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Supporting Information

Mesoporous polydopamine nanoparticles for sustained release of rapamycin and reactive oxygen species scavenging to synergistically accelerate neurogenesis after spinal cord injury

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1. Materials and methods

1.1 Chemicals

Pluronic F127, 1,3,5-trimethylbenzene were purchased from Sigma-Aldrich, USA. Tris(hydroxymethyl)aminomethane and dopamine hydrochloride were purchased from Sinopharm Chemical Reagent Co., Ltd, China. Rapamycin was obtained from Medchemexpress, New Jersey, USA. Primary antibodies including anti-GFAP antibody (ab7260), anti-Tuj1 antibody (ab7751), anti-NeuN antibody (ab177487) were purchased from Abcam, UK. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were gained from Gibco, USA. Cell counting kit-8 (CCK8) was purchased from Target Molecule Corp, USA. All other reagents were of analytical grade and were used as received if without a specific description. Water used in all experiments was purified by the Milli-Q system.

1.2 Synthesis and characterization of mPDA

The mPDA particles were prepared by a one-pot synthesis according to previous reported method [1]. Typically, 0.36 g of F127 and 0.36 g of 1,3,5-trimethylbenzene were first dissolved in a mixture of H_2O (65 mL) and ethanol (60 mL). After 30 min of stirring, a solution of 90 mg of tris(hydroxymethyl)aminomethane (TRIS) dissolved in 10 mL of H_2O was introduced to the mixture, followed by the addition of 60 mg of dopamine hydrochloride. The reaction mixture was stirred at room temperature for 24 h, and then the product particles were separated by centrifugation. Ethanol and acetone were used to wash the centrifuged particles. The template removal was performed by extraction, where the samples were treated in a mixed solution of ethanol and acetone (2:1 v/v) with sonication (three times, 30 min every time). The final product was suspended in ethanol for further use. Dynamic light scattering (DLS) measurements were performed to measure the hydrodynamic diameter of NPs using a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd, Malvern, UK). The morphology of NPs was characterized using transmission electron microscopy (TEM, HT7700, Hitachi, Tokyo, Japan).

1.3 Loading efficiency and drug release of mPDA@Rapa

Rapamycin (Rapa) was loaded into mPDA NPs. Firstly, to determine the adsorption amount of Rapa into mPDA NPs, 1 mL various concentrations of Rapa (200 to 1000 μg/mL) in THF were added to 4 mL 250 μg/mL mPDA solutions under stirring at room temperature for 12 h. After THF was volatilized, the mixtures were centrifuged to remove the unloaded rapamycin and washed with deionized water by centrifugation. Rapa was extracted by THF and quantified by using UV-visible spectroscopy (Shimadzu UV2550, Japan) at absorbance of 278 nm. The results were normalized with a calibration curve of free rapamycin.

To test the release profile of rapamycin, mPDA@Rapa NPs were suspended in phosphate-buffered saline (PBS) at 37°C under continuous stirring. At designed time points, the suspension was centrifuged at 10,000 × g for 30 min. A 2 mL aliquot of the supernatant was obtained and replaced with an equal volume of fresh PBS. The rapamycin concentrations in the supernatant were determined using UV-visible spectroscopy.

1.4 Free radical scavenging ability of mPDA and mPDA@Rapa

For H_2O_2 scavenging, a series of mPDA or mPDA@Rapa with different concentrations were incubated with 5 mg/mL 3,3',5,5'-tetramethylbenzidine, 100 mM H_2O_2 in 25 mM pH=6.5 HAc/NaAc buffer for 3 h. The ascorbic acid (AA) was set as positive control.

For •OH scavenging, different concentrations of mPDA or mPDA@Rapa were added into 25 mM pH=6.5 PBS buffer containing 5 mM p-phthalic acid (TA), 100 mM H_2O_2. TA was used as a probe, which could easily react with •OH to form a highly fluorescent product. After 12 h reaction, the fluorescence spectra of the samples were collected using fluorescence spectroscopy (PTI QM-40, USA) at Ex/Em = 320/425 nm. The AA was set as positive control.

For $O_2^{\bullet-}$ scavenging, all the procedure were followed by the manufacturer's instructions of total superoxide dismutase (SOD) assay kit with WST-1 (Beyotime Biotechnology, China). The SOD enzyme was set as positive control.

