Electronic Supplementary Material (ESI) for Nanoscale Horizons.

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Electronic Supporting Information

Of

ROS Self-Generation and Hypoxia Self-Enhanced Biodegradable Magnetic Nanotheranostics for Targeted Tumor Therapy

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Experiment section

Chemicals and Materials

Unless specified, chemicals are purchased and used without further purification. Vitamin c (Vc), $K_3[Fe(CN)_6]$, 1-tetradecanol (PCM), (3-aminopropyl)triethoxysilane (APTES, 99%), methylene blue (MB), rhodamine 6G (R6G) and folic acid (FA, 97%) are bought from Alfa Aesar Co., Ltd (Tianjin, China). HOOC-PEG-NH_2 (Mw $\approx$ 5000) is provided by Jenkem Co., Ltd. Folic acid (FA, 97%), tert-butoxycarbonyl (t-Boc) and Polyvinyl Pyrrolidone (PVP) are supplied by TCI (Japan). p-hydroxyphenylacetic acid (PHPA) and 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO) are obtained from Sigma-Aldrich Co., Ltd (St. Louis, America). Other conventional biochemical reagents are provided by Aladdin Co., Ltd (Shanghai, China).

Fabrication of PBNCs

The PBNCs was prepared by a previous report.[1] Typically, under continuous stirring condition, $K_3[Fe(CN)_6]$ (131.7 mg) and PVP (3.0 g) were dissolved into a teflon-lined stainless steel autoclave (50 mL), and the pH of the mixture solution was adjusted to 3.0 by hydrochloric acid (HCl, 0.1 M) with stirring for 10 min. Then the mixture solution was placed at 80°C for 20 hours in oil bath. After cooling to room temperature, the blue products were collected and washed with alcohol and distilled water each for 8 times and then dried at 80 °C. It was named as PBNCs.

Preparation of MMNCs

In the process, to synthesize mesoporous magnetic iron nanocubes, the PBNCs were used as the precursor.[2] Briefly, PBNCs nanopowers (100.0 mg) were placed into a porcelain crucible and then calcined up to 250 °C at a heating rate of 1 °C per min for 4 h to ensure the completed thermal oxidization decomposition. Then the products were cooled to room temperature inside the muffle furnace naturally. The brown nanoparticles were collected with a magnet and washed with alcohol for 8 times and then dried at 80 °C. And the obtained products were noted as MMNCs.

PCM and Vc loading in MMNCs

To obtain the promising PCM and Vc loaded MMNCs, 1.2 g of 1-tetradecanol (PCM) was melted completely in a three-necked flask at 80 °C with an oil bath. When the flask was cooled to 50 °C, various concentrations of Vc (0.1-20 mM) were added to the flask and further stirred for 2 h. After that, 0.5 g of MMNCs dispersed in 100.0 mL of methyl alcohol was added into the above mixture. The resulting nanoparticles were gathered with a magnet and washed with distilled water for several times at room temperature.

PEG and FA coating

Firstly, the HOOC-PEG-FA was fabricated by a previous report.[3] Typically, EDC•HCl (9.2 mg), NHS (5.5 mg) and FA (amino group is protected by tert-butoxycarbonyl (t-Boc), 10.5 mg) were dissolved into PBS (pH 7.4, 20 mL) under continuous stirring in dark at room temperature for 4 h. Then HOOC-PEG-NH_2 aqueous solution (10.0 mM) was added into the above reaction mixture with continuously stirring at room temperature

in the dark for another 24 h. Next, the mixture was purified by dialysis (4500 Da) for 3 d in PBS (20×10^{-3} M, pH 7.4) to remove any free FA, NHS, and EDC. The yellow products were then dried to obtain carboxyl PEG-FA product (HOOC-PEG-FA).

After that, the above obtained Vc and PCM loading MMNCs (1.0 g) nanoparticles were dispersed in ethanol (100.0 mL) and deionized water (2.0 mL) with ultrasonication for 10 min, the pH was adjusted to 5.0 whit acetic acid. Next, (3-aminopropyl)triethoxysilane (APTES, 99%, 0.25 mL) was added to the above mixture and continuously stirred under nitrogen protection at room temperature for 6 h. Then under the atmosphere of nitrogen, 160.0 mg HOOC-PEG-FA was dissolved into 20.0 mL DMSO, followed by adding of 42.4 mg EDC•HCl and 12.4 mg NHS and 12 h continuously stirring in dark at room temperature. The mixture was further purified by dialysis to remove any unreacted HOOC-PEG-FA. The finial products were washed with ethanol, distilled water, and dried in refrigerated drying chamber, which were named as MMNCs@PCM@Vc.

Materials characterization

The morphologies of the as-synthesized nanoparticles were characterized by scanning electron microscopy (SEM, FEINova-400, Philips, Netherlands, operated at 200 kV) and transmission electron microscope (TEM, LIBRA 200-FEG, Zeiss, Germany). The crystalline structure of the PBNCs and MMNCs nanocubes was recorded with an X-ray diffraction (D/max 2500-PC, Rigaku, Japan) with Cu Kα radiation (λ = 1.54 Å), the scan

range was performed from 30° to 90° (2θ). The surface charges of particles were measured by a potentiometric analyser (BIC-ZetaPALS, Brookhaven, USA).

Thermogenesis of MMNCs@PCM@Vc

To verify the possibility of MMNCs@PCM@Vc for magnetic hyperthermia, the calorigenic behaviour of the magnetic nanoparticle was evaluated by using an external AMF. Typically, MMNCs@PCM@Vc nanoparticle with different concentrations (0.5, 1.25, 2.5, 5.0 and 7.5 mg/mL) in PBS was treated with AMF (4 kW, 300 A & 200 kHz). To prove the thermogenesis stability of the as-synthesized nanoparticles, the MMNCs@PCM@Vc (7.5 mg/mL) was re-exposed to AMF (4 kW, 300 A & 200 kHz) for 6 on-off cycles.

