Supporting Information for *Advanced Materials*

Engineered Nano-Platelets for Enhanced Treatment of Multiple Myeloma and Thrombus

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Methods

Materials

All chemicals in this study were purchased from Sigma-Aldrich and were used without further purification. Bortezomib, biotin and streptavidin were obtained from Fisher Scientific Inc. Fluorescein isothiocyanate (FITC)-NHS, rhodamine-NHS and Cy5.5-NHS were purchased from Life Technologies (Grand Island, NY, USA). Coumarin-6, tissue plasminogen activator (tPA), thrombin, fibrinogen and thromboplastin were purchased from Sigma-Aldrich.

Modification of dextran and preparation of nanoparticle

The pendant acetal modified dextran (*m*-dextran) was synthesized as previously reported.[1] Briefly, 10 mL of anhydrous DMSO was added to 1 g of dextran (molecular weight: 9-11 kDa), followed by the addition of pyridinium p-toluenesulfonate (PPTS, 15.6 mg, 0.062 mmol) and 2-ethoxypropene (4.16 mL, 37 mmol). The mixture was stirred at room temperature for 30 min, and then 1 mL of triethylamine was added to stop the reaction. The resulted *m*-dextran was precipitated by washing with water (pH~8), collected by centrifugation and lyophilized for future use.

The *m*-dextran nanoparticle was prepared *via* the nanoprecipitation method.[2] Briefly, 25 mg *m*-dextran was dissolved in 2 mL acetone, followed by the addition of 4 mL pure water. The mixture was then kept under vacuum overnight to remove acetone. The resulted nanoparticles were centrifuged at 1000g to remove large nanoparticles. The bortezomib-loaded nanoparticle was made with the same procedure.

Preparation of PM-NP

Platelet membrane-coated nanoparticle (PM-NP) was prepared as described before.[3] In brief, whole blood was collected from the mice, centrifuged at 200 g to achieve platelet-rich plasma and further centrifuged at 800g to obtain the platelets. After lysis, the mixture of platelet membrane and nanoparticles was treated with sonication and then stirred overnight.

In order to conjugate tPA on the surface of PM-NP, the PM-NP was first reacted with NHS-LC-biotin at a 1:5 molar ratio in PBS, pH 7.4 at room temperature for 2 hours. Afterwards, the streptavidin was added to biotinylated PM-NP at a ratio of 5:1 and incubated for 1 h. Then, biotinylated tPA was mixed with streptavidin-biotinylated PM-NP to form tPA-PM-NP. The concentration of the conjugated tPA was determined by tPA ELISA kit (Abcam, USA). The decoration of Ald on PM-NP was made through the sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) linker.

Characterization of PM-NP

The sizes and zeta potentials of NP and PM-NP were investigated by the Zetasizer (Nano ZS, Malvern). The morphologies were characterized by Transmission Electron Microscopy (TEM) (JEM-2000FX, Hitachi) after stained with 1% (w:v) phosphotungstic acid.

***In vitro* stability of PM-NP and bortezomib release**

The stability of PM-NP and tPA-Ald-PM-NP were studied in PBS (pH = 7.4) and 10% FBS solution. The size change was monitored at pre-determined time intervals using dynamic light scattering (DLS).

For bortezomib release profile study, 0.5 mL of NP-bortezomib and PM-NP-bortezomib were added into a dialysis tube (3K MWCO) embedded into 14 mL of the PBS buffer solution

(containing 0.1% Tween) at different pH, and gently shaken at 37 ^{o}C in a shaker (New Brunswick Scientific) at 100 rpm. At predetermined time points, 25 μL buffer solution was withdrawn, followed by replacing with 25 μL of fresh buffer solution with the same pH. The concentration of bortezomib released was measured *via* High Performance Liquid Chromatography (HPLC) (Agilent, Japan). Methanol/water (80/20, v/v) was used as the mobile phase at a flow rate of 1.0 mL per minute. Bortezomib was separated by a C18 column and detected at 254 nm.

Cell culture

NCI-H929 cells were purchased from American Type Culture Collection (ATCC) and cultured in Roswell Park Memorial Institute (RPMI)-1640 Medium with 10% (v:v) FBS, 0.05 mM 2-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin.

Cellular association

Coumarin-6 was selected as the fluorescence probe to investigate the subcellular location of the PM-NP after internalization. NCI-H929 cells (1×10^5 cells/well) were seeded in a confocal microscopy dish (MatTek) pretreated with lysine. After culture for 24 h, the cells were washed with PBS to remove non-adherent cells and incubated with courmarin-6-PM-NP for 1 h and 4 h. Thereafter, the cells were washed with PBS, stained with lysosome tracker and Hoechst, and observed by CLSM (LSM 710, Zeiss).