1.5 ROS scavenging ability and inflammatory cytokines and mTOR protein inhibition *in vitro*

Rat microglia cell line (HAPI) was used to verify the ROS scavenging effect of the mPDA. HAPI cells were purchased from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in the DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

To evaluate the ROS scavenging in vitro, HAPI cells were seeded onto 24-well plates at a density of 10^5 cells/well for 12 h. Then, cells were stimulated by 200 μM H_2O_2 and incubated with 100 μg/mL mPDA or mPDA@Rapa or 3.4 μg/mL Rapa (equivalent to the total amount of Rapa released in mPDA@Rapa) for 24 h. The cells were washed with PBS twice and then incubated with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) probe at 37°C for 30 min. DCFH-DA fluorescence in the cells was visualized by using a confocal fluorescence microscope (Leica TCS SPE, Leica, Germany). The cells without H_2O_2 treatment were utilized as a control. Similarly, cells were seeded onto 6-well plates at a density of 10^6 cells/well for 12 h. The same method was used to treat cells, and cells were collected for flow cytometry. Data were analyzed using the FlowJo software. The same method was used to measure the level of inflammatory cytokines and mTOR protein in cells by PCR method (SYBR Green QPCR system) according to manufacturer's protocol. Primers were shown in Table S2.

1.6 Cytotoxicity of mPDA@Rapa *in vitro*

CCK8 kit was applied to evaluate the HAPI cell viability after treated with mPDA or mPDA@Rapa. Cells were seeded onto 96-well plates (10^4 cells/well) for 12 h and then incubated with mPDA or mPDA@Rapa (0, 10, 20, 50, 100 and 200 μg/mL) for another 24 h. After washing the wells with PBS for 3 times, CCK8 solution was added and incubated for another 1.5 h at 37°C. Subsequently, the absorbance for each sample at 450 nm was measured using Microplate Reader (TECAN, Infinite M200).

1.7 *In vivo* evaluation of therapeutic efficiency of mPDA@Rapa

The animal experiments were approved by the ethics committee of the first affiliated hospital, College of Medicine, Zhejiang University (No. 20201066). Healthy female Sprague-Dawley (SD) rats (180-200 g) were supplied by Zhejiang Academy of Medical Sciences and maintained in a pathogen-free environment under controlled temperature (24°C). All the handling, maintenance, and procedures of animals were approved and carried out in accordance with the "Guidelines for Animal Experimentation" by the Institutional Animal Care and Use Committee, Zhejiang University.

To generate the animal model of SCI, an intraperitoneal injection of 10% chloral hydrate was used for anesthesia. After T7-T8 spinal cord was exposed, a metal rod (3 mm, 10 g) falling freely from 5 cm high hit onto spinal cord. SD rats with SCI were randomly divided into 4 groups. The needle was inserted about 2-3 mm from the lateral uninjured position between T7-T8 of the spinal cord to the injured position. After injected 10 μL of PBS, or 10 μL of 5 mg/mL mPDA or mPDA@Rapa, or 10 μL of 170 μg/mL Rapa, the wound was sewn back. SCI rats had to manually urinate three times a day, until the spinal cord was substantially repaired at 21 days post-injury. 3 rats in each group were used for histological analysis at 21 days post-injury. The spinal cords were obtained after heart perfusion 4% formaldehyde solution. After fixed with 4% formaldehyde 24 h under 4°C and embedded by paraffin, spinal cord tissues were cut into thickness of 15 μm slices for Nissl, and Masson's trichrome staining and 5 μm slices for immunofluorescence staining by vertical slices. The nuclei were visualized with DAPI. The spinal cord cavity was measured using Image J software.

1.8 Recovery of motor behavior

In order to evaluate the recovery of motor ability of rats, the behavioral scores were performed on day 1, 7, 14 and 21 after treatment according to the Basso Beattie & Bresnahan locomotor rating scale (BBB scale). Detailed scores are shown in Table S1.

1.9 Statistical analysis

The results are averaged from three parallel samples without specific description. Data are reported as mean ± SD. The differences among groups, for all the biological results, were determined using the one-way ANOVA analysis followed by Tukey's post hoc test. The significant difference was set at the $p < 0.05$ level, $p < 0.05$ as "*", $p < 0.01$ as "**", $p < 0.005$ as "***", $p < 0.001$ as "****".

