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# ESI

**A tumour microenvironment-responsive polymeric complex for targeted depletion of tumour-associated macrophages (TAMs)**

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**Supplementary Experimental Section**

*Cell culture and antibodies.* Human umbilical vein endothelial cells (HUVEC), mouse macrophage cell line Raw 264.7, human acute monocytic leukaemia cell line (THP-1), mouse sarcoma cells (S180) and mouse hepatoma solidity cells (Heps) were all purchased from the American Type Culture Collection (ATCC, VA, USA). HUVEC and THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS). Raw 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. All cells were maintained at 37 °C with 5% $CO_2$. All reagents used in cell culture were purchased from Gibco Life Technology (MD, USA).

Rat anti-mouse F4/80 antibody (Cat. No. 123102) and rat anti-mouse CD8a antibody (Cat. No. 100801) were purchased from Biolegend (USA).Rabbit anti-mouse PECAM-1 antibody (Cat. No. M-185; sc-28188) and goat anti-mouse IFN-γ antibody (Cat. No. D-17; sc-9344) were provided by Santa Cruz (USA). Alexa Fluor 488-conjugated donkey anti-rat IgG (H+L; Cat. No. A-21208), Alexa Fluor 488-conjugated donkey anti-goat IgG (H+L; Cat. No. A-11055) and Alexa Fluor 546-conjugated donkey anti-rabbit IgG (H+L; Cat. No. A10040) were purchased from Life Technology (USA).

*Characterisation of $P^3AB$*. We analysed the particle sizes of AB and $P^3AB$ (suspended in $dH_2O$, 100 μg/ml) with Nanoparticle Tracking Analysis system (NS500, Nanosight, UK) at 25°C and measured their zeta-potential with Zetasizer Nano Z (Malvern, UK). To examine the structure of $P^3AB$ in a visualised manner, we labelled PPP shell with Cy5 during preparation. Once the first emulsion was obtained, it was slowly poured into PVA containing Cy5 (0.5 mg/ml). The fluorescent-labelled particles including PPP (Cy5), AB (FTSC) and $P^3AB$ (Cy5+FTCS) were observed under confocal microscopy, during which z-axis scanning was performed to examine the formation of the spherical structure. Further, we observed the morphology of $P^3AB$ treated with or without MMP-2 with transmission electron microscopy (TEM, JEM 1200EX, JOEL, Japan). Samples were incubated

in Tween 80 aqueous solution (0.5 %, w/w) with or without MMP-2 (5 ng/μl) followed by centrifugation (15,000 rpm) and rinsing before sample processing for TEM observation.

*In vitro cytotoxicity of PPP, AB and P³AB.* Cells were pre-seeded in 96-well plates for 24 h ($1.0 \times 10^4$ per well) and were treated with the AB core and the P³AB complex at various concentrations (0.01 – 10 μM, equivalent to that of ALN) in medium with 0.5% FBS. Another group, the P³AB incubated with MMP-2 (5 μg/ml) for 48 h was also added to evaluate the cytotoxicity. After 24-hour incubation, CCK-8 solution (10 μl/well) was added to wells, the plates were incubated for 4 h, and the absorbance of each well at 450 nm was recorded by Multilabel Counter. The relative cell viability of each group was calculated as below,

$$x = \frac{a - b}{c - b} \times 100\%$$

Where $a$ is the absorbance of assay group, $b$ is the absorbance of blank groups and $c$ is the absorbance of control groups. Blank groups were the wells with medium only, and control groups were the wells with medium and cells.

*Cellular uptake assay.* To study the cellular uptake of AB-FTSC released from P³AB, RAW264.7 cells ($1.0 \times 10^5$ cells/well) were seeded in 96 wells plate and incubated with 100 μL P³AB (100 μg/ml, in DMEM/0.5% FBS) with or without MMP-2 (5 μg/ml) for 2 h, 6 h, 12 h and 24h, respectively. After that, the culture medium was removed and washed three times with cold PBS, and Triton X-100 (0.5% in 0.2 M NaOH) solution was added to lyse the cells. Fluorescent Spectrometer (Lumina, Thermo Scientific, USA) was used to measure the fluorescence intensity.

The relative percentage of cellular uptake was calculated as below,

$$y = \frac{F_a}{F_t} \times 100\%$$

Where $F_a$ is the fluorescence intensity of assay group, $F_t$ is the fluorescence intensity of total AB-FTSC.

