Molecular Pharmaceutics **Supporting Information**

Targeting of Tumor-Associated Macrophages Made Possible by PEG-Sheddable, Mannose-Modified Nanoparticles

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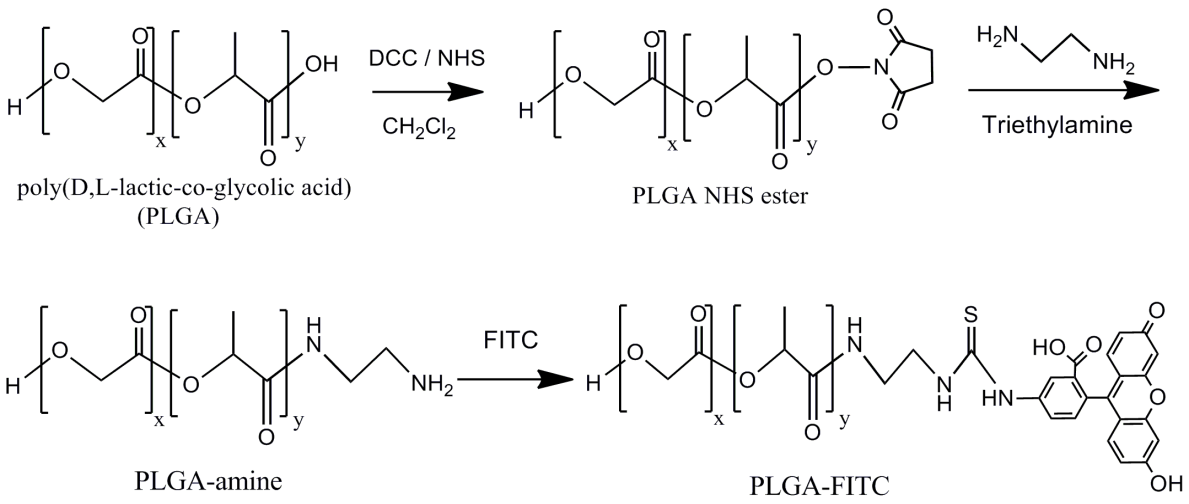
1. Materials

Stearoyl chloride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI), poly(D,L-lactic-*co*-glycolic acid) (PLGA) 752H, ethylenediamine (EDA), fluorescein isothiocyanate (FITC), N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC) and Lugol's solution were from Sigma-Aldrich (St. Louis, MO, USA). D-(+)-mannose was from TCI America (Portland, OR, USA). All solvents used in chemical synthesis were analytical grade.

2. Synthesis and characterization of PLGA-FITC

FITC-labeled PLGA752H was synthesized following a previously reported method with modification (Scheme S1).[1] Briefly, PLGA 752H (400 mg, 42.1 μmol) was dissolved in 10 mL of anhydrous methylene chloride (DCM), followed by the addition of NHS (15 mg, 126.4 μmol) and DCC (26 mg, 126.4 μmol). The reaction was stirred at room temperature overnight. The precipitate was removed by filtration, and the filtrate was added to 5 mL of DCM containing 25.4 mg of EDA (422.6 μmol). After another 24 h of reaction at room temperature, the solvent was evaporated under reduced pressure, and the white solid obtained was washed with diethyl ether (20 mL × 3) to give PLGA-amine, which was further reacted with FITC (65 mg, 166.9 μmol) in 4.5 mL of anhydrous dimethyl sulfoxide (DMSO) at room temperature overnight. The reaction mixture was dialyzed against DMSO and water (MWCO = 1000) sequentially, and lyophilized to give a yellow powder (195.7 mg, 46.8% yield).

As seen in Figure S1, the unlabeled PLGA 752H showed no absorbance within the range of 200 nm to 600 nm, while PLGA-FITC showed a UV absorbance profile similar to that of FITC, indicating the successful conjugation of FITC to PLGA.



Scheme S1. Synthesis of PLGA-FITC.

