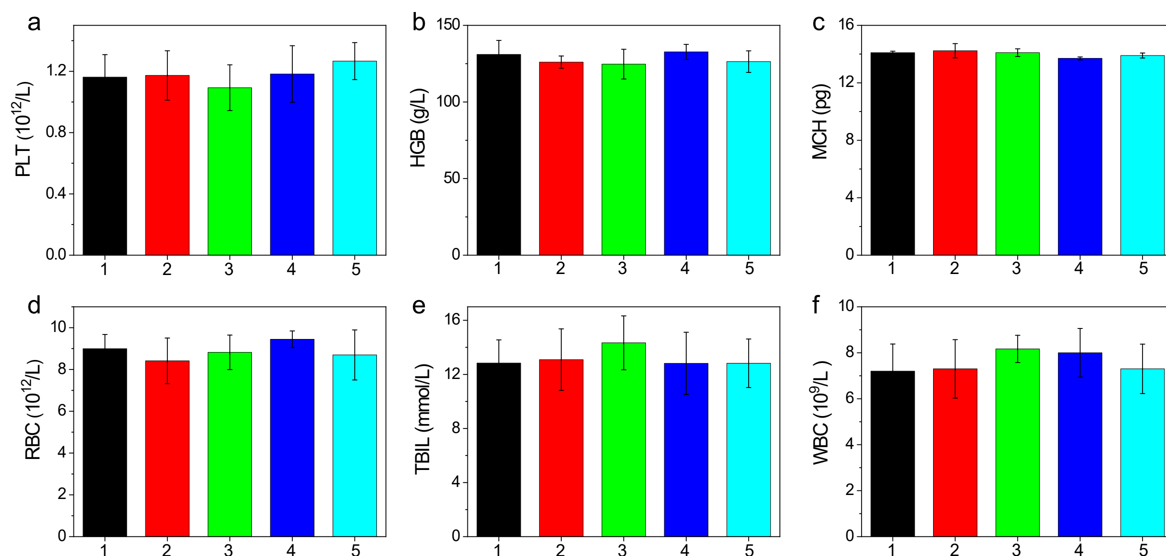

Figure S14. Blood biochemistry analysis of mice administrated with different formulas: (1) saline (as a control), (2) NMN (800 mg/kg/day), (3) PDA-Lf-NMN (8 mg/kg/day), (4) NMN (400 mg/kg/day), (5) PDA-Lf-NMN (4 mg/kg/day). Data are mean ± SD (n = 5).

Table S1. DLS measures, polydispersity index (PDI) values and zeta potential analysis of PDA.

	Average size (nm)	PDI	Zeta Potential (mv)
PDA	131.2±4.60	0.29±0.4	-26.41±0.74