Table S1. Basso Beattie & Bresnahan locomotor rating scale

Behavior	score
No observable hindlimb (HL) movement	0
Slight movement of one or two joints, usually the hip or knee	1
Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint	2
Extensive movement of two joints	3
Slight movement of all three joints of the HL	4
Slight movement of two joints and extensive movement of the third	5
Extensive movement of two joints and slight movement of the third	6
Extensive movement of all three joints of the HL	7
Sweeping with no weight support or plantar placement of the paw with no weight support	8
Plantar placement of the paw with weight support in stance only or occasional, frequent, or consistent weight supported dorsal stepping and no plantar stepping	9
Occasional weight supported plantar steps, no forelimb (FL)–HL coordination	10
Frequent weight supported plantar steps and no FL–HL coordination	11
Frequent weight supported plantar steps and occasional FL–HL coordination	12
Frequent weight supported plantar steps and frequent FL–HL coordination	13
Consistent weight supported plantar steps, consistent FL-HL coordination; and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance or frequent plantar stepping, consistent FL-HL coordination, and occasional dorsal stepping	14
Consistent plantar stepping and consistent FL-HL coordination; and no or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact	15
Consistent plantar stepping and consistent FL-HL coordination; and toe clearance during occurs frequently forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off	16

Consistent plantar stepping and consistent FL-HL coordination; and toe clearance during occurs frequently forward limb advancement; predominant paw position is parallel at initial contact and at lift off	17
Consistent plantar stepping and consistent FL-HL coordination; and toe clearance during occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off	18
Consistent plantar stepping and consistent FL-HL coordination; and toe clearance during occurs consistently forward limb advancement; predominant paw position is parallel at initial contact and lift off; and tail is down part or all of the time	19
Consistent plantar stepping and consistent FL-HL coordination; and toe clearance during occurs consistently forward limb advancement; predominant paw position is parallel at initial contact and lift off; and tail consistently up; and trunk instability	20
Consistent plantar stepping and consistent FL-HL coordination; and toe clearance during occurs consistently forward limb advancement; predominant paw position is parallel at initial contact and lift off; and tail consistently up	21

Table S2. Primers used for PCR.

Gene	Forward sequence	Reverse sequence
mTOR	5'-ACGCTGTCATCCCTTTATC-3'	5'-CTTCTTCTTCTCCCTGTAGTC-3'
TNF-α	5'-ATCTCACAGCAGCATCTC-3'	5'-TAGCAGGTCGTCATCATC-3'
IL-1β	5'-CTTCTCATTCCTGCTCGTGG-3'	5'-CTCCGCTTGGTGGTTTGC-3'
GAPDH	5'-GGAGTCTACTGGCGTCTTCAC-3'	5'-ATGAGCCCTTCCACGATGC-3'

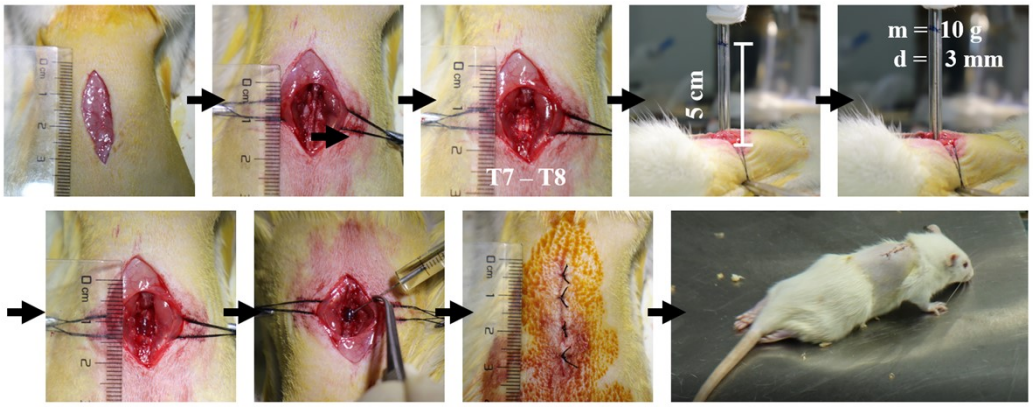

Figure S1 Scheme of animal experimental design. A metal rod (m = 10 g, d = 3 mm) free fall to exposed T7-T8 spinal cord of rats from a height of 5 cm. Then,

mPDA@Rapa were intrathecally injected within 1 min after weight drop.

