Supplementary Information

Biofunctional Janus Particles Promote Phagocytosis of Tumor Cells by Macrophages

Ya-Ru Zhang[a], Jia-Qi Luo[b], Jia-Xian Li[a], Qiu-Yue Huang[a], Xiao-Xiao Shi[a], Yong-Cong Huang[b], Kam W Leong[b,f], Wei-ling He*[d], Jin-Zhi Du*[a,c,e]

[a]Guangzhou First People's Hospital, and Institutes for Life Sciences, School of Medicine, South China University of Technology
Guangzhou, 510006, China
E-mail: djzhi@scut.edu.cn
[b]School of Biomedical Sciences and Engineering, Guangzhou International Campus, South China University of Technology
Guangzhou, 510006, China
[c]National Engineering Research Center for Tissue Restoration and Reconstruction, Key Laboratory of Biomedical Materials and Engineering of the Ministry of Education, Key Laboratory of Biomedical Engineering of Guangdong Province, and Innovation Center for Tissue Restoration and Reconstruction, South China University of Technology, Guangzhou 510006, China
[d]Department of Gastrointestinal Surgery, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong, 510080, China
E-mail: hewling@mail.sysu.edu.cn
[e]Guangzhou Regenerative Medicine and Health Guangdong Laboratory, Guangzhou 510005, China
[f]Department of Biomedical Engineering Columbia University New York, NY 10027, USA

Materials. propargylamine, didodecyldimethylammonium bromide (DDAB), rhodamine B (RB), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), *N*-Hydroxysuccinimide (NHS), fluorescein isothiocyanate (FITC) were purchased from Shanghai Aladdin Bio-Chem Technology Co.,LTD (Shanghai, China). DBCO-PEG_4-NHS and DBCO-PEG_4-Mal were purchased from Click Chemistry Tools (Scottsdale, AZ, USA). Ferritin, bovine serum albumin (BSA), and transferrin (Tf) were purchased from Sigma-Aldrich. CFSE were purchased from Shanghai Biyuntian Bio-Technology Co., Ltd. (Shanghai, China). Anti-signal regulatory protein alpha antibodies (aSIRPα) and eFluor 670 were purchased from eBioscience. APC-tagged anti-F4/80 were purchased from BioLegend. Azide-functionalized silica nanoparticles were prerated according to the methods reported previously.[1] PEG_{2k}-Saline was synthesized as reported.[2] Other chemicals were purchased from Sinopharm Chemical Reagent Co. and used as received unless otherwise specified.

Characterizations. The particle size and zeta potential were recorded by dynamic light scattering (DLS) on Anton Paar Litesizer 500 Particle Analyzer (Anton Paar Corporation, Austria). The morphology of the particles was observed by a field emission scanning electron microscope (SEM, JSM-7001F, JEOL, Ltd. Japan) and a transmission electron microscope (TEM JEM-2100F, JEOL Ltd., Japan). FTIR spectroscopy was performed on a Dig lab FTS 3000 instrument. Fluorescence images were obtained using an Axiovert 200 M inverted microscope with a 40× and 100× objectives (Zeiss, Oberkochen, Germany) equipped with a FluoArc lamp. The concentration of nanoparticles was quantified by inductively coupled plasma-atomic emission spectrometry (Thermo Fisher Scientific, iCAP 7000 Plus, USA). Flow cytometry was performed using the FACS Calibur (BD Biosciences) and analyzed by the FlowJo software package (version 10.0.7; TreeStar, USA). Confocal laser microscopy (Nikon, A1+, Japan) and high-content analysis system (PerkinElmer, operetta CLS, USA) were used to study the phagocytosis behavior.

Synthesis of FITC-labeled SPA1. One gram of SPA1 was ultrasound dispersed in 10 mL of DMF, 0.03 molar fold propargylamine was added. Then, a solution of $CuSO_4$ (6.225×10^{-8} mol) in 1 mL of water and sodium ascorbate (12.450×10^{-8} mol) in 1 mL of water were added orderly into the solution. The reaction was run for 24 h under inert atmosphere at room temperature. After that, the particles were isolated *via* centrifugation at 9000 rpm and rinsed with a solution of the sodium salt of EDTA and a water/ethanol (1/1, v/v) mixture to remove the copper catalyst and obtain the amino-modified silica nanoparticles (SPM1). The obtained SPM1 were dispersed in carbonate buffer, to which FITC was added. The reaction was stirred overnight at room

temperature. Then, the particles were washed by carbonate buffer and water to remove FITC to yield the FITC-labeled SPA1.