The "on-off" drug release profiles

To investigate the encapsulation and ''on-off'' drug release behavior of the MMNCs@PCM, two commonly used dyes with different solubility, hydrophilic methylene blue (MB) and hydrophobic Rhodamine 6G (R6G) were used as model drugs.[4] Typically, MMNCs@PCM@MB and MMNCs@PCM@R6G were treated with AMF (4 kW, 300 A & 200 kHz), the release profiles were recorded with ultraviolet spectrophotometer. To investigate the stability of MMNCs@PCM system, the drug release profiles of MMNCs@PCM@MB and MMNCs@PCM@R6G were measured after 30 days storage.

Temperature-dependent drug release

To study the temperature responsive release behavior of the as-synthesized nanoparticles, the MMNCs@PCM@Vc nanoagents were dissolved in PBS (Vc concentration: 5.0 mM) with different temperatures (20 °C, 37 °C, 38 °C, 39 °C, 40 °C, 45 °C, 50 °C and 55 °C), the release behavior of Vc was recorded with high performance liquid chromatography.

Cell culture

Hela cells were cultured with 1640 medium containing 10% fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL streptomycin at 37 °C under 5% CO_2 atmosphere. The culture medium was changed every two days.

Cytotoxicity assay

The cytotoxicity of the as-synthesized MMNCs@PCM@Vc was evaluated with MTT assay. Typically, Hela cells were seeded in 96-well plates with cell density of 7.5×10^3 cells/cm^2. When cells confluence reached around 80%, the culture medium containing MMNCs@PCM@Vc with different concentrations (0, 0.5, 1.25, 2.5, 5.0 and 7.50 mg/mL) was added to each well and treated with AMF (4 kW, 300 A & 200 kHz) for 10 min. Then the Hela cells were cultured for another 12 h. After that, 50 μL of MTT (2.0 mg/mL) solution was added to each well and incubated at 37 °C for another 3 h. Next, 150 μL of DMSO was added to each well and incubated for 10 min. Finally, the culture medium was collected and recorded by a microplate reader (Bio Rad-680, USA) at a wavelength of 570 nm.

H_2O_2 release and detection

The H_20_2 endpoint assay for H_20_2 release from Hela cells was adapted by a substituted phenolic compound, p-hydroxyphenylacetic acid (PHPA). In the presence of H_20_2, PHPA dimerizes to fluorescent 2,2'-dihydroxy-biphenyl-5,5'-diacetate [$(PHPA)_2$]. Typically, Hela cells (5×10^5) were rinsed in a tube containing 1.0 ml of PBS (37 ^oC, pH 7.4). Then, 1.0 ml of PBS supplemented with Vc (5.0 mM), MMNCs@PCM@Vc (0.1 mL, 5.0 mg/mL) and 1.6 mM PHPA was added, followed by incubation for 1 h at 37 ^oC in air. AMF treatment was taken and the baseline fluorescence was determined by fluorescence spectrophotometry (λ_{ex} = 320 nm and λ_{em} = 400 nm, 5 nm slit width). To correct for instrument fluctuations, a solid pyrex fluorescence standard (Fluoroskan Ascent^{TM} FL, Thermo Scientific, America) was recorded at intervals during the fluorescence measurements.

Hydroxyl radicals (•OH) generation and monitoring

The amount of •OH was determined by using 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO) as the •OH specific spin-trapping agent. Briefly, Vc (5.0 mM) and MMNCs@PCM@Vc (0.1 mL, 5.0 mg/mL) solution at pH 7.4 in PBS (1.0 mL, pH 7.4, 10 mM, contain Hela cell: 5×10^5), followed by incubation at 37 ^oC for 1 h. Then DMPO (0.05 mL, 0.5 M) was added as the spin--trapping agent, and the 1:2:2:1 multiplicity characteristic peaks of DMPO-OH adducts were recorded by ESR immediately.

Study of cellular uptake mechanism

To test whether the phagocytosis processes of MMNCs@PCM@Vc nanoparticles is an active process that requires energy, we used the low temperature (4 °C) and sodium azide (0.1%, v/v) and 50 mM deoxyglucose (NaN_3/DOG) to inhibit ATP generation during the MMNCs@PCM@Vc nanopaticles addition. To study the uptake pathways of MMNCs@PCM@Vc, Hela cells were pre-incubated with the following inhibitors: sucrose, chlorpromazine, filipin, methyl-β-cyclodextrin (Mβ-CD), colchicines and wortmannin. Prior to the inhibition experiment, the toxicity of each endocytosis inhibitor at a defined concentration was assessed by MTT assay. Briefly, cells were seeded into 96-well plates at a 1×10^4 cell/well density and then incubated with the following inhibitors: 150 mg/mL sucrose, 10 μg/mL chlorpromazine, 4 μg/mL filipin, 5 mM Mβ-CD, 50 nM wortmannin and 40 μg/mL colchicines at 37 °C. After 48 h incubation, the cells were washed with PBS and then 100 μL MTT (5 mg/mL) was added to each well, and the plates further incubated for 4 h. The medium in each well was removed and 100 μL DMSO was added to dissolve the internalized purple formazan crystals. Cell viability was measured on a microplate reader (Thermo, USA).

Animal model

All animal studies were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (Chongqing, revised in April 2006) and adhered to the Guiding Principles in the Care and Use of Animals of the American Physiological Society. In this study, all protocols involving animals were in

accordance with ethical standards and approved by the Institutional Animal Care and Use Committee of China, Xinqiao Hospital affiliated to Third Military Medical University (SYXK-PLA-20120031). 4 weeks female nude mice (average weight: about 23.0 g) were purchased from the animal experimental centre of Xinqiao Hospital (Chongqing). The mice were subcutaneously injected with 100 μL cell suspension containing 2×10^6 Hela cells to develop the tumor model.

***In vivo* synergistic photothermal radiotherapy studies**

To investigate the synergistic tumor suppression of photothermal radiotherapy, the tumor-bearing mice were randomly divided into 8 groups (five mice per group): treated with PBS (Group I), PBS + AMF (Group Ⅱ), Vc (Group Ⅲ), Vc + AMF (Group Ⅳ), MMNCs (Group Ⅴ), MMNCs +AMF (Group Ⅵ), or MMNCs@PCM@Vc (Group Ⅶ), MMNCs@PCM@Vc + AMF (Group Ⅷ). When the volume of tumor reached to about 60 mm^3, the nanoparticles (5.0 mg/mL, 100 μL) injected mice were treated with AMF (4 kW, 300 A & 200 kHz). The volume of tumor was calculated with the formula: V_{tumor} = length × $width^2/2$. The relative volume was defined as (V/V_0 (V: current volume, V_0: initial tumor volume).