For flow cytometry assay, the released AB-FTSC was separated by centrifugation (6000 rpm). The pre-treated $P^3$AB (500 μl/well) was added into 6-well plates pre-seeded with Raw 264.7 cells ($1.0 \times 10^6$ cells/well). After 24 hours, cells were scraped and collected by centrifugation at 1000 rpm for 5 min, washed with ice-cold PBS for three times and tested with BD Accuri C6 flow cytometry. Cells were gated with forward-versus-side scatter to exclude dead cells and debris, and data collected with 10,000 cell counts. For confocal microscopy assay, Raw 264.7 cells were pre-seeded in Petri dishes (35-mm glass-bottom, SPL, Korea, $2.0 \times 10^6$ cells/dish) and incubated with the addition of pre-treated $P^3$AB (500 μl/well) for 24 h. Cells were then stained with Hoechst 33258 (1 μg/ml) for 30 min and washed with ice-cold PBS for three times before the photos were taken under a Leica TCS SP 8 Laser confocal microscope.

*Measurement of cytokines and AST/ALT.* Samples of blood and tumour tissue were collected from mice treated with saline, AB or $P^3$AB. The blood samples were incubated and centrifuged to harvest serum for the measurement of aspartate transaminase (AST) and alanine transaminase (ALT) activity, by using microplate-based assay kits following the instructions from the manufacturer (Nanjing Jiancheng Bioengineering Institute, China). The tumour samples were weighed, homogenised in a tissue extraction reagent (Novex, Life Technologies, USA; 10 ml per 1 g tissue), and centrifuged to collect the supernatant. The levels of interleukin-10 (IL-10), interferon γ (IFN-γ), and vascular endothelial growth factor-A (VEGF-A) in the supernatant were determined with corresponding ELISA kits (R&D Systems, USA, for IL-10 and VEGF-A; Abcam, UK, for IFN-γ). Each group in each test contains six parallel samples.

**Supplementary figure legends**

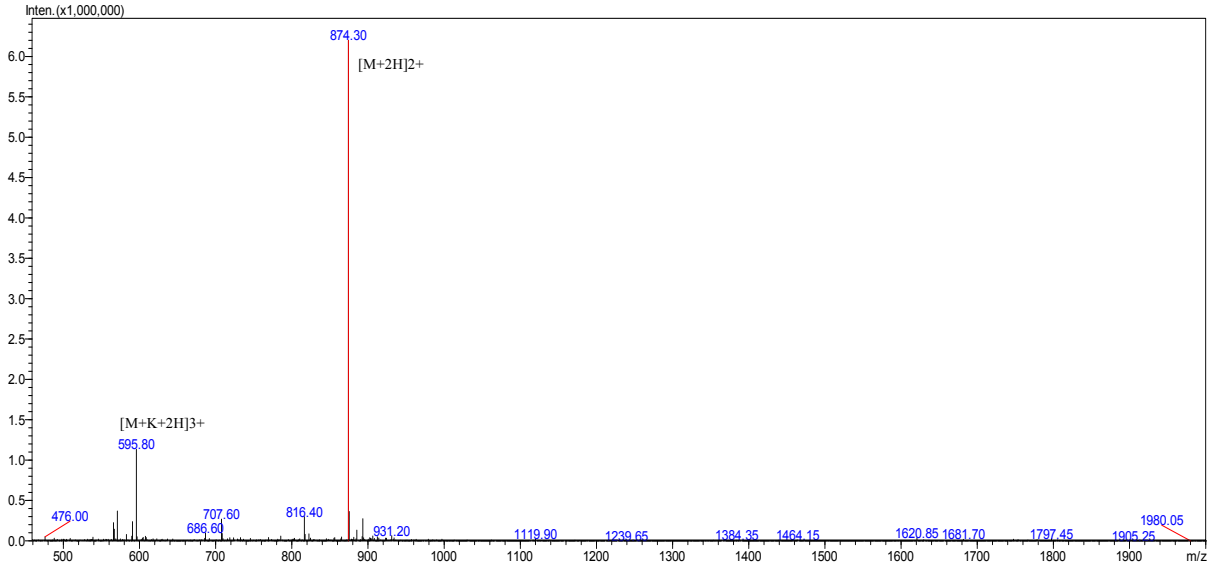
**Figure S1**. Mass spectrometry report for the synthesised, MMP-sensitive peptide.