Synthesis of RB-labeled SPA3. RB labeled SPA3 was synthesized by conjugating the carboxylic group of RB with the amino-modified silica nanoparticles. In brief, 10 mg of NHS and 40 mg of EDC were added in RB (50 mg) dissolved in deionized water (2 mL). Over 2 h of stirring, SPM3, which was prepared similarly to SPM1, was added. Then, the mixture was stirred for 24 h at room temperature. After washing with water to remove excess RB, RB-labeled SPA3 was obtained.

Preparation of wax colloidosomes by Pickering emulsion. The wax colloidosomes were prepared using Pickering emulsion according to Granick group with some modifications.[3] Freshly, 27 mg of SPA1 (36 mg for SPA2, 43 mg for SPA3) were homogenously dispersed in water, and then mixed with 216 mg of paraffin, this entire setup was heated to 80 °C to melt the wax. The wax suspension was then mixed with DDAB, and the mixture was subjected to vigorous agitation by using an Ultra Turrax homogenizer at 10000 rpm for 90 s, and then cooled to room temperature for the solidifcation of the wax. Finally, the obtained colloidosomes were filtered and washed with deionized water to remove excess DDAB and unbound small particles. Colloidosome of FITC-labeled SPA1 or RB-labeled SPA3 was prepared following similar procedures.

Synthesis of native PEG-SPA1 Janus nanoparticles (PEG-SPA1 JNPs). Firstly, 100 mg of the wax colloidosomes stabilized by SPA1 were dispersed in 9 mL of absolute ethanol. 8 mg mL^{-1} PEG_{2k}-Saline solution in ethanol was slowly added to this mixture under N_2 bubbling at room temperature for 1 h. Then, the colloidosomes were washed thrice with ethanol to remove PEG_{2k}-Saline residues. Cyclohexane was added to dissolve the wax, and after centrifuge, the native PEG-SPA1 JNPs was obtained and used for subsequent protein conjugation.

Synthesis of native Mal-SPA Janus particles (Mal-SPA JPs). The colloidosomes were dispersed in PBS buffer (pH = 7.4), to which was added DBCO-PEG_4-Mal solution in dimethyl sulfoxide (DMSO) to achieve a final molar ratio of azide groups to DBCO-PEG_4-Mal around 1:5. The reaction was allowed to proceed for 4 h at room temperature and the coupled product were then washed thrice with ethanol and water. Next, cyclohexane was added to these wax droplets overnight at room temperature to dissolve the wax to obtain native Mal-SPA JPs. The native Mal-SPA JPs have azide groups and maleimide groups on opposing faces of the particles and were used for subsequent protein conjugation. A variety of native Mal-SPA JPs with different sizes, including Mal-SPA1 JPs, Mal-SPA2 JPs, Mal-SPA3 JPs, FITC-labeled Mal-SPA1 JPs, RB-labeled Mal-SPA3 JPs, were prepared following the same procedures.

Synthesis of FITC-labeled BSA (FITC-BSA) or RB-labeled BSA (RB-BSA). For FITC-BSA, 6 mg of FITC were dispersed in carbonate buffer solution (pH = 9), then BSA was were added and stirred for 12 h in dark at room temperature. After that, the products were purified by using a centrifugal filter device (Amicon Ultra-0.5, Millipore Co, German) to remove the excess FITC, FITC-BSA was obtained. RB-labeled BSA was prepared similarly to RB-labeled SPA3 by conjugating the carboxylic group of RB with BSA.

Protein modification with DBCO-PEG$_4$-NHS linker. For the bio-functionalization conjugation, a variety of proteins including ferritin, BSA, Tf, and aSIRPα need to be modified with DBCO-PEG$_4$-NHS linker in advance. Firstly, a 50-fold molar excess of DBCO-PEG$_4$-NHS linker was added to ferritin solution and incubated at 4 °C overnight. Then the solution was filtered using a centrifugal filter device at 7000 rpm at 4 °C for 30 min and suspended in water to obtain DBCO-PEG$_4$-ferritin. DBCO-PEG$_4$-Tf, RB-labeled DBCO-PEG$_4$-BSA, FITC-labeled DBCO-PEG$_4$-BSA, DBCO-PEG$_4$-aSIRPα were prepared following similar procedures.