***In vivo* multimodal imaging**

After *i.v.* injection of MMNCs@PCM@Vc (5.0 mg/mL, 100 μL), the infrared thermal images under AMF (4 kW, 300 A & 200 kHz) were recorded with an IR-thermal camera. The T_2-weighted MR images were recorded witha 3 T MRI scanner (Bruker Biospin

Corporation, ClinScan, USA). The PAI images were measured with a photoacoustic 3D tomographic imaging system (Veco®LAZR, VisualSonics, Canada) at 1, 2 and 6 h.

***In vivo* pharmacokinetics**

Mice were administrated with MMNCs@PCM@Vc (4.0 mg/kg) via tail vein injection. At different time points (0, 0.5, 1, 1.5, 2, 4, 6, 12, 24 h), 50 μL whole blood were collected through eyeball extirpating. Fe content in each sample were recorded by ICP-AES. The pharmacokinetics parameters were calculated with the model as reported.[5]

***In vivo* biodistribution studies**

After *i.v.* injection of MMNCs@PCM@Vc, mice were ultimately killed at 1 and 48 h. Heart, liver, spleen, lung, kidney, skin, muscle, bone, stomach and tumor from each mouse were collected after tissue homogenation and manually grounding and transferred into test tubes. Then the Fe content in main organs was detected by ICP-AES.

***In vivo* systematic toxicological evaluation**

To investigate the hemocompatibility of MMNCs@PCM@Vc, hemolysis ratio (HR) and coagulation time were recorded. In this study, a hemolysis ratio (HR) test was taken to detect hemoglobin release *in vitro*. Typically, five healthy mice were i.v. injected with MMNCs@PCM@Vc (100 mg/kg^{-1}), while *i.v.* injection of saline was used as control. At the 1st, 14th, and 28th day post *i.v*. injection, the blood samples from mice were collected for blood biochemical test and hematology analysis. Meanwhile, the tumors and main

organs including heart, liver, lung, spleen and kidney were fetched for staining with haematoxylin & eosin (H&E).

Statistical analysis

All data were presented as means with standard deviation (SD). The statistical analysis was analyzed by using software of OriginPro (version 8.0) via one-way analysis of variance (ANOVA) and Students's t-test. The confidence levels were set as 95% and 99%.

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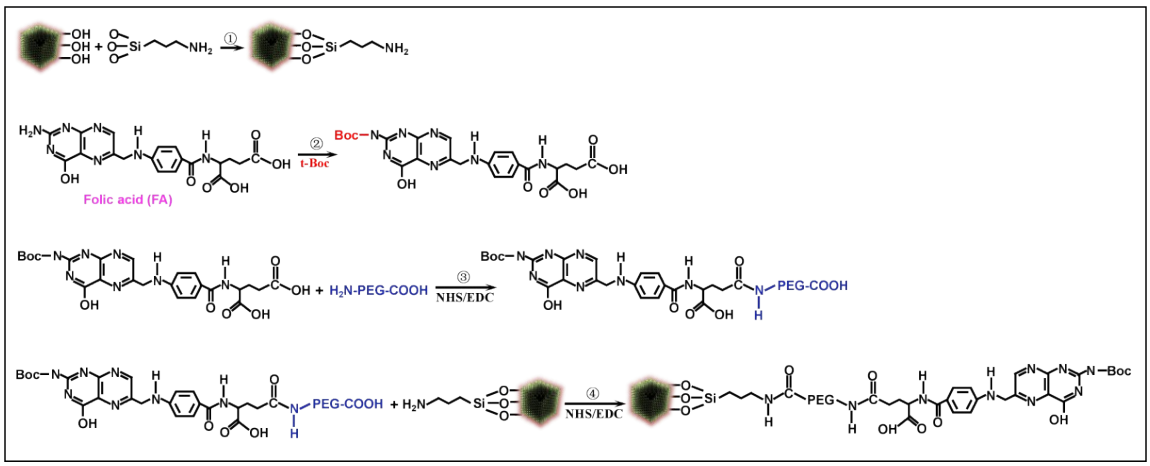

Scheme S1. Chemical reaction scheme for the fabrication of PEG and FA coated MMNCs@PCM@Vc nanoparticles.