Apoptosis assay.

Apoptosis of NCI-H929 cells were detected using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) and APO-BrdU TUNEL Assay Kit (Life Technologies), respectively. The

cells were seeded in the 6-well plates at the density of 1×10^5 cells/well. Twenty-four hours later, the cells were incubated with NP-bortezomib, PM-NP-bortezomib, Ald-PM-NP-bortezomib for 12 h. Drug-free RPMI 1640 medium served as the control. Afterwards, the cells were treated according to the manufacturer's protocol, respectively. For Annexin V-FITC apoptosis detection, the cells were analyzed by flow cytometry (BD FACS Calibur). For TUNEL assay, the cells were observed by fluorescence microscope (IX71, Olympus).

In vitro cytotoxicity

NCI-H929 cells (5×10^3 cells/well) were seeded in the 96-well plates and incubated for 24 h. Then the cells were incubated with NP-bortezomib, PM-NP-bortezomib, Ald-PM-NP-bortezomib for 24 h with different bortezomib concentrations ranging from 1 ng/mL to 50 ng/mL. Then the cells were added with 10 μL of the CCK8 solution. After 4 h of incubation, the absorbance was measured at the wavelength of 450 nm by a microplate reader.

In vitro hydroxyapatite and bone fragment targeting

1 mL coumarin-6-loaded NP, PM-NP and Ald-PM-NP were incubated with 10 mg hydroxyapatite for various time intervals. Afterwards, the mixture was centrifuged at 2000 rpm and the coumarin-6 concentration in supernatant was detected *via* HPLC at 465nm. The methanol/water (96/4, v/v) was used as the mobile phase. For the bone fragment targeting assay, the coumarin-6-loaded NP, PM-NP and Ald-PM-NP was incubated with mice femurs for 1 h. After washed for three times, the femurs were subjected to fluorescence microscope for observation.

In vitro thrombolysis activity assay of tPA-Ald-PM-NP

The bioactivity of tPA after conjugation on PM-NP was evaluated by studying the capability of hydrolyzing tripeptide chromogenic substrates. In brief, various concentrations of free tPA and tPA-PM-NP was incubated with S2288 substrate for 30 min at 37 ^{o}C. Afterwards, the reaction was stopped by acetic acid and the absorbance was detected at the wavelength of 405 nm.

The *in vitro* thrombolysis activity of tPA-Ald-PM-NP was investigated by evaluating clot lysis capability. Fibrinogen (3 mg) labeled with FITC was pre-incubated with 0.5 mL PBS, free tPA, tPA-NP, tPA-PM-NP and tPA-Ald-PM-NP at tPA concentration of 5 μg/mL and 10 μg/mL. Ten minutes later, $CaCl_2$ and thrombin were added in to mixture at the final concentrations of 20 mM and 0.2 units/ml final concentrations, respectively. After incubation for 20 min, the fluorescence intensity in the supernatants was measured in a microplate reader. To further test the tPA activity after blood circulation, the mice was intravenously injected with free tPA, tPA-NP, tPA-PM-NP and tPA-Ald-PM-NP at tPA concentration of 0.3 mg/kg *via* the tail vein. After 10 or 20 min, 100 μL blood was withdrawn and mixed with Cy5.5-labeled fibrinogen, followed by the addition of $CaCl_2$ and thrombin. The fluorescence intensity in the supernatants was detected.

***In vivo* bone targeting assay**

To evaluate the *in vivo* targetability of Ald-PM-NP, Nine nude mice were randomly divided into three groups and intravenously injected with Cy5.5-labeled NP, Cy5.5-labeled PM-NP and Cy5.5 labeled Ald-PM-NP at Cy5.5 dose of 30 nmol/kg. Images of the mice were taken on IVIS Lumina imaging system (Caliper, USA) at 24, 48 and 72 h post-injection. The whole body fluorescence intensity of each mouse were analyzed by Living Image Software. Thereafter, the mice

were euthanized at 72 h post injection. The leg bones were harvested and subjected for *ex vivo* imaging. The fluorescence intensities of region-of-interests (ROI) were analyzed by Living Image Software.