Synthesis of thiol-functionalized protein. Firstly, 10 mg of FITC-BSA was dissolved in 1 mL sodium phosphate EDTA buffer (pH 8.0, 0.1 M), to which 1 mg of 2-iminothiolane HCl was added. The solution was then reacted at 4°C for 1 h. After ultrafiltration using a centrifugal filter device at 7000 rpm at 4 °C for 30 min, thiol-functionalized FITC-BSA was obtained. Thiol-functionalized Tf and RB-BSA were prepared following similar procedures.

Synthesis of ferritin and PEG functionalized Janus nanoparticles (PEG-SPA1-ferritin JNPs). For the ferritin conjugation, the DBCO-PEG$_4$-ferritin was conjugated to native PEG-SPA1 JNPs *via* copper-free click chemistry according to protocol described previously[4] to generate PEG-SPA1-ferritin JNPs.

Synthesis of FITC-BSA or RB-BSA functionalized Janus microparticles (FITC-SPA3 JMPs, RB-SPA3 JMPs). 10 mg of native Mal-SPA3 JPs were dispersed in PBS (pH=7.4) buffer, and thiol-functionalized FITC-BSA or RB-BSA were added. This mixture was stirred at room temperature for 12 h to obtain FITC-SPA3 JMPs or RB-SPA3 JMPs.

Synthesis of FITC-BSA and RB-BSA functionalized Janus microparticles (FITC-SPA3-RB JMPs). For dual-color FITC-SPA3-RB JMPs, RB-labeled DBCO-PEG$_4$-BSA were attached to the surface of FITC-SPA3 JMPs *via* copper-free click chemistry.

Synthesis of Tf and BSA functionalized Janus nanoparticles (Tf-SPA1-BSA JNPs). For the Tf conjugation, thiol-functionalized Tf was conjugated to FITC-labeled native Mal-SPA1 JPs as mentioned above to generate the intermediate product of Tf-SPA1 JNPs. Then, DBCO-PEG$_4$-BSA was conjugated to Tf-SPA1 JNPs via copper-free click chemistry to generate FITC-labeled Tf-SPA1-BSA JNPs.

Synthesis of FITC-BSA and RB-BSA functionalized uniform nanoparticles (FITC-SPA3-RB UNPs). As a control, we prepared nanoparticles coated with a uniform distribution of FITC-BSA and RB-BSA. SPA3 were co-incubated with FITC-labeled DBCO-PEG_4-BSA and RB-labeled DBCO-PEG_4-BSA for 12 h at 4 °C *via* copper-free click chemistry.

Synthesis of Tf and BSA functionalized uniform nanoparticles (Tf-SPA1-BSA UNPs). As a comparison, we prepared FITC labeled SPA1 that were uniformly coated with Tf and BSA. Firstly, FITC labeled SPA1 were incubated with DBCO-PEG_4-Tf at RT for 12 h via click chemistry, the particles were collected by centrifugation at 9000 rpm for 10 min, and supernatant was collected. And then obtained intermediate product were incubation with DBCO-PEG_4-BSA at RT for an additional 12 h. The final product was centrifuged and washed with free H_2O and supernatant was collected.

Synthesis of Tf and aSIRPα functionalized Janus nanoparticles (Tf-SPA1-aSIRPα JNPs). For the Tf and aSIRPα conjugation, thiol-functionalized Tf and DBCO-PEG_4-aSIRPα were sequentially bonded to the surface of the native Mal-SPA1 JPs *via* click chemistry to obtain Tf-SPA1-aSIRPα JNPs. The supernatant generated during the preparation process was collected.

Synthesis of Tf and aSIRPα functionalized uniform nanoparticles (Tf-SPA1-aSIRPα UNPs). As a control, we prepared nanoparticles coated with a uniform distribution of Tf and aSIRPα. SPA1 were co-incubated with DBCO-PEG_4-Tf and DBCO-PEG_4-aSIRPα for 12 h at 4 °C *via* click chemistry. Finally, the particles were collected by centrifugation at 9000 rpm for 10 min, and the supernatant was collected.