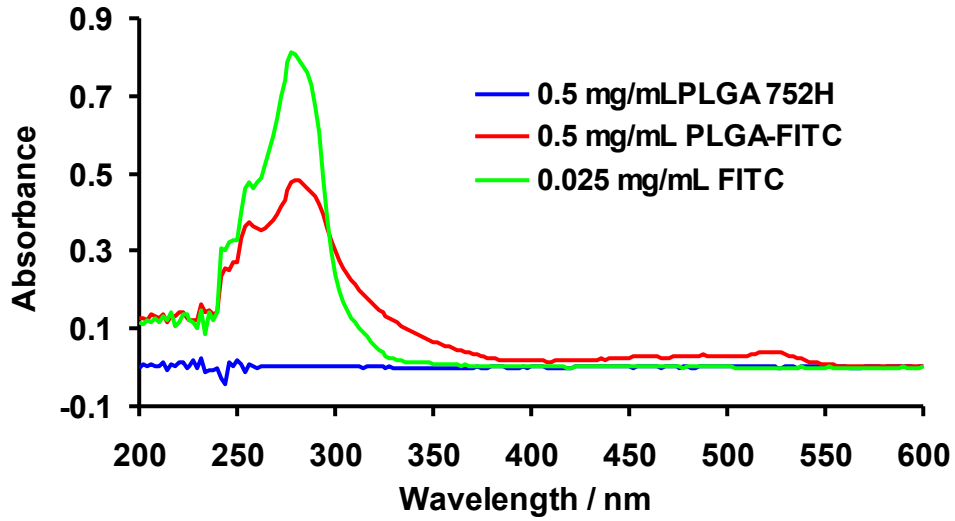
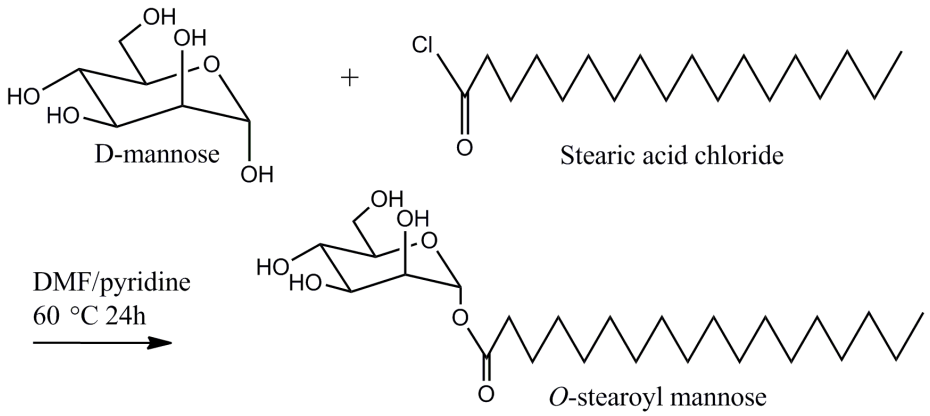


Figure S1. UV-Vis spectra of PLGA 752H, PLGA-FITC and FITC in DMSO.

3. Synthesis and characterization of *O*-stearoyl mannose

O-stearoyl mannose (M-C18) was synthesized by esterification of mannose with stearoyl chloride following a previously reported method with modifications (Scheme S2).[2, 3] Briefly, mannose (600 mg, 3.33 mmol) was dissolved in 15 mL of anhydrous dimethylformamide (DMF). To the resultant solution, 2.1 mL of anhydrous pyridine and 1.5 mL of anhydrous DMF

containing stearoyl chloride (165 mg, 0.545 mmol) were added subsequently, and the resultant mixture was stirred at 60°C for 24 h in the presence of argon. The reaction mixture was then dispersed into 60 mL of water and 90 mL of DCM. The collected DCM layer was re-extracted with water (100 mL × 2), washed with brine (100 mL × 2), dried with anhydrous Na_2SO_4 and concentrated. The crude product obtained was purified on a silica gel column ($CHCl_3$/methanol, 5:1) to give *O*-stearoyl mannose (97.6 mg, 38.6% yield) as a white solid. $R_f = 0.52$ ($CHCl_3$/methanol, 5:1); ^{1}H NMR (300 MHz, $CDCl_3$): 5.4-3.0 ppm (H from mannose), 2.30 ppm (t, 2H, COCH_2), 1.59 ppm (m, 2H, COCH$_2$CH_2), 1.26 ppm (m, 28H, (CH_2)$_{14}$), 0.88 ppm (t, 3H, CH_3). ESI-MS $[M+Na]^+$ *m/z* found: 469.3 (Figure S2).