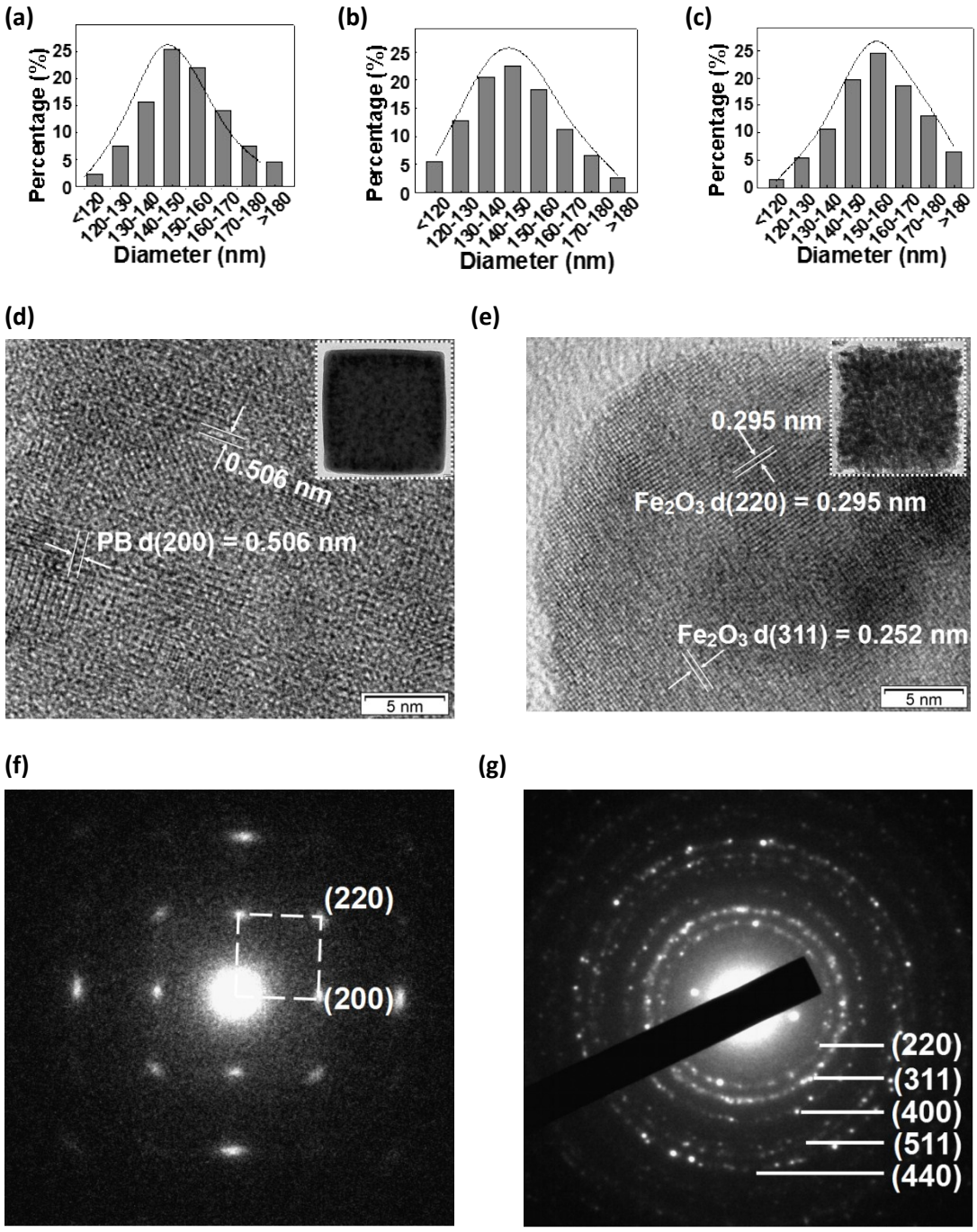

Figure S1. Particle size distributions of as-synthesized nanoparticles: (a) PBNCs, (b) MMNCs and (c) MMNCs@PCM@Vc, respectively. High resolution TEM images of (d) PBNCs and (e) MMNCs. Fourier transition patterns of (f) PBNCs and (g) MMNCs.

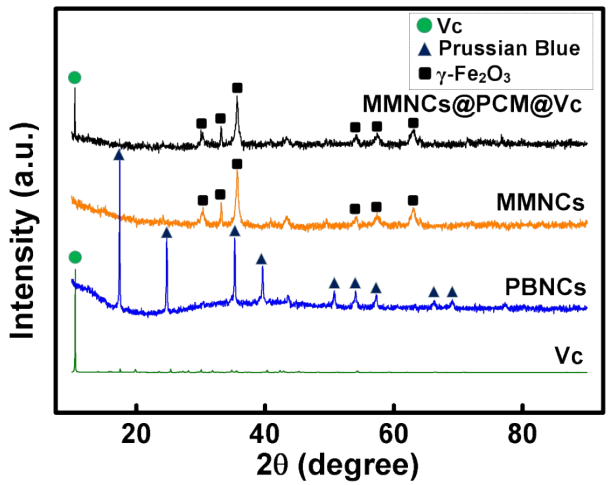

Figure S2. X-ray diffraction patterns of the obtained PBNCs, MMNCs and MMNCS@PCM@Vc, respectively.

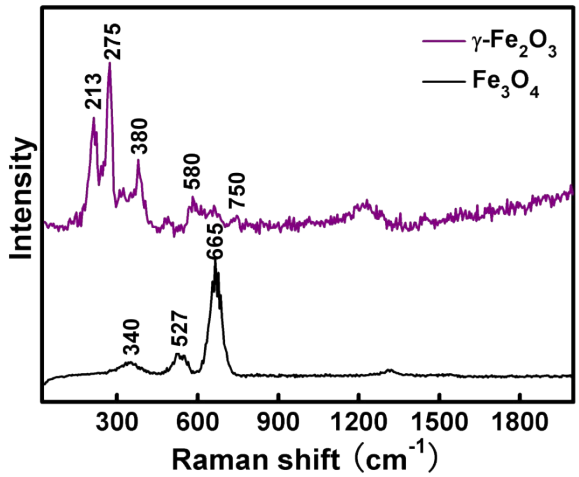

Figure S3. Raman spectrum of the as-prepared γ-Fe_2O_3 nanoparticles.

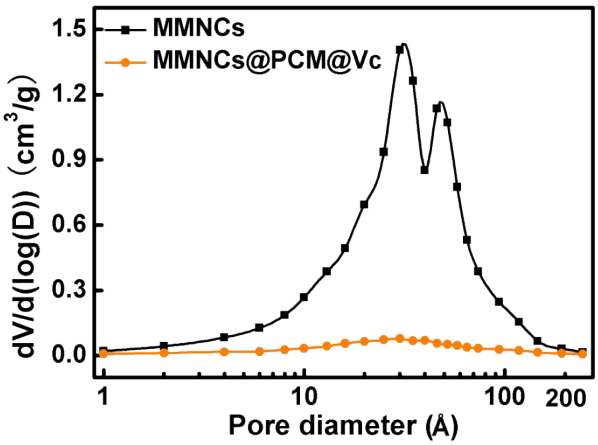

Figure S4. Pore diameter isotherms for the as-prepared MMNCs and MMNCs@PCM@Vc.

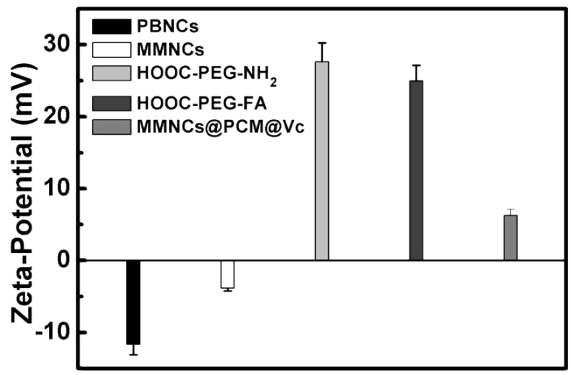

Figure S5. Zeta potentials of the as-synthesized nanoparticles at different steps during the fabrication process (n = 5).