Synthesis of Tf and aSIRPα functionalized Janus microparticles (Tf-SPA3-aSIRPα JMPs). Tf-SPA3-aSIRPα JMPs were prepared following similar procedures of Tf-SPA1-aSIRPα JNPs.

Synthesis of Tf and aSIRPα functionalized uniform microparticles (Tf-SPA3-aSIRPα UMPs). Tf-SPA3-aSIRPα UMPs were prepared following similar procedures of Tf-SPA1-aSIRPα UNPs.

Quantification of conjugated proteins on particles. The conjugated proteins were quantified by calculating the difference from feeding amount and supernatant amount. The unbound Tf and BSA concentrations were measured in the supernatant with standard protein-concentration curves (QuantiPro BCA Assay Kit, Beyotime). The concentrations of unbound aSIRPα were measured with an immunoglobulin G-1 (IgG1) ELISA Kit (Neobioscience). Concentrations of particles-bound proteins were calculated according to the following formula: bound proteins = total feeding proteins - unbound proteins in solution.

Cell lines. The murine B16F10 melanoma cell line was purchased from American Type Culture Collection (ATCC), cells were cultured in the Dulbecco's minimum essential medium (Gibco) containing 1% penicillin (Invitrogen) and 10% fetal bovine serum (Gibco) at 37 °C in 5% CO_2. Macrophages were extracted from the bone marrow of C57/BL6 mice femurs according to a previously established protocol,[5] and maintained in Dulbecco's minimum essential medium supplemented with a 30% L929 cell-conditioned medium, 10% fetal bovine serum (FBS), and 1% penicillin.

Tf-SPA1-BSA JNPs target to B16F10 cells. To study the targeting ability of Tf-SPA1-BSA JNPs, B16F10 cells were treated with FITC labelled SPA1, Tf-SPA1-BSA JNPs or Tf-SPA1-BSA JNPs pretreated with 100 μg/mL of free Tf, respectively, at 37 °C and 5% CO_2 for 1 h. After PBS rinsed to remove unbound nanoparticles, flow cytometry and confocal microscope were used to study the cellular uptake behavior.

Binding-avidity assay. B16F10 cells were plated in 24-well plates at a population of $5x10^4$ cells/well. Then, cells were incubated in 0.45 mL of DMEM containing various concentrations of FITC labeled free Tf, Tf-SPA1-BSA JNPs or Tf-SPA1-BSA UNPs at 37 °C and 5% CO_2 for 1 h. After PBS rinsed to remove unbound nanoparticles, flow cytometry was used to quantify the mean fluorescence intensity (MFI) at 488 nm. A Scatchard analysis gave the dissociation constant (K_D) of free Tf, Tf-SPA1-BSA JNPs and Tf-SPA1-BSA UNPs from Tf receptors of B16F10 cells.