Scheme S2. Synthesis of *O*-stearoyl mannose.

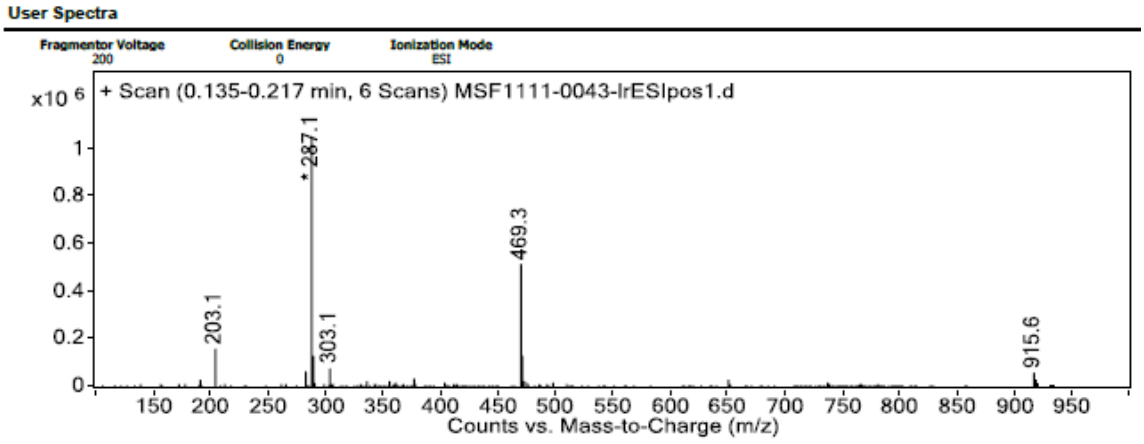
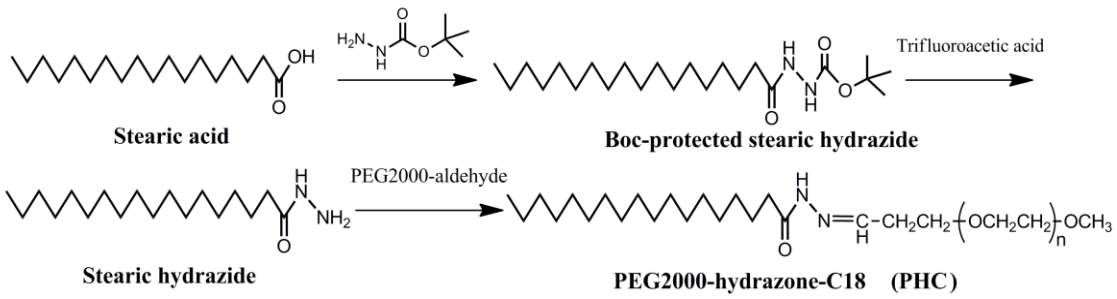


Figure S2. Mass spectrum of *O*-stearoyl mannose.

4. Synthesis and characterization of PEG-hydrazone-C18 (PHC)

PHC was synthesized according to Scheme S3 following our previously reported method and characterized by ^{1}H NMR.[4] Electrospray ionization mass spectrometry (ESI-MS), but not the more commonly used matrix-assisted laser desorption/ionization (MALDI-MS), was used to further characterize the PHC, because the acidic matrix used in the MALDI-MS led to quick a hydrolysis of the PHC. As seen in Figure S3, the PHC with two ions showed an *m/z* value of 1294.6 (Na^+ and K^+ adduct), by which the molecular weight of PHC was calculated to be 2527.2. Based on the molecular weight of the starting materials of PEG2000-CHO (Figure S4, 2269.1 Na^+ adduct) and stearic hydrazide (298.5), the expected molecular weight of the product should be 2526.6, which is in agreement with the ESI-MS result of PHC.



