Supporting information

Conductive photothermal non-swelling nanocomposite hydrogel patch accelerating bone defect repair

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Characterization

The storage moduli (G′) of GM/Ac-CD/rGO hydrogels was evaluated by a time sweep test with 1% constant strain and a constant frequency of 10 rad/s at 37°C. 300 μL of GM/Ac-CD/rGO hydrogel was placed between 20 mm parallel plates.[1]

For the uniaxial compression stress-strain, GM/Ac-CD/rGO hydrogel samples were prepared into a cylindrical shape (~9 mm high × 1.2 mm in diameter). The test was investigated using a rheometer with a speed of 50 mm/min to determine the compressive properties of GM/Ac-CD/rGO hydrogels.

For cyclic compression stress strain test, GM/Ac-CD/rGO0.6 hydrogel samples were prepared into a cylindrical shape (~9 mm high × 1.2 mm in diameter). The test was investigated using a rheometer with a speed of 50 mm/min at a pre-determined strain (20%, 40%, and 60%) and then recovered to 0% strain. This cycle was repeated 10 times to determine the cyclic compressive and recovery properties of GM/Ac-CD/rGO hydrogels.

The swelling behavior of GM/Ac-CD/rGO hydrogels were determined by the gravimetric method.[2] The temperature was set at 37°C throughout the experiment to simulate body temperature. For the weight ratio tests of GM/Ac-CD/rGO hydrogels, the completely gelled wet hydrogel with the same volume was immersed in 20 mL PBS (0.01 M, pH 7.4) in sealed vials. The hydrogel samples were taken out from PBS solution at each pre-set time interval and a filter paper was used to remove the superficial water. Subsequently, the hydrogels were weighed. The weight ratio of the hydrogels was calculated using the following equation:

$$\text{Weight ratio} = W_t/W_0 \times 100\%$$

where W_0 and W_t represented the initial weight of the wet hydrogels and the weight after swelling pre-set time, respectively. The test was repeated three times.

The lyophilized GM/Ac-CD/rGO hydrogel samples were sprayed with a thin layer of gold on the surface and then the morphologies were observed by a field emission scanning electron microscope (FE-SEM; QUTAN FEG 250, FEI). The pore diameters of GM/Ac-CD/rGO hydrogels were calculated by employing NIH ImageJ software.

For the in vitro degradation evaluation of GM/Ac-CD/rGO hydrogels,[3] GM/Ac-CD/rGO hydrogel bulks, with the same volume, were put into 30 mL PBS solution at 37^{o}C with shaking at 100 rpm. At each pre-set time interval, the samples were taken out and rinsed with DI water to remove excess salinity. The hydrogel samples were lyophilized and the weights were recorded. The weight remaining ratio % of the hydrogels was defined by the following equation:

$$\text{Weight remaining ratio of hydrogel (\%)} = W_t/ W_0 \times 100\%$$

where W_t and W_0 are the lyophilized weight of the remaining hydrogels after degradation at different time points and the dry weight of the initial hydrogels, respectively. The test was repeated three times.

The conductivity of GM/Ac-CD/rGO hydrogels were measured at 25℃ by using a conventional four-point probe method (Agilent B2900A digital 4-probe tester).

For the photothermal test of the hydrogel, firstly, 300 μL of GM/Ac-CD/rGO hydrogel disks with a diameter of 10 mm were prepared in the mold. Then, the as-prepared hydrogel was exposed to a NIR laser (MDL-III-808 nm-1000 mW, Changchun New

Industries Optoelectronics Tech Co., Ltd.) with a power density of 2.4 or 2.8 W cm^{-2} for 10 min. The heat maps of the GM/Ac-CD/rGO hydrogels were recorded using an infrared (IR) thermal camera.

For the photothermal antibacterial test of the hydrogel,[4-6] 300 μL of GM/Ac-CD/rGO hydrogel disks with a diameter of 10 mm were prepared in the mold and then equilibrated with sterilized Dulbecco's phosphate buffered saline (DPBS). 10 μL of bacterial suspension in sterilized DPBS (10^6 CFU mL^{-1}) was added on the surface of the hydrogel disks. Then, the hydrogel was exposed to NIR laser light (808 nm, 2.8 W cm^{-2}) for 10 min, respectively. 10 μL of bacterial suspension (10^6 CFU mL^{-1}) was used as a negative control, which was also exposed to NIR laser light. After allowing all the groups to contact with bacteria for preset time, 990 μL of sterilized DPBS was added into each well to re-suspend any bacterial survivor. Then, 10 μL of the above bacterial survivor resuspension was added onto agar plate. The colony-forming units on the agar plate were counted after incubated for 18 to 24 h at 37°C. All the tests were carried out with 3 times of repetition.