In vivo lung thrombus targeting and treatment efficacy

The mouse lung thrombus model was established as described.[4] For lung thrombus targeting evaluation, the mice were intravenously injected with fibrinogen (2 nmol/30 g body weight) and 1 min later, with the various Cy 5.5-labeled NP formulations, followed by induction of thrombosis with thromboplastin (4 μL/g body weight) 20 min later *via* the tail vein. The mice were sacrificed 30 min later. The lungs were collected, washed with PBS, and imaged using the IVIS Lumina imaging system. For the lung thrombus treatment efficacy, the mice were intravenously administrated with Cy5.5-labeled fibrinogen. One minute later, saline, tPA, tPA-NP, tPA-PM-NP and tPA-Ald-PM-NP were injected, followed by thromboplastin 20 min later. After 30 minutes, the lungs were collected and imaged. The fluorescence intensities of region-of-interests (ROI) were analyzed by Living Image Software.

In vivo bone marrow targeting capability

The *in vivo* multiple myeloma-bearing mice model was created as previously reported.[5] Female Nod/SCID mice were injected with 4×10^6 NCI-H929 cells per mouse *via* the tail vein. Three weeks later, the mice were intravenously administrated with saline, coumarin-6-loaded Ald-NP, coumarin-6-loaded PM-NP, coumarin-6-loaded Ald-PM-NP and coumarin-6-loaded tPA-Ald-PM-NP. After 6 hours, the femurs of the mice were collected and the bone marrow was extracted, frozen and sectioned. After staining with Hoechst, the bone marrow was subjected to a confocal microscope for observation.

In vivo multiple myeloma and lung thrombus treatment

Female Nod/SCID mice were injected with 4×10^6 NCI-H929 cells per mouse and randomly divided into 6 groups (n = 6). After two weeks, the mice were administrated with saline, bortezomib, Ald-NP-bort, PN-NP-bort, Ald-PM-NP-bort, and tPA-Ald-PM-NP-bort at the bortezomib concentration of 0.5 mg/kg and a tPA concentration of 0.1 mg/kg every other day for 2 weeks. Afterwards, the survival time of the mice were recorded. Furthermore, the femurs and organs of the mice were collected and sectioned for analysis. For the hematoxylin and eosin (H&E) staining, the slides were observed by optical microscope (DM5500B, Leica). For the TUNEL apoptosis assay, the fixed sections were stained by the *In Situ* Cell Death Detection Kit (Roche Applied Science) according to the manufacturer's protocol and observed by fluorescence microscope. The fluorescence signals in the images were quantitatively analyzed by Image J software. For *in vivo* lung clot thrombolysis evaluation, the mice treated with Ald-NP-NP-bort and tPA-Ald-PM-NP-bort were further induced with a lung clot by the addition of fibrinogen and thromboplastin. The lungs were collected, fixed, and sectioned for H&E staining and observed by optical microscope.

In vivo pharmacokinetics investigation

Nine mice were randomly divided in to three groups (n=3) and injected with free bortezomib, NP-bort and PM-NP-bort at the bortezomib dose of 5 mg/kg *via* the tail vein. At pre-determined time intervals (0.25, 0.5, 1, 3, 6, 12, 24, and 48 h), blood samples were collected and centrifuged at 800 g for 10 min. The concentration of bortezomib was then analyzed by HPLC.

Statistics

All results presented are Mean ± s. d. Statistical analysis was performed using Student's *t*-test. With a P value < 0.05, the differences between experimental groups and control groups were considered statistically significant.

Reference

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[2] Z. Fan, H. Zhou, P. Y. Li, J. E. Speer, H. Cheng, *Journal of Materials Chemistry B* **2014**, *2*, 8231-8238.
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[4] Z.-G. She, X. Liu, V. R. Kotamraju, E. Ruoslahti, *ACS Nano* **2014**, *8*, 10139-10149.
[5] A. Swami, M. R. Reagan, P. Basto, Y. Mishima, N. Kamaly, S. Glavey, S. Zhang, M. Moschetta, D. Seevaratnam, Y. Zhang, *Proceedings of the National Academy of Sciences* **2014**, *111*, 10287-10292.