Scheme S3. Synthesis of PEG-hydrazone-C18 (PHC).

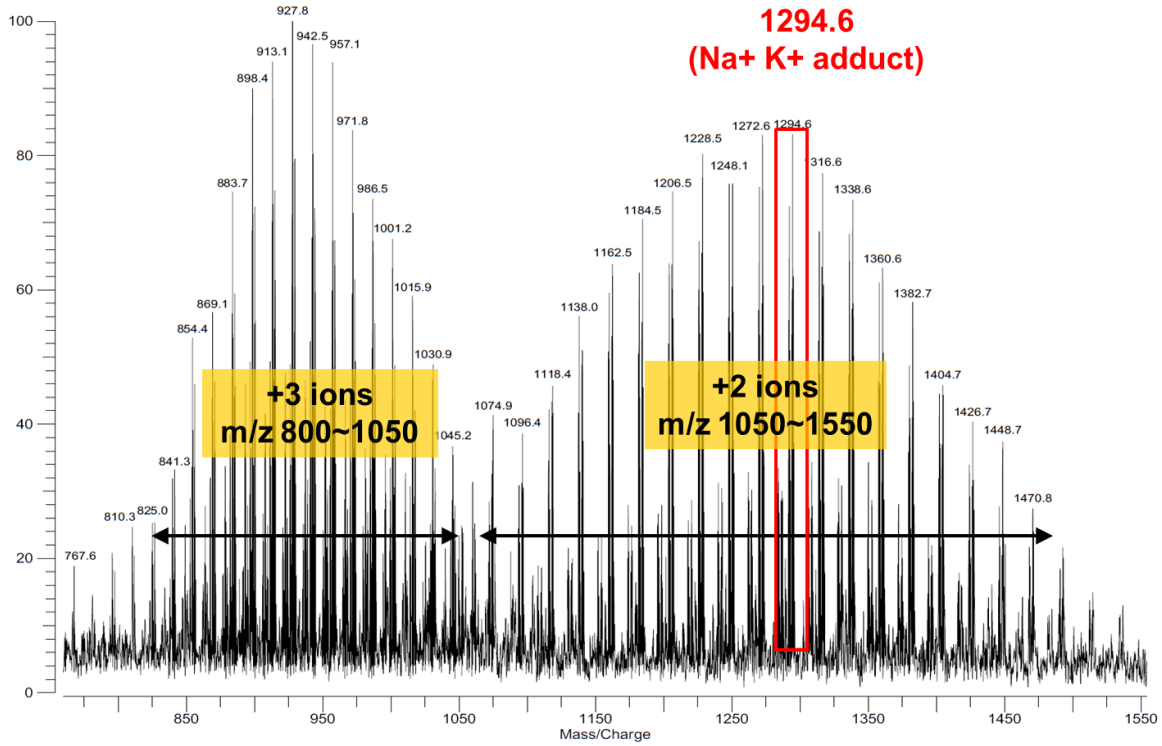


Figure S3. ESI-MS spectrum of PHC.