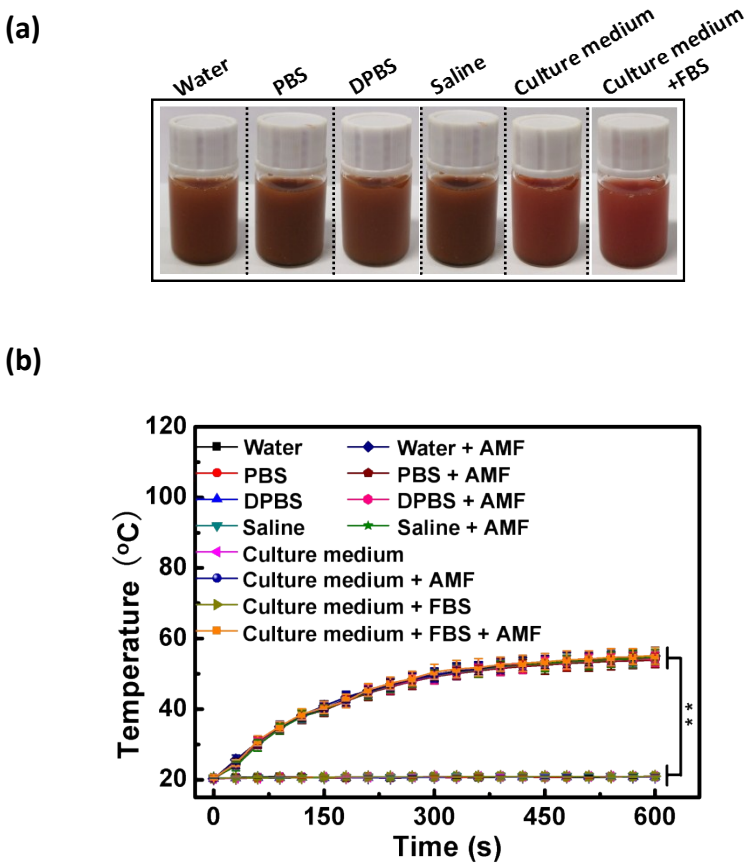

Figure S6. (a) Digital photographs of MMNCs@PCM@Vc dispersion in different solvents after one day. (b) Thermogenesis capacity of MMNCs@PCM@Vc (7.5 mg/mL) treated with AMF (4 kW, 300 A & 200 kHz) in different solvents.

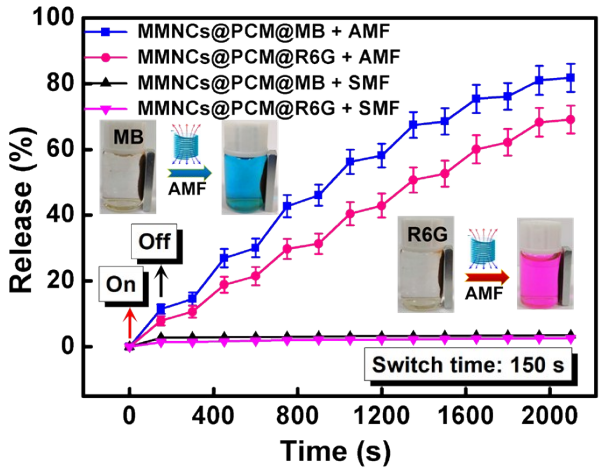

Figure S7. The "on-off" model drug release profiles of MMNCs@PCM@MB and MMNCs@PCM@R6G within an external alternating magnetic field (AMF, 300 A, 200 kHz, 4 kW) and static magnetic field (SMF, 0.3 T).

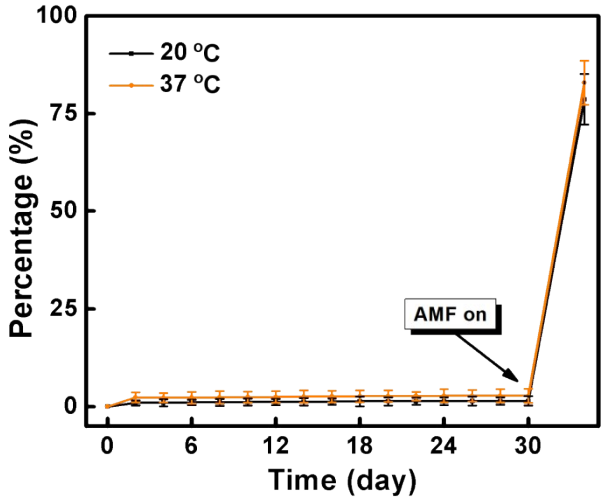

Figure S8. Delayed dug release of MMNCs@PCM@Vc (5.0 mg/mL, Vc: 5.0 mM) at different storage temperatures.

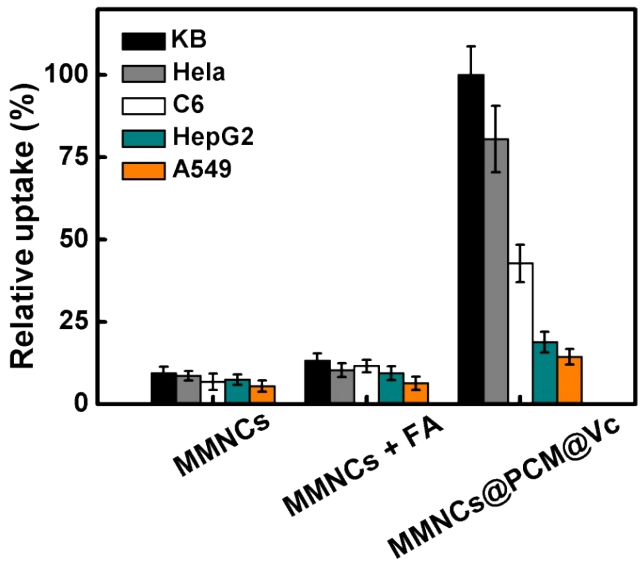

Figure S9. Targeting uptake of the nanoparticles: (a) Folic acid-receptor mediated targeting capacity of MMNCs@PCM@Vc evaluated with FR positive (KB, Hela, C6) and negative (HepG2, A549) cancer cells.