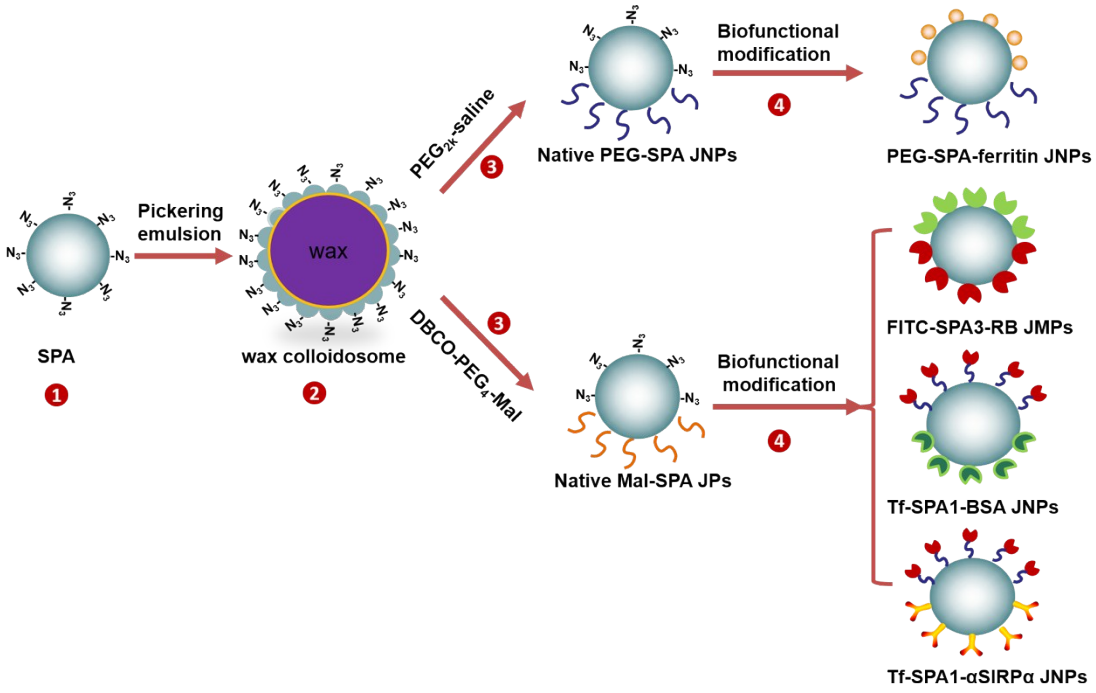
Tf-SPA1-aSIRPα JNPs target to tumor cells or macrophage. FITC-tagged Tf-SPA1-aSIRPα JNPs or Tf-SPA1-aSIRPα UNPs with same protein conjugation density were incubated with eFluor 670 labeled B16F10 cells or anti-F4/80 labeled BMDMs. After 1 h incubation, the cells were washed with cold PBS. Flow cytometry were used to study the binding ability to B16F10 cells or BMDMs.

Phagocytosis assay. eFluor 670 labelled BMDMs were plated in 12-well plates at 5 x 10^4 cells/well. Then, 3.5 x 10^5 CFSE labelled B16F10 cells were added and co-cultured with the cancer cells for 6 h at 37 °C with the different treatments in medium. The treatment conditions were as follows: PBS, Tf+aSIRPα+SPA1 (free Tf (4 μg mL^{-1}), free aSIRPα (8 μg mL^{-1})), Tf-SPA1-aSIRPα JNPs, Tf-SPA1-aSIRPα UNPs (normalized to free aSIRPα/Tf dose), respectively. After 6 h incubation, cells were then collected and washed twice with cold PBS. Flow cytometry and confocal microscope were used to study the phagocytosis behavior of cancer cells by BMDMs.

Tracking the time course of phagocytosis. To observe the time-course of phagocytosis, $1×10^4$ eFluor 670 labelled BMDMs were plated in 12-well plates for 12 h, after which $3×10^4$ CFSE

labelled B16F10 cells and Tf-SPA3-aSIRPα JMPs were added and the co-cultures incubated for an additional 90 min at 37 °C. Then, phagocytosis process was observed with high-content confocal imaging system.

Results



Scheme S1. Schematic representation of the synthesis route of biofunctional JPs. Step 1: Unfunctionalized silica particles (SPs) were modified with azide group. Step 2: Formation of wax colloidosomes stabilized by the SPA. Step 3: The colloidosomes are functionalized with DBCO-PEG_4-Mal or PEG_{2k}-Saline and the wax was further dissolved to yield native JPs. Step 4: Native JPs with distinct groups on their respective sides were functionalized with different types of proteins or antibodies *via* click chemistry.