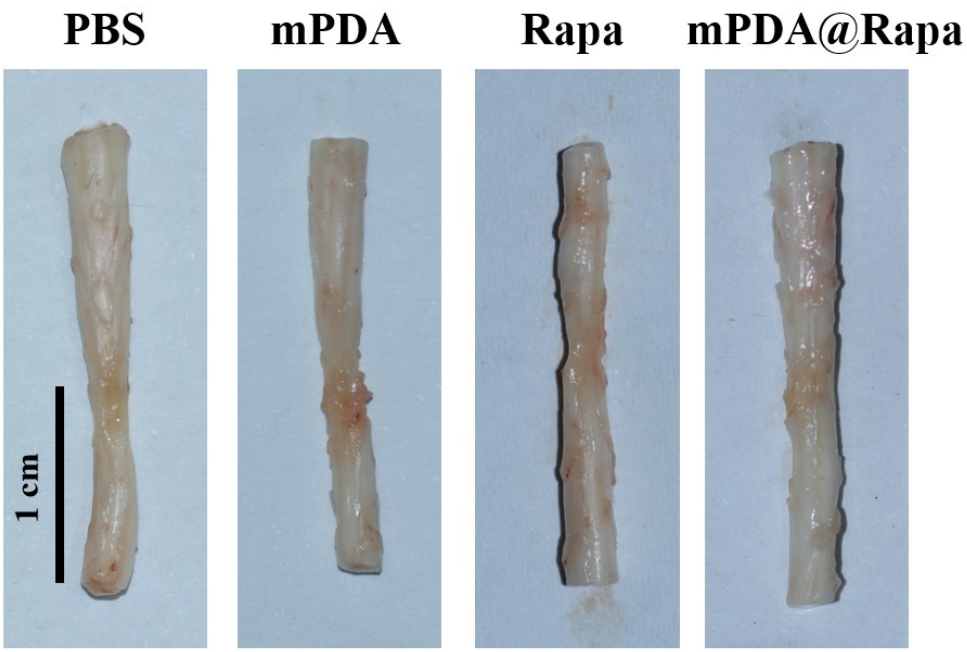

Figure S2 Typical macroscopic images of the entire spinal cord of PBS, mPDA, Rapa, mPDA@Rapa groups. Scar bar = 1 cm.

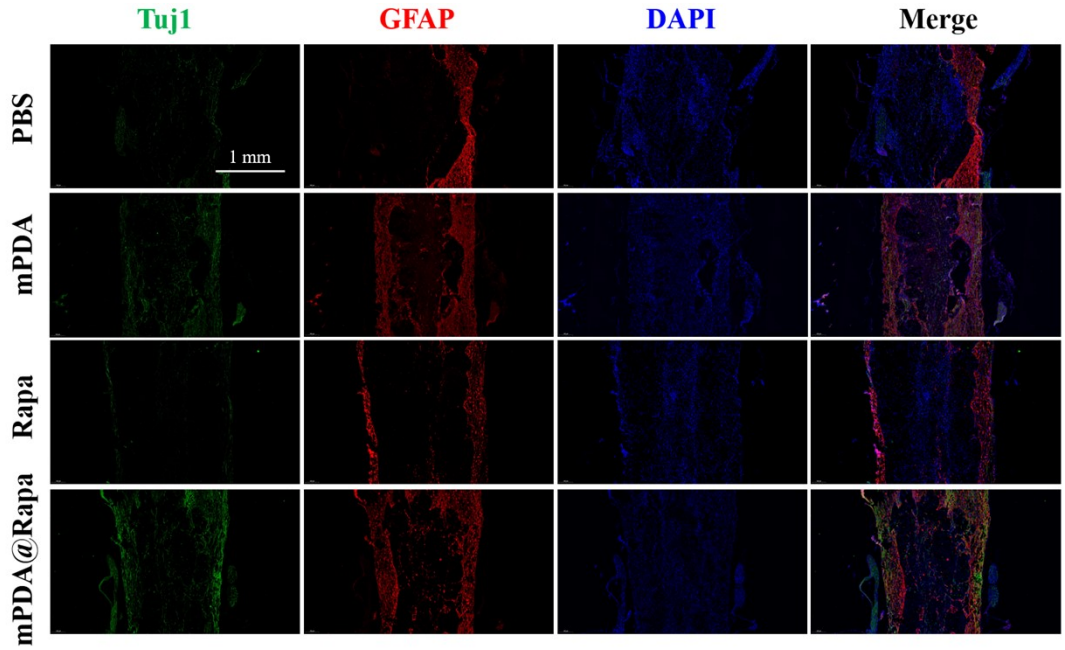

Figure S3 Representative immunohistochemical staining images of Tuj-1 (green), GFAP (red), and DAPI (blue) in the spinal cord tissues of rats treated with PBS, mPDA,

rapamycin and mPDA@Rapa at day 21 post surgery. Scale bar = 1 mm.