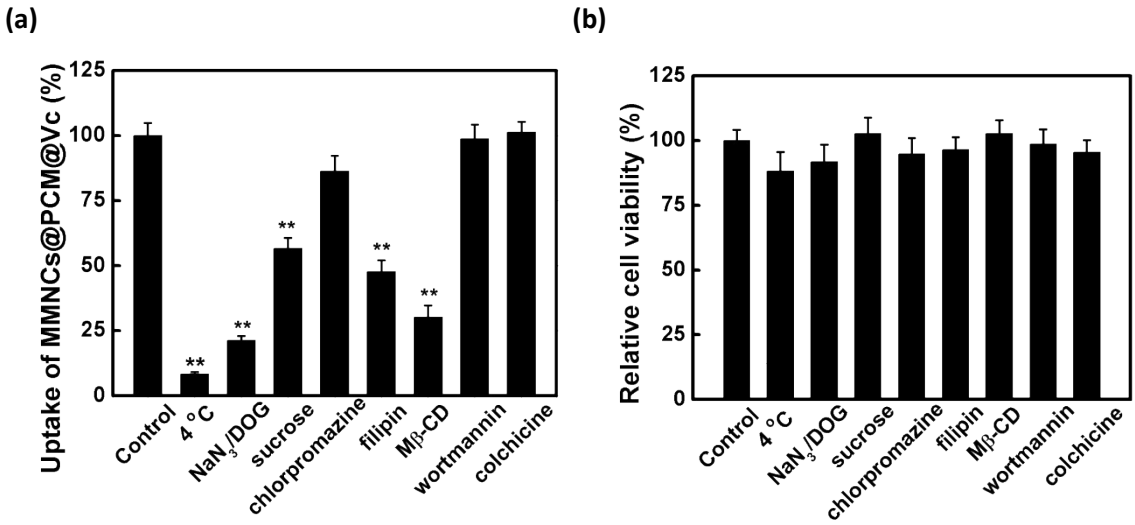

Figure S10. Cell endocytosis mechanism assay. (a) Relative uptake percentages of MMNCs@PCM@Vc nanoparticles after treatments with different uptake inhibitors. (b) Relative cells viability when incubating with corresponding different uptake inhibitors only served as control. Data are presented as means ± SD (n=5). **Indicated $p<0.01$.

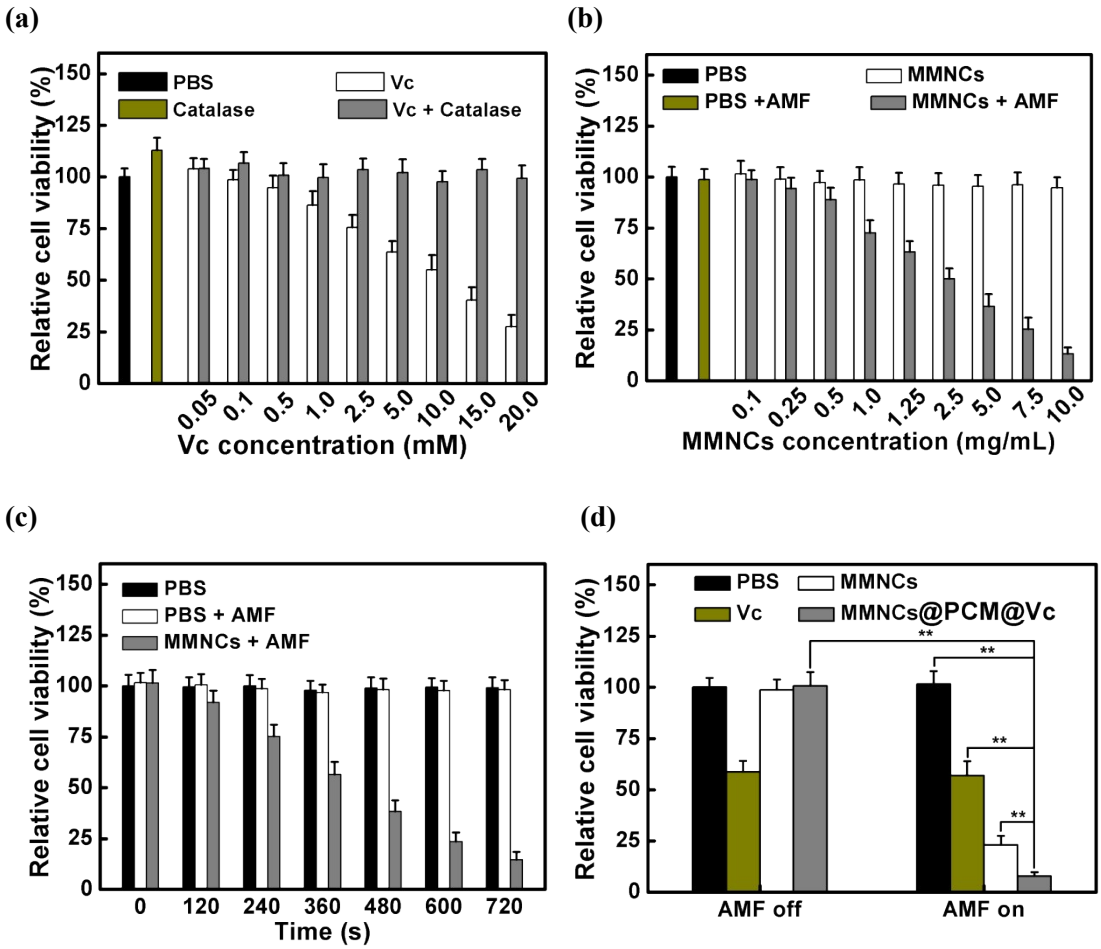

Figure S11. (a) Relative cells viabilities of Hela cells after incubation with various concentrations of Vc for 24 h (n=5); (b) Relative cell viabilities of Hela cells after incubation with different concentrations of MMNCs nanoparticles under AMF (4 kW, 300 A & 200 kHz) for 10 min; (c) Relative viabilities of Hela cells treated with AMF (4 kW, 300 A & 200 kHz) at different periods. (d) Relative cell viabilities after the synergistic magnetic hyperthermia and ROS therapy with the as-synthesized MMNCs@PCM@Vc (5.0 mg/mL, Vc: 5.0 mM) under AMF (4 kW, 300 A & 200 kHz) for 10 min.