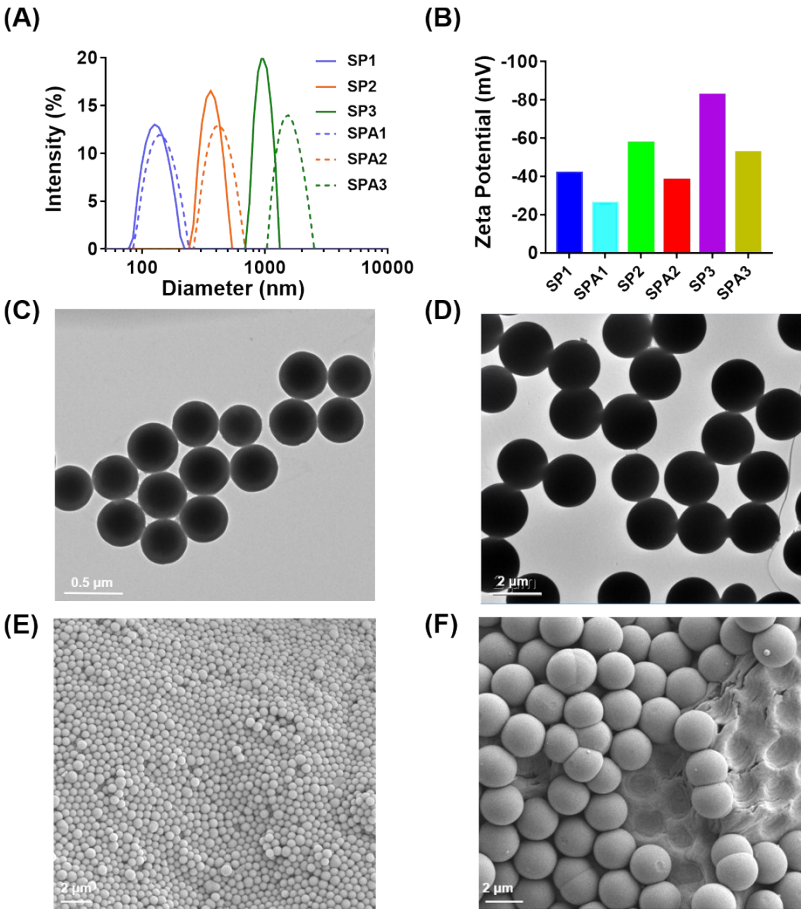


Figure S1. (A) Dynamic light scattering (DLS) measurements showing size distributions of SP and SPA. (B) Zeta potential measurements for different SP and SPA. Data are presented as mean ± SD (n = 3). TEM micrographs of the prepared SPA2 (C) and SPA3 (D). SEM micrograph of wax colloidosome stabilized by SPA2 (E) and SPA3 (F), respectively.

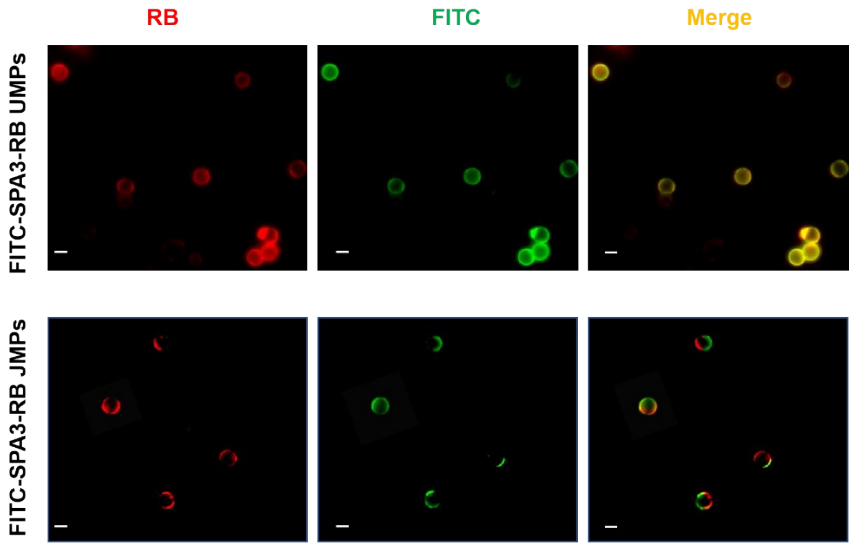


Figure S2. Fluorescence images of FITC-SPA3-RB UMPs and FITC-SPA3-RB JMPs. Scale bar, 2 μm.