Hemocompatibility test

For the hemocompatibility test of GM/Ac-CD/rGO hydrogels, the erythrocytes were obtained by centrifuging (1000 rpm) of the mouse blood for 10 min. PBS was used to wash the obtained erythrocytes for three times, and then the purified erythrocytes were further diluted to a final concentration of 5% (v/v). After that, 500 μL of the erythrocytes were mixed with 500 μL of GM/Ac-CD/rGO hydrogel in 24-well culture plate. After placed at 37°C for 1 h with a shaking speed of 100 rpm, all the microplate

well contents were centrifuged at 1000 rpm for 10 min to remove hemolysis-free erythrocytes. A volume of 100 μL of the supernatants was carefully transferred into a new 96-well clear plate. The absorbance of the solutions at 540 nm was read using a microplate reader (Molecular Devices). 0.1% Triton X-100 served as the positive control and DPBS served as the negative control.

Hemolysis ratio was calculated as the following equation：

$$\text{Hemolysis ratio (\%)} = (\text{ODt-ODn})/(\text{ODp-ODn}) \times 100\ \%$$

where ODt, ODn, and ODp were the absorbance values of samples, negative control (PBS) and positive control (Triton x-100), respectively. All the tests were carried out with 3 times of repetition.

Cell culture

The mouse preosteoblast cell line (MC3T3-E1 Subclone 14), purchased from Procell Life Science & Technology Co., Ltd, was used to study the bioactivity test of the hydrogel system. The MC3T3-E1 cells were maintained in a-minimum Eagle's medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (100 Uml^{-1}) and streptomycin (100 mg ml^{-1}) in a humidified atmosphere of 5% CO_2 at 37°C. The culture medium was refreshed every 2 days, and the cells were passaged at a ratio of 1:3 to 1:4 once reaching 90% density. To observe the proliferation and osteogenic differentiation of cells, MC3T3-E1 cells were seeded on the hydrogel in 24-well plate, which was sterilized under an ultraviolet lamp for 2 h before cell seeding.

Cell proliferation assay

The cell proliferation on the hydrogel was measured using a cell counting kit-8 (CCK-8)

following the manufacturer's protocol (Biosharp, Beijing, China). MC3T3-E1 cells were seeded on the hydrogels (GM/Ac-CD/rGO0, GM/Ac-CD/rGO0.2, GM/Ac-CD/rGO0.6 and GM/Ac-CD/rGO1.0) and the tissue culture plate (TCP) at a density of 1×10^4 cells/well, and After 1, 3, and 7 days of culture, the original culture was discarded, and 1 ml of 10% CCK-8 solution in a-MEM was added to each well (n=3). After incubated for 4 h, 300 ul of the culture medium from each well was transferred to 3 wells of a 96-well plate; each well had a volume of 100 ul, and the optical density (OD) values at 450 nm was measured in a microplate reader (Gene Company Limited).

Live/dead staining assay

In order to further observe the growth of cells on the hydrogel, the cells on GM/Ac-CD/rGO hydrogels were stained by a Calcein-AM/PI viability/cytotoxicity assay kit (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China) after 7 days of culture. And the status of the live/dead cells was observed by inverted fluorescence microscopy (Olympus CKX41, Tokyo, Japan).

Protein adsorption

Protein adsorption was performed by incubating the hydrogel films in phosphate buffered saline (PBS, 0.1 M, pH=7.4) containing 2.5% FBS. The films with dimensions of 8 mm were used. Before incubation in the medium containing FBS, the films were treated by ultraviolet light for 30 min and then washed by PBS three times under gentle shaking. Films were then put into 24-well culture plates and 1.5 mL FBS/PBS solution was added to each well. The specimens were incubated at 37°C for 20 h. The

concentration of the protein in the FBS/PBS solution was then quantified with BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China), using bovine serum albumin (BSA) standards. The amount of absorbed proteins was determined by subtracting the amount of proteins left in the FBS/PBS solution after adsorption from the amount of proteins in FBS/PBS solution (0 h) under the same incubation conditions in the same specimens.