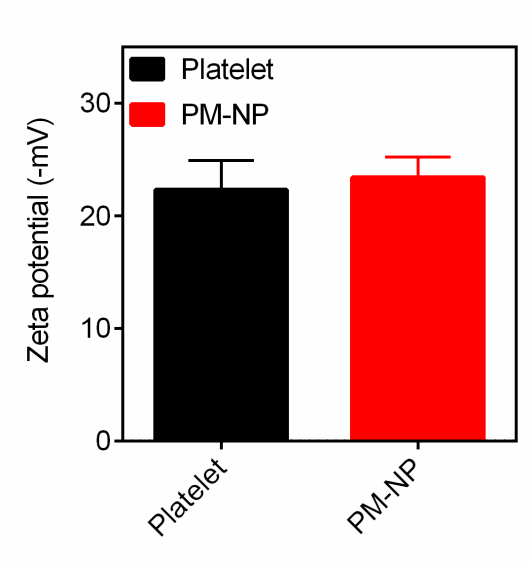

Figure S1. Zata potentials of platelet and PM-NP. Error bars indicated s.d. (*n*=3).

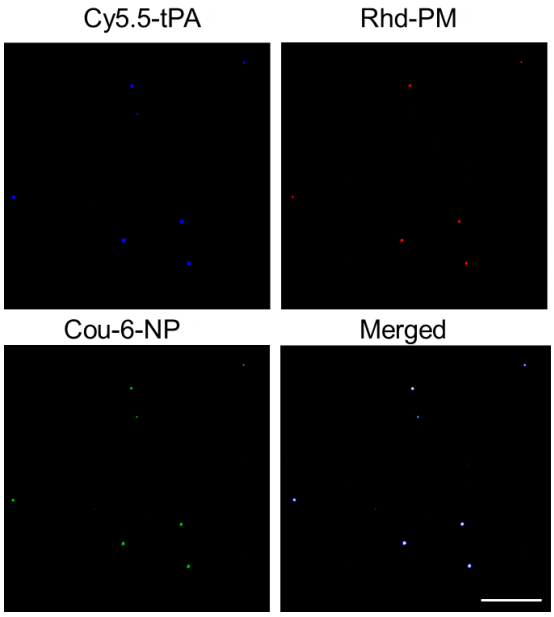

Figure S2. CLSM images of tPA-PM-NP. NP was loaded with coumarin-6, PM was labeled with rhodamine and tPA was conjugated with Cy5.5 for imaging. Scale bar: 50 μm.

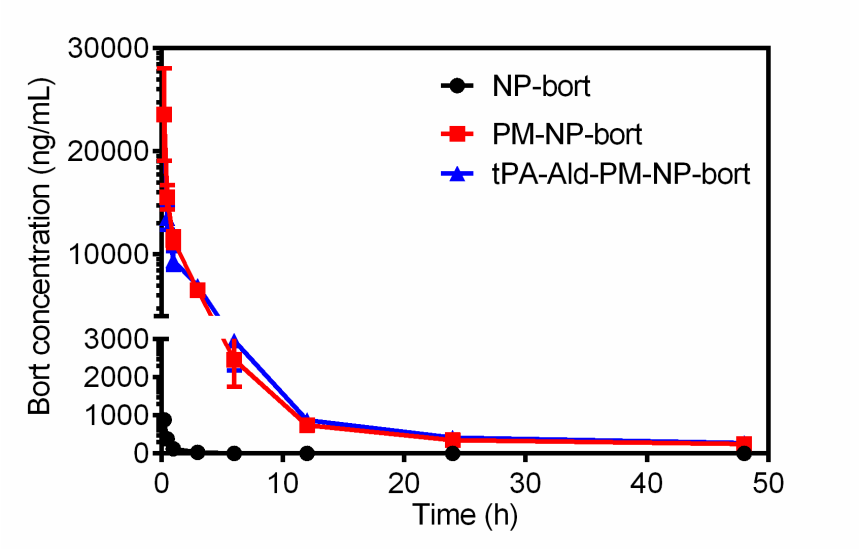

Figure S3. The plasma bortezomib concentration curves of the NP-bort, PM-NP-bort and tPA-Ald-PM-NP-bort. Error bars indicated s.d. (*n*=3).