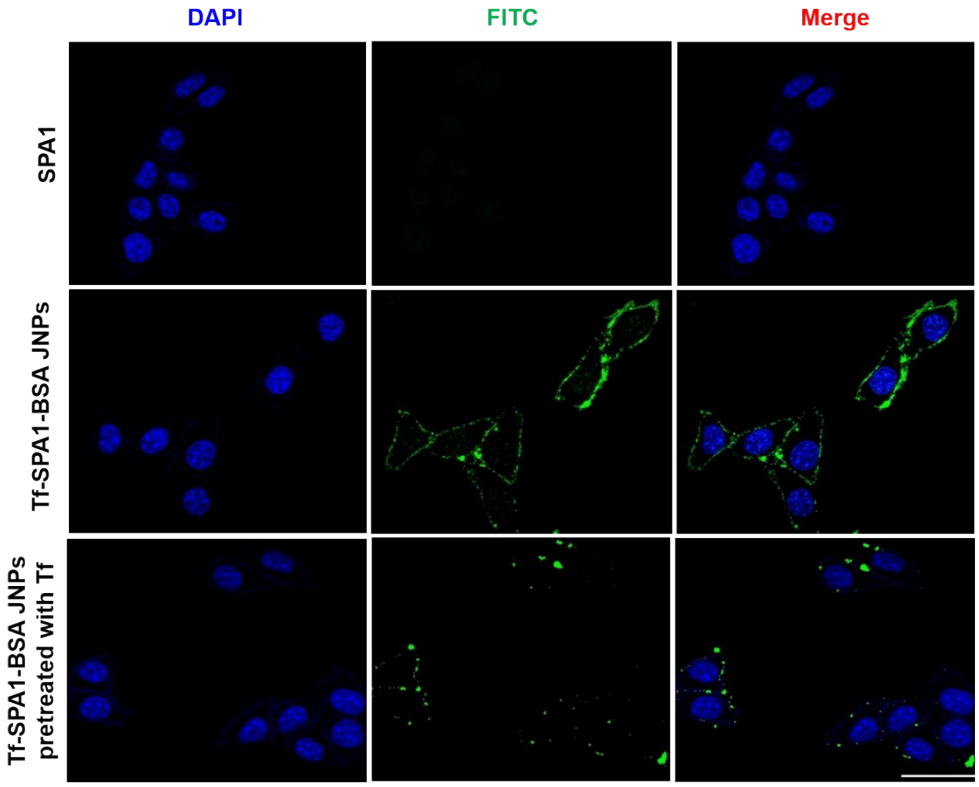


Figure S3. Representative confocal images of B16F10 cells treated with FITC-labeled SPA1 (upper) and FITC-labeled Tf-SPA1-BSA JNPs in the absence (middle) or presence (lower) of free Tf. Scale bar, 50 μm.

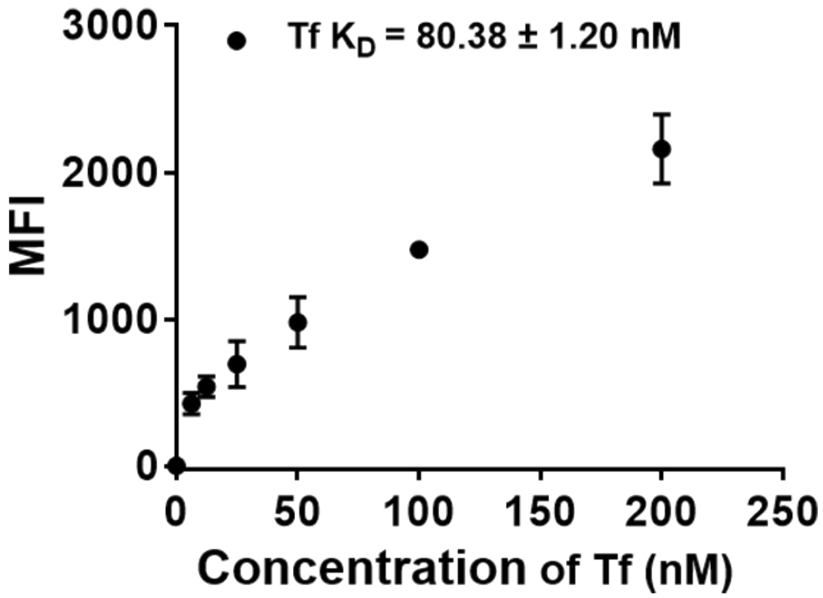


Figure S4. Saturation binding of Tf to B16F10 cells.