The host inflammatory response tests

The biocompatibility was also assessed by the host inflammatory response tests. In brief, four groups (GM/Ac-CD/rGO0, GM/Ac-CD/rGO0.2, GM/Ac-CD/rGO0.6 and GM/Ac-CD/rGO1.0) of hydrogels were implanted subcutaneously in rats (3 in each group). After 7 days and 28 days, the rats were sacrificed, and the samples covered with hydrogels were removed, and then H&E and TB-staining were carried out.

Osteogenic characteristics in vitro

MC3T3-E1 were seeded on the hydrogels and a control group at a density of 2×10^4 cells/ mL in 24-well plate and incubated in a cell culture incubator (37°C, 5% CO_2 and saturated humidity). When 80% of the cells were confluent, the culture medium was replaced with an osteogenic medium, α-MEM containing 50 μg/mL of L-ascorbic acid, 10×10^{-3} M of β glycerophosphate, and 100×10^{-9} M of dexamethasone, to induce differentiation for a certain time. The medium was refreshed every 2 days. At each time point, the samples were collected for further osteogenic differentiation test.

ALP activity, staining and alizarin red S staining

The ALP activity of MC3T3-E1 cultured with the hydrogels for 7 and 14 days was

evaluated by the ALP kit and bicinchoninic acid (BCA) protein kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the instructions. Briefly, the MC3T3-E1 cells were lysed by Cell lysis buffer for Western and IP without inhibitors (Beyotime Institute of Biotechnology). The protein concentration was determined by the BCA protein assay and P-nitrophenyl phosphate was used to determine the ALP activity from the absorbance measured at 405 nm. The levels of ALP activity were normalized to the total protein content of MC3T3-E1. And ALP staining was also performed by BCIP/NBT Chromogen Kit (Solarbio, Beijing, China) following the instruction after co-culturing for 7 and 14 days in the control group and each hydrogel group. Mineralization was shown in the cells cultured on the hydrogels for 21 days using an Alizarin Red S (ARS) Staining Kit for Osteogenesis (Beyotime Institute of Biotechnology). The stained samples were photoed with a high-definition digital camera, and the ARS results were also observed in inverted microscope.

RT-PCR analysis

Real-time quantitative polymerase chain reaction (RT-PCR) was used to evaluate the effect of the hydrogels on the expression of osteogenic genes in MC3T3-E1 cells. Detailed experiments were carried out according to the standard protocol. The total RNA was extracted using the Trizol kit (Takara, American). The mRNA expression of MC3T3-E1 cell marker genes (ALP, OCN, Runx2, Col I) were detected using β-actin as internal reference. Primer sequences are shown in Table S2. 500 ng RNA was reverse transcribed by PrimeScript RT Master Mix (Takara, American). SYBR Premix Ex Taq II (2 ×) (Takara, American) was used as PCR reagent and 7500 Fast Real-Time PCR

System was used for quantitative PCR detection. All experiments were performed three times and analyzed using the 2-ΔΔCT method.

Western blot assay

The expressions of Runx2, Col I and OCN proteins were measured used Western blot after cells cultured with the hydrogels for 7 and 14 days. For Western blot analysis, cells were lysed with RIPA buffer (Beyotime Institute of Biotechnology). Briefly, proteins were separated by SDS-PAGE and then transferred to PVDF membranes (Millipore, USA) which were then blocked with skim milk (5%) in TBST for 1 h. Next, the membranes were incubated with β-actin, Runx2, Col I and OCN antibodies. After incubation with secondary antibodies, bound proteins were visualized by enhanced chemiluminescence (ECL, Millipore) and detected using a Tannon 5200 Multi system (Tannon). Blots were performed in triplicate, and band density was quantified by image J software.