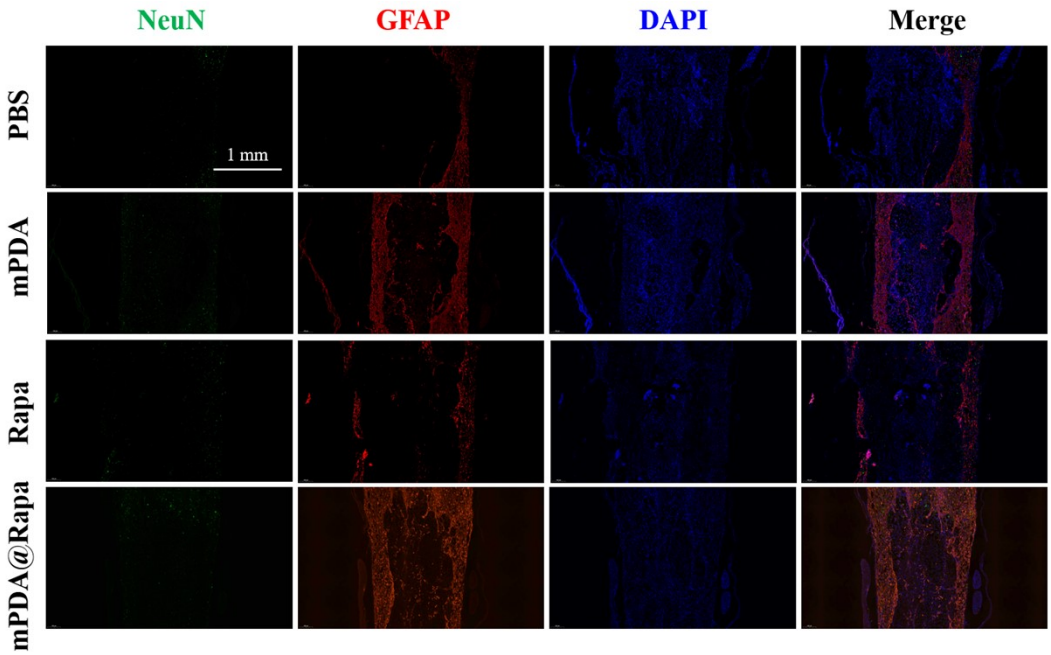

Figure S4 Representative immunohistochemical staining images of NeuN (green), GFAP (red), and DAPI (blue) in the spinal cord tissues of rats treated with PBS, mPDA, rapamycin and mPDA@Rapa at day 21 post surgery. Scale bar = 1 mm.

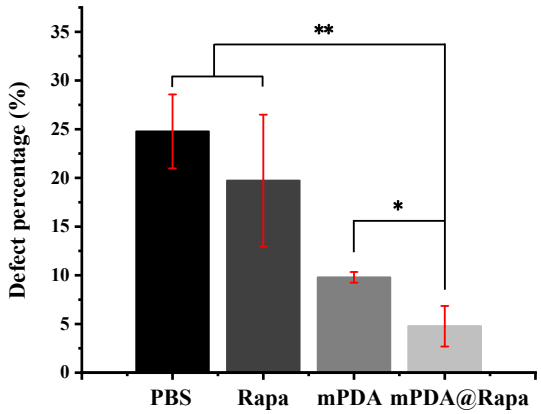

Figure S5 The spinal cord cavity (n=3). *p < 0.5, **p < 0.01.

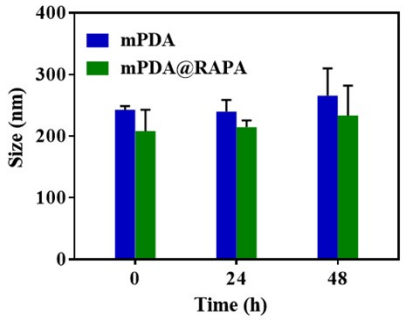

Figure S6 Hydrodynamic diameter of mPDA and mPDA@Rapa in PBS containing 5% bovine serum albumin at 0, 24, 48 h.

References

[1] F. Chen, Y. Xing, Z. Wang, X. Zheng, J. Zhang, K. Cai, Nanoscale Polydopamine (PDA) Meets pi-pi Interactions: An Interface-Directed Coassembly Approach for Mesoporous Nanoparticles, Langmuir 32(46) (2016) 12119-12128.