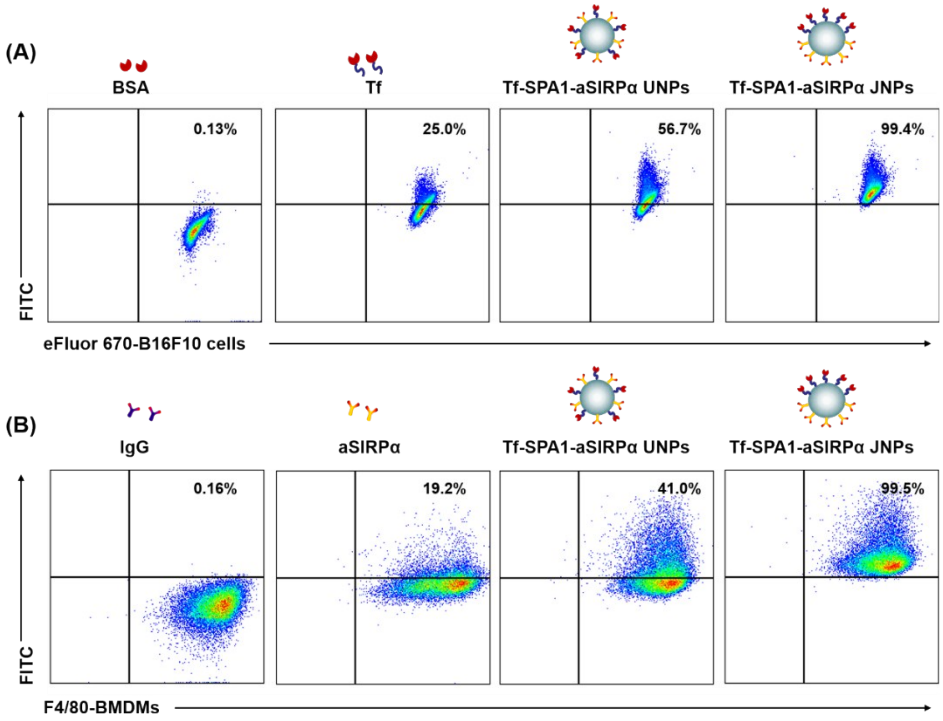


Figure S5. Representative FACS plots showing the binding ability of different treatments to B16/F10 cells (A) and BMDMs (B). B16/F10 cells were labelled with eFluor 670, BMDMs were labelled with anti-F4/80.

Table S1. Size and zeta potential analysis of SPA1 and SPA3 with different biofunctionalities.

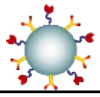
Sample	Nanoparticles	Zeta potential (mV)	Size (nm)
SPA1		-26.0 ± 1.1	150 ± 5
PEG-SPA1-ferritin JNPs		-25.0 ± 0.7	165 ± 5
FITC-SPA3-RB JMPs		-60.0 ± 2.0	2652 ± 64
Tf-SPA1-BSA JNPs		-36.0 ± 1.2	184 ± 1
Tf-SPA1-BSA UNPs (control)		-33.0 ± 3.0	165 ± 6
Tf-SPA1-aSIRPα JNPs		-22.0 ± 1.6	187 ± 5
Tf-SPA1-aSIRPα UNPs (control)		-24.0 ± 1.0	188 ± 4
SPA3		-52.0 ± 0.2	2300 ± 15
Tf-SPA3-aSIRPα JMPs		-44.0 ± 0.4	2538 ± 60
Tf-SPA3-aSIRPα UMPs (control)		-50.0 ± 5.4	2413 ± 22

Table S2. The conjugation amount of BSA, Tf and aSIRPα on the surface of prepared biofunctional JNPs

Particle	Tf per Particle (Tf/NP)	BSA per Particle (BSA/NP)	aSIRPα per Particle (aSIRPα/NP)
Tf-SPA1-BSA JNPs	12 ± 5	15 ± 4	-
Tf-SPA1-BSA UNPs	15 ± 4	14 ± 4	-
Tf-SPA1-aSIRPα JNPs	12 ± 5	-	17 ± 5
Tf-SPA1-aSIRPα UNPs	15 ± 4	-	15 ± 6
Tf-SPA3-aSIRPα JMPs	4795 ± 95	-	4772 ± 105
Tf-SPA3-aSIRPα UMPs	4655 ± 112	-	4895 ± 92

Reference:

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