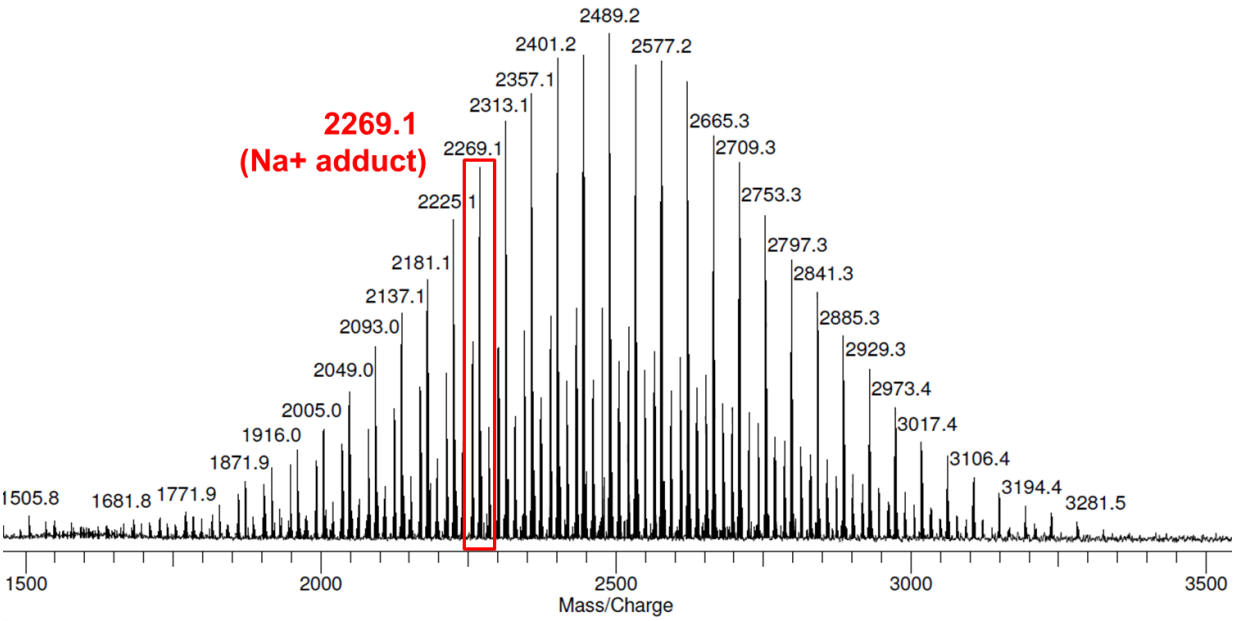


Figure S4. MALDI-MS spectrum of PEG2000-aldehyde.

5. Preparation and characterization of the nanoparticles

A nanoprecipitation method was used to prepare PLGA nanoparticle with various surface modification.[5] Briefly, 0.3 mL of tetrahydrofuran (THF) containing PLGA 752H (2.85 mg) and PLGA-FITC (0.15 mg) was added drop-wise into 1.5 mL of water under stirring. The nanoparticles were collected by centrifugation (13 000 × *g*, 10 min, 4°C) after the evaporation of THF. For the purpose of surface modification, various amounts of M-C18, PAC or PHC were incorporated into the formulations. Particle size and zeta potential of the nanoparticles were determined using a Malvern Zeta Sizer Nano ZS (MA, USA). The PEG on the surface of the nanoparticles was determined using an iodide staining method. The nanoparticles incubated at pH 6.8 for different periods of time were collected by centrifugation and mixed with Lugol's solution before measuring the $OD_{500\ nm}$ using a BioTek Synergy HT Multi-Mode Microplate Reader (Winooski, VT, USA).

6. *In vitro* stability study

The stability of the nanoparticles was investigated by evaluating the changes in particle size after incubation in 10% fetal bovine serum (FBS) for 18 h. As seen in Table S1, no significant change in the particle sizes was observed after 18 h incubation with 10% FBS. It should be noted that the particle size of the nanoparticles decreased from ~180 nm to around ~ 150 nm after mixing with 10% FBS, which is likely due to the serum proteins that have a small size.

Table S1. Sizes of the nanoparticles after 18 h of incubation with 10% FBS (n = 3). (0 h, sizes of nanoparticles measured immediately after diluted with 10% FBS).

	Particle size (nm)	
	0 h	**18 h**
NP	152.4 ± 1.6	156.8 ± 2.3
M-NP	156.2 ± 2.2	152.5 ± 2.5
AI-M-NP	149.9 ± 2.8	154.0 ± 2.3
AS-NP	148.3 ± 3.6	151.8 ± 7.0
AS-M-NP	152.2 ± 2.0	149.9 ± 5.2

7. Fluorescence intensities of nanoparticles

To exclude the possibility that the different fluorescence intensities observed in cells and animal tissues were due to the different fluorescence intensities of the nanoparticles added to the cell culture or injected into mice, we diluted the nanoparticle suspensions and measured their fluorescence intensities using a BioTek Synergy HT Multi-Mode Microplate Reader (Ex = 485 nm, Em = 528 nm). As seen in Table S2, all nanoparticles showed similar fluorescence intensities at the same dilution.