Model of skull defect in rats

The skull defect model of rats with 8 mm diameter was established using the previously reported protocol. In brief, after anesthesia and shaving, the rats were fixed on the fixator, and then a 3 cm incision was made in the middle above the skull, and then the subcutaneous tissue and the periosteum was separated to both sides. Then an 8 mm defect was created by 8 mm trepanation, during which physiological saline was dropped around to cool down, and attention was paid to protect the meninges to avoid damage. The hydrogel films (diameter × thickness = 7 × 1 mm) were implanted in the defect area, and then the periosteum was sutured with absorbable thread. 50,000 units of

penicillin was injected intramuscularly to prevent postoperative infection up to 3 days after surgery.

Micro-CT analysis

The skull specimens were obtained and fixed in 10% formalin for a certain period of time. The three-dimensional (3D) structures of the regenerated bone tissue within the skull defect area were scanned using Micron X-ray 3D Imaging System (Y. Cheetah, Germany) at a resolution of 1.5 μm. The bone volume to total volume (BV/TV) and number of bone trabeculae (Tb.N) in the defects were analyzed by the VG Studio 2.1V.

Treatment of the specimens before TPLSM

Before TPLSM detection, the skulls were processed simply as follows: all soft tissues including periosteum were removed from the surface of the specimens. After that, the specimens were placed on a glass slide, and the bone defect repair area was covered with a glass of similar size, then placed in an examination box for further detecting.

Histomorphology and immunohistochemistry analysis

Histological tests were performed to analyze bone formation. The skull defect tissue specimens were decalcified and embedded in paraffin. Then the skull defect was cut into 5 μm thick sections from the cross section of the central area of each defect. Hematoxylin eosin (H&E) staining, Masson's trichromatic (MT) staining, and immunofluorescence staining (Runx2, Col I and OCN) were performed to observe the new bone regeneration. The slides were observed, and photographs were captured by a microscope. The bone formation areas for H&E and MT staining were evaluated quantitatively by Image J. And the average expression levels of Runx2, Col I and OCN

were calculated according to standard protocols.

Results and Discussion

Sample	GelMa mg/mL	AC-CD mg/mL	GO-CD mg/mL
GM/Ac-CD0	80	0	0
GM/Ac-CD6	80	60	0
GM/Ac-CD/rGO0	80	60	0
GM/Ac-CD/rGO0.2	80	60	0.2
GM/Ac-CD/rGO0.6	80	60	0.6
GM/Ac-CD/rGO1.0	80	60	1.0

Table S1. The detailed parameters of each GM/Ac-CD/rGO hydrogel.

Primer	Sequence (5′ to 3′)
ALP (F)	CGGCGTCCATGAGCAGAACTAC
ALP (R)	CAGGCACAGTGGTCAAGGTTGG
Runx2 (F)	GATGATGACACTGCCACCTCTGAC
Runx2 (R)	TGAGGGATGAAATGCTTGGGAACTG
OCN (F)	CAAGCAGGAGGGCAATAAGGTAGTG
OCN (R)	CATACTGGTCTGATAGCTCGTCACAAG
Col I (F)	GACAGGCGAACAAGGTGACAGAG
Col I (R)	CAGGAGAACCAGGAGAACCAGGAG