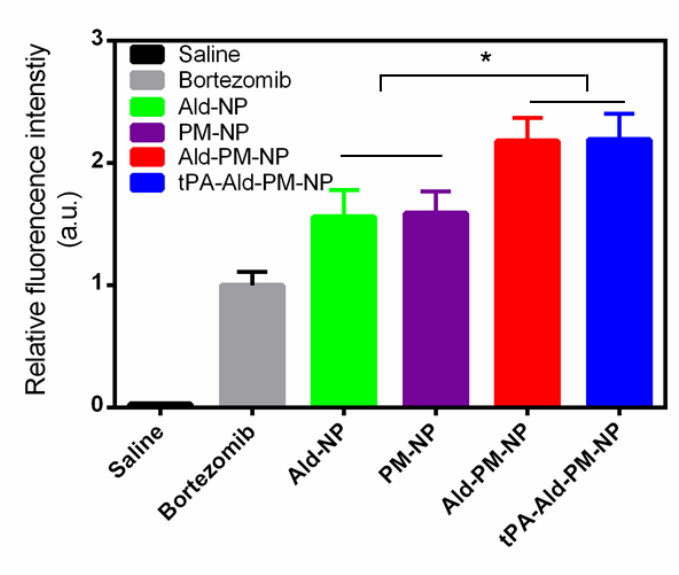

Figure S4. Quantitative analysis of the fluorescence intensities in the TUNEL assay, based on the imaging results of Figure 5D. All the fluorescence signals were normalized to the bortezomib group. Error bars indicate s.d. (*n*=3). **P* <0.05 (two-tailed Student's *t*-test).

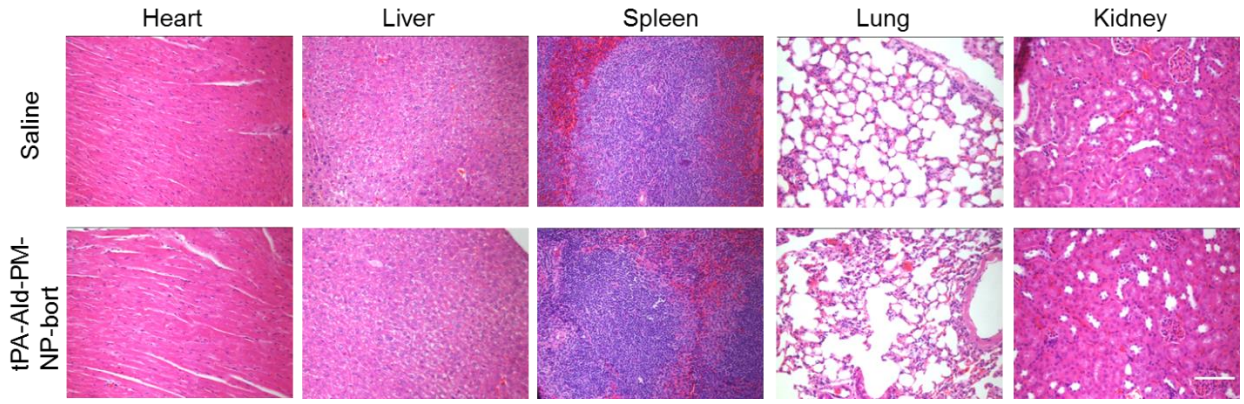

Figure S5. Histological observation of the organs collected from the MM-bearing Nod/SCID mice after the treatment. The organ sections were stained with H&E. Scale bar: 100 μm.

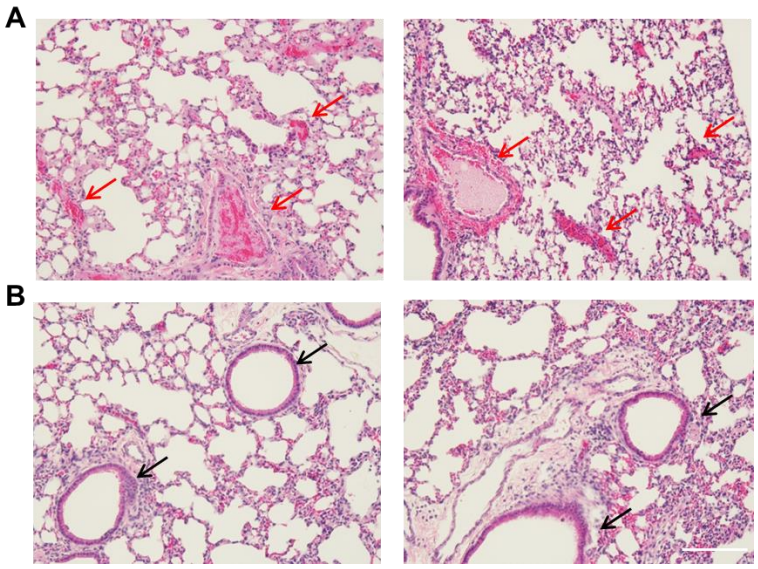

Figure S6. H&E staining of the lungs of the mice treated with Ald-PM-NP-bort (A) and tPA-Ald-PM-NP-bort (B) after induction of lung thrombus. Black arrows indicate the blood vessel. Red arrows indicate the blocked sites. Scale bar: 100 μm.