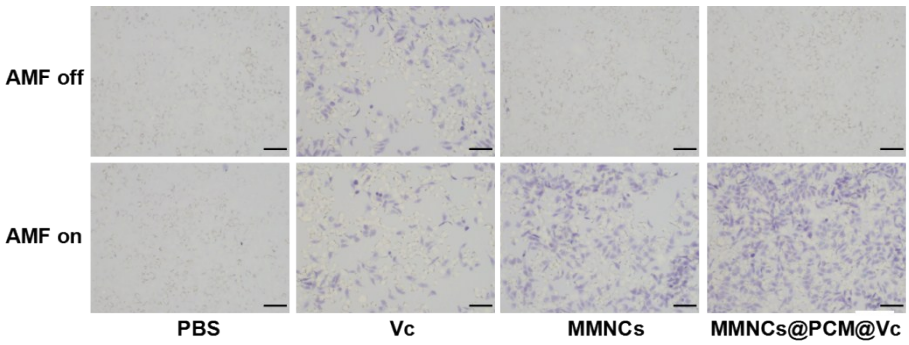

Figure S12. Optical graphs of Hela cells with different treatments in presence or absence of AMF (4 kW, 300 A & 200 kHz). Dead cells are reflected by blue color staining with trypan blue (scale bar: 100 μm).

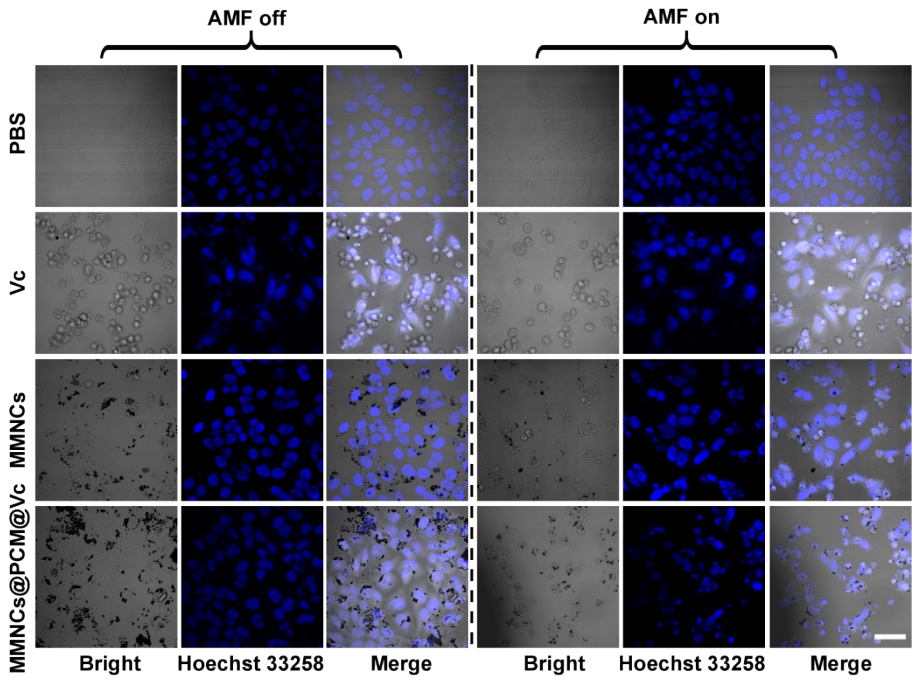

Figure S13. CLSM images of Hela cells treated with different administrations in presence or absence of AMF (4 kW, 300 A & 200 kHz, 10 min). Scale bar: 50 μm.

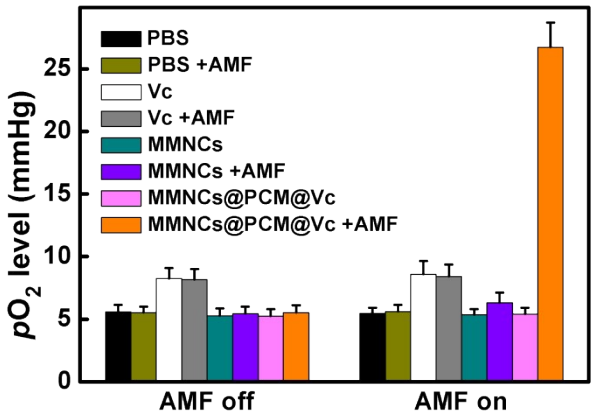

Figure S14. Corresponding pO_2 level of tumor treated with different administrations in presence or absence of AMF (4 kW, 300 A & 200 kHz, 10 min).

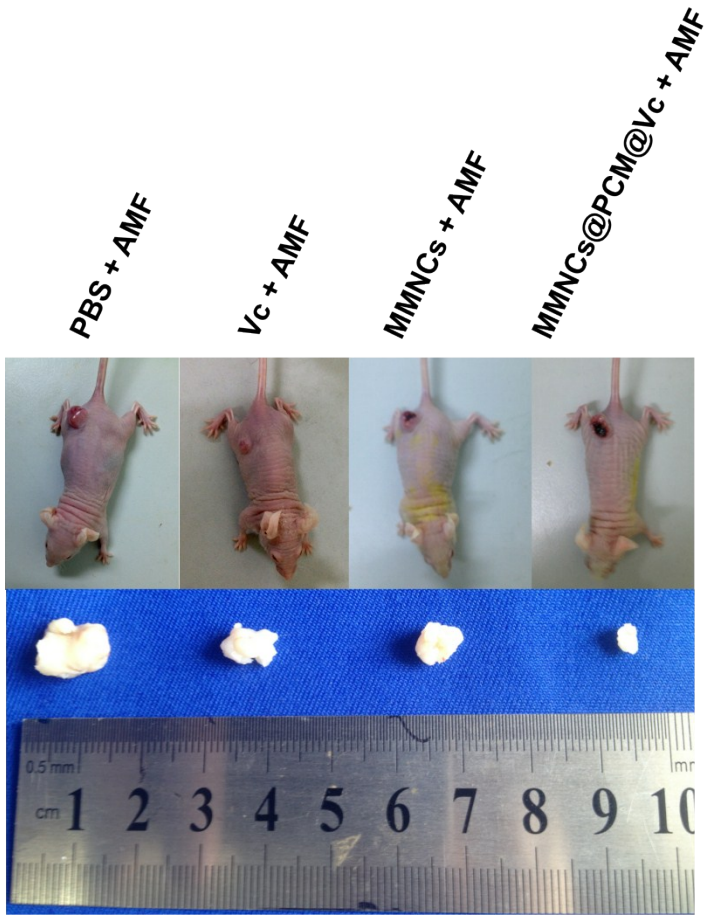

Figure S15. Digital photos of Hela-tumor bearing mice and their tumor regions with different treatments for 16 days.