Table S2. Primer sequences of the genes

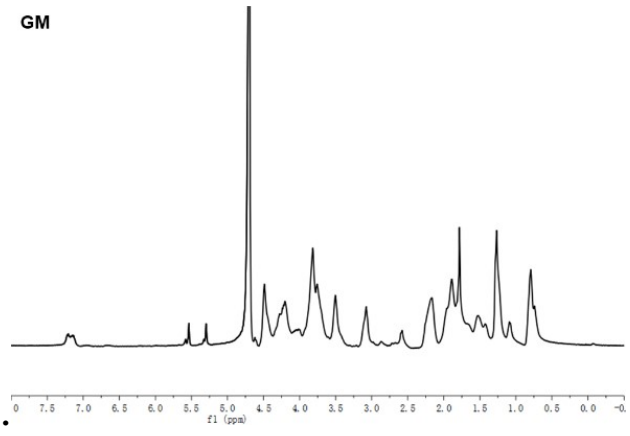

Figure S1. ^{1}H NMR spectra of GM

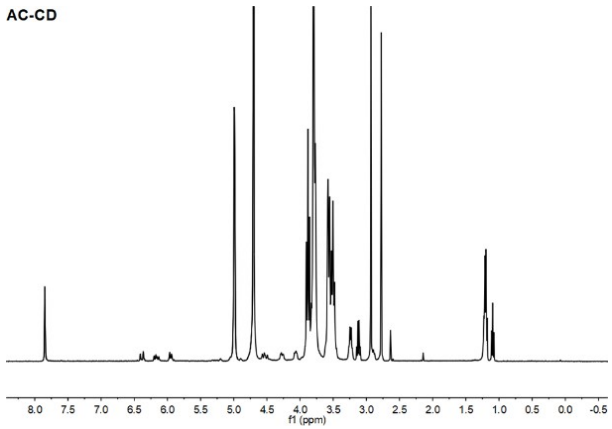

Figure S2. ^{1}H NMR spectra of Ac-CD

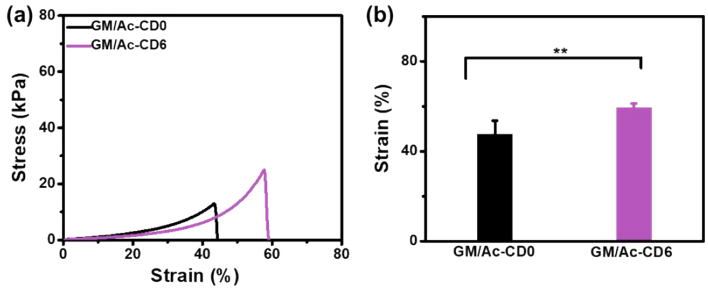

Figure S3. (a) The uniaxial compression stress–strain curves of GM/Ac-CD0 and GM/Ac-CD6 hydrogels; (b) the maximum compression strain of GM/Ac-CD0 and GM/Ac-CD6 hydrogels.

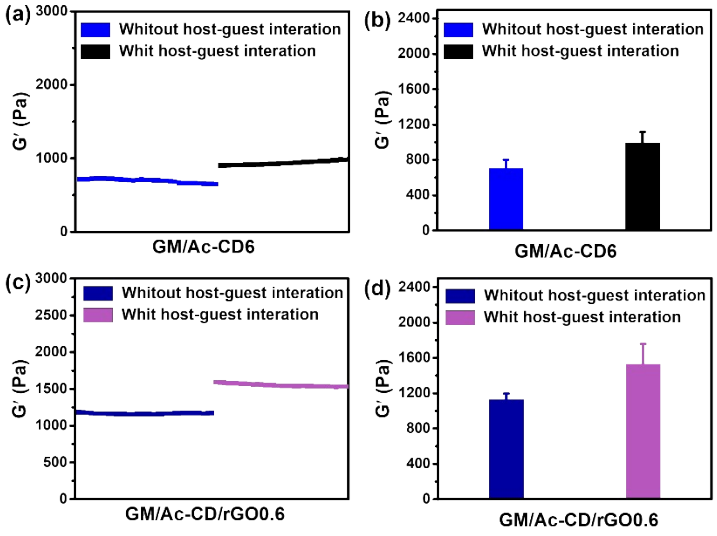

Figure S4. (a) The G′ of GM/Ac-CD6 hydrogel with or without host-guest interaction,

(b) the average G′ of GM/Ac-CD6 hydrogels with or without host-guest interaction; (c) the G′ of GM/Ac-CD/rGO0.6 hydrogel with or without host-guest interaction; (d) the average G′ of GM/Ac-CD/rGO0.6 hydrogels with or without host-guest interaction. "Without host-guest interaction" refers to adding APS/TEMED immediately after mixing GM and Ac-CD solution or GM, Ac-CD, and rGO precursor complex solution, "with host-guest interaction" means that GM and Ac-CD or GM, Ac-CD, and rGO are continuously stirred vigorously at room temperature for 24 hours to obtain the GM/AC-CD or GM/AC-CD/rGO inclusion complex solution, and then APS/TEMED is added.

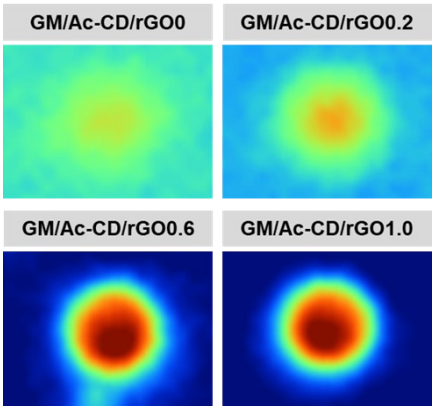

Figure S5. Near-infrared image of each GM/Ac-CD/rGO hydrogels after 10 min of irradiation.