Table S2. Fluorescence intensities of various nanoparticles (n = 3).

	NP	**M-NP**	**AI-M-NP**	**AS-NP**	**AS-M-NP**
Fluorescence intensity	131.7 ± 0.6	130.7 ± 1.2	133.0 ± 3.6	131.7 ± 2.5	129.0 ± 1.7

8. *In vitro* cellular uptake

Murine macrophage cell line J774A.1 (American Type Culture Collection, Manassas, VA, USA) were seeded in a 24-well plate (2×10^5 cells/well) and incubated with FITC-labeled nanoparticles for 1 h. After incubation, cells were washed twice with cold phosphate buffered saline (PBS, 10 mM, pH 7.4) and lysed with a lysis buffer (Sigma-Aldrich). The fluorescence intensity of the cell lysates was measured (Ex = 485 nm, Em = 528 nm). To study the effect of mannose on the uptake of the nanoparticles, J774A.1 cells were pre-incubated in a medium that contained 2 mg/ml mannose for 45 min prior to the addition of the FITC-labeled nanoparticles (i.e., M-NP or AS-M-NP). Finally, to study the effect of the PEGylation of the nanoparticles on the uptake of the nanoparticles, the nanoparticles were pre-incubated in a pH 6.8 solution for 6 h to facilitate the shedding of the PEG chains before they were added into the cell culture medium.

9. Accumulation of nanoparticles in the liver and spleen

All care and handling of animals were performed in accordance with National Institutes of Health guidelines, and the animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. Female C57BL/6 mice (n = 3, Charles River Laboratories, Wilmington, MA, USA) were intravenously (i.v.) injected with nanoparticles (15 mg PLGA/mouse). The liver and spleen were collected 6 h later and cryosectioned. The resultant 5-μm tissue sections were fixed, counterstained with 4',6-diamidino-2-phenylindole (DAPI) and visualized under an Olympus fluorescence microscope (Center Valley, PA, USA).

10. Colocalization of nanoparticles with TAMs

Female C57BL/6 mice were s.c. injected in the right flank with B16-F10 murine melanoma cells (5 x 10^5 per mouse). When tumors reached 6-8 mm in diameter, mice (n = 3) were i.v. injected with nanoparticles (15 mg PLGA/mouse). Tumors were dissected 12 h after the injection, and 5-μm frozen sections were fixed and stained with phycoerythrin (PE)-conjugated rat anti-mouse CD68 antibody (BioLegend, San Diego, CA, USA). The slides (3 slides per mouse) were then examined under an Olympus fluorescence microscope.

References

1. Kim, S. H.; Jeong, J. H.; Chun, K. W.; Park, T. G. Target-specific cellular uptake of PLGA nanoparticles coated with poly(L-lysine)-poly(ethylene glycol)-folate conjugate. *Langmuir* **2005,** *21*, 8852-8857.
2. Singodia, D.; Verma, A.; Verma, R. K.; Mishra, P. R. Investigations into an alternate approach to target mannose receptors on macrophages using 4-sulfated N-acetyl galactosamine more efficiently in comparison with mannose-decorated liposomes: an application in drug delivery. *Nanomedicine* **2012,** *8*, 468-77.
3. Hammerli.U; Westphal, O. Synthesis and Use of O-Stearoyl Polysaccharides in Passive Hemagglutination and Hemolysis. *Eur J Biochem* **1967,** *1*, 46-50.
4. Zhu, S.; Wonganan, P.; Lansakara-P, D. S.; O'Mary, H. L.; Li, Y.; Cui, Z. The effect of the acid-sensitivity of 4-(N)-stearoyl gemcitabine-loaded micelles on drug resistance caused by RRM1 overexpression. *Biomaterials* **2013,** *34*, 2327-2339.
5. Dong, Y. C.; Feng, S. S. Methoxy poly(ethylene glycol)-poly(lactide) (MPEG-PLA) nanoparticles for controlled delivery of anticancer drugs. *Biomaterials* **2004,** *25*, 2843-2849.