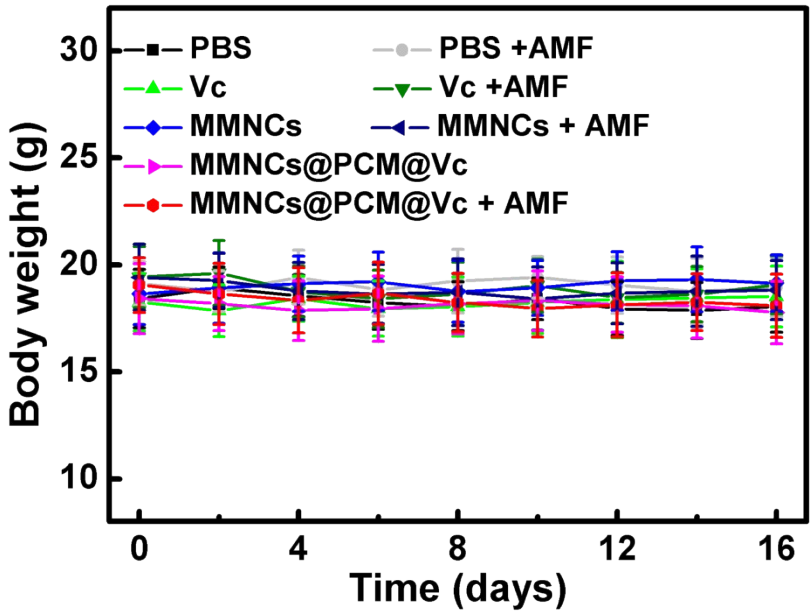

Figure S16. Body weight change of mice in the different treatment groups.

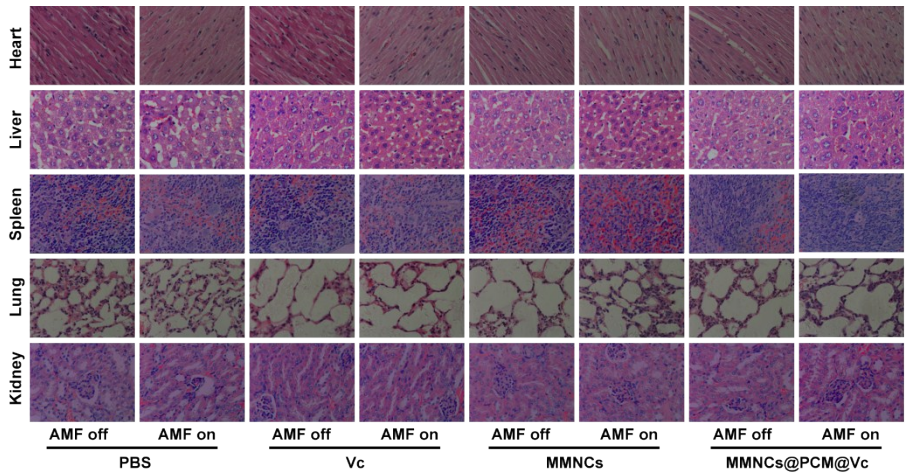

Figure S17. H&E staining images of the major organ (heart, liver, spleen, lung and kidney) of the nude mice treated with MMNCs@PCM@Vc on the 1th day post-injection (scale bars: 100 μm).

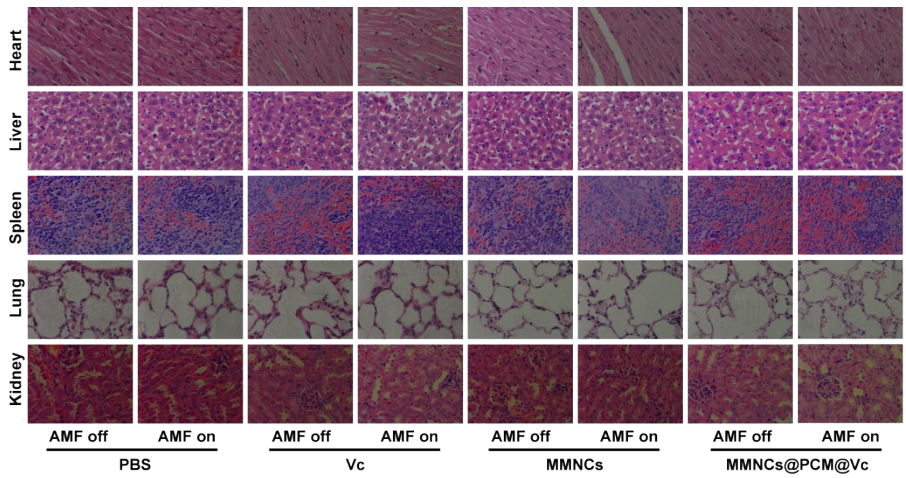

Figure S18. H&E staining images of the major organ (heart, liver, spleen, lung and kidney) of the nude mice treated with MMNCs@PCM@Vc on the 14th day post-injection (scale bars: 100 μm).

Table S1. Hemolytic ratio (HR), prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) of the as-synthesized nanoparticles (n = 5).

Materials	**HR(%)**	**PT(s)**	**APTT(s)**	**TT(s)**
Control	2.35± 0.31	11.4 ± 2.1	33.1 ± 3.2	14.8 ± 2.2
Vc	2.92 ± 0.64	13.7 ± 1.6	34.7 ± 4.6	15.8 ± 1.7
MMNCs	3.78 ± 0.41	11.7 ± 3.2	33.6 ± 3.4	13.7 ± 2.4
MMNCs@PCM@Vc	2.83 ± 0.76	12.9 ± 3.1	34.2 ± 2.8	15.1 ± 1.9