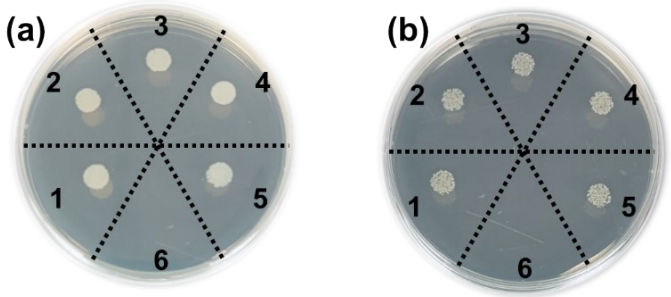

Figure S6. Photographs of in vitro photothermal antibacterial performance of GM/Ac-

CD/rGO hydrogels against E. coli (a) and S. aureus (b), with 1, 2, 3, 4, 5, and 6 represent the PBS, PBS + NIR, GM/Ac-CD/rGO0, GM/Ac-CD/rGO0+NIR, GM/Ac-CD/rGO0.6, GM/Ac-CD/rGO0.6 + NIR group, respectively.

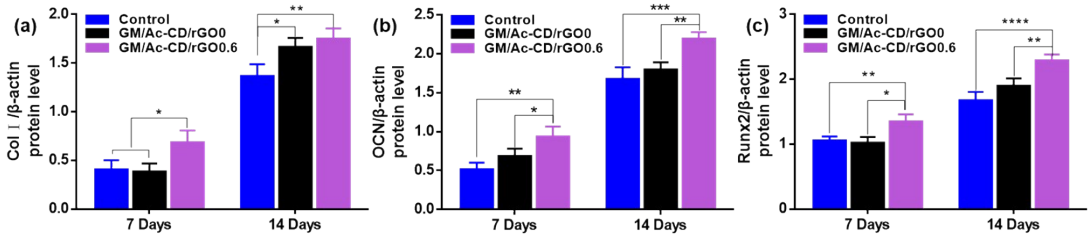

Figure S7. Protein level of the Col I (Figure a), OCN (Fiugre b) and Runx2 (Fiugre c) in control, GM/Ac-CD/rGO0 and GM/Ac-CD/rGO0.6 groups. *P <0.05, **P < 0.01, ***P < 0.005, **** P < 0.001.

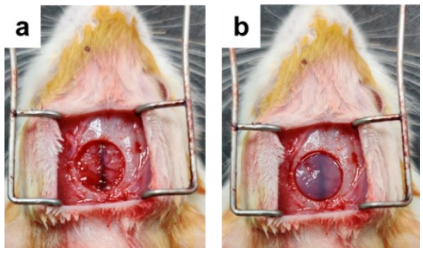

Figure S8. Construction of rat skull defect model (a) and implantation with the GM/Ac-CD/rGO hydrogel patch (b).

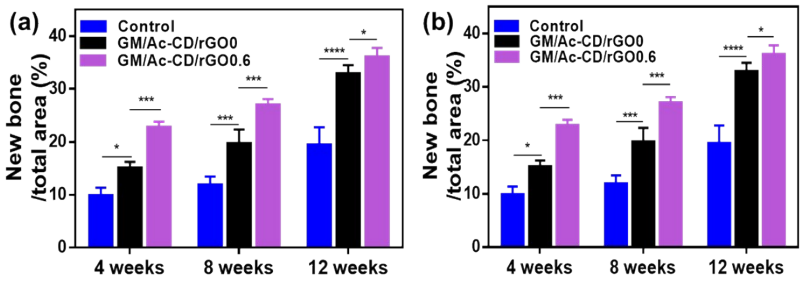

Figure S9. Quantitative analysis of the bone formation areas for H&E staining (a) and

Masson's trichrome staining (b) in control, GM/Ac-CD/rGO0 and GM/Ac-CD/rGO0.6 groups. *P <0.05, **P < 0.01, ***P < 0.005, **** P < 0.001.

Reference

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