**Figure S2**. Schematic diagram of the preparations of (A) oxidised BSP; (B) AB and (C) FTSC-conjugated AB.

**Figure S3**. Fluorescent spectra of 5-FTSC, AB and FTSC-conjugated AB.

**Figure S4**. Flow cytometric analysis to compare the expression level of MMR in differently polarised Raw 264.7 cells.

## Figure S1




| | | | | | |
|---|---|---|---|---|---|
| Sample Description | | Instrument | SHIMADZU LCMS-2010 | | |
| Analyzed date: | 2015-1-24 | Probe: | ESI | Probe Bias: | +4.5kv |
| Analyst: | QIU | Nebulizer Gas Flow: | 1.5L/min | Detector: | 1.5kv |
| Sample: | Ac-GCRDGPQGIWGQDRCG | CDL: | -20.0v | T. Flow: | 0.2ml/min |
| M.W.: | 1746.90 | CDL Temp.: | 250 °C | B. Conc.: | 50%H2O/50%ACN |
| Lot. No.: | P150114-HX115233 | Block Temp.: | 200 °C | | |

# Figure S2

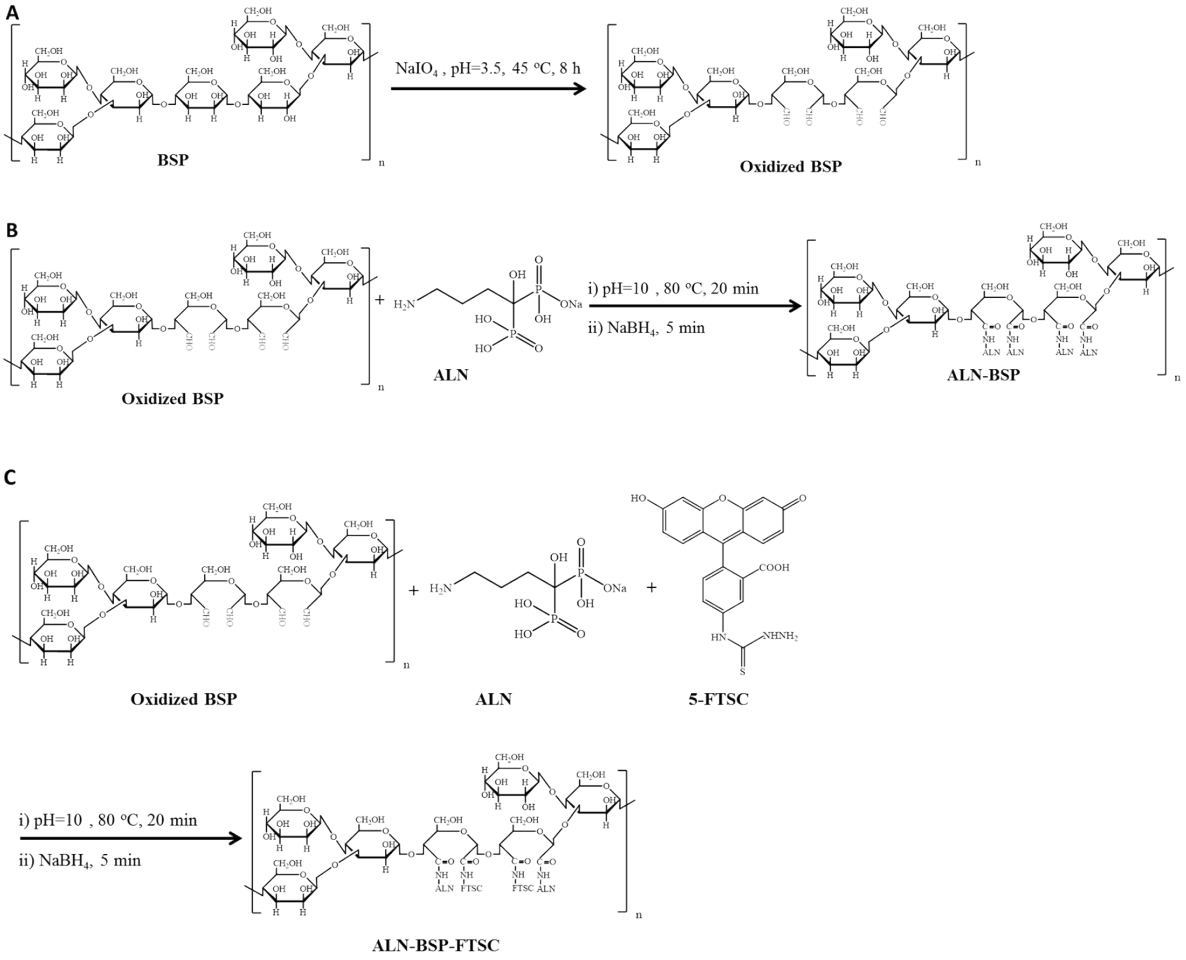

A
BSP
NaIO4 , pH=3.5, 45 ºC, 8 h
Oxidized BSP
B
Oxidized BSP
ALN
i) pH=10 , 80 ºC, 20 min
ii) NaBH4, 5 min
ALN-BSP
C
Oxidized BSP
ALN
5-FTSC
i) pH=10 , 80 ºC, 20 min
ii) NaBH4, 5 min
ALN-BSP-FTSC

**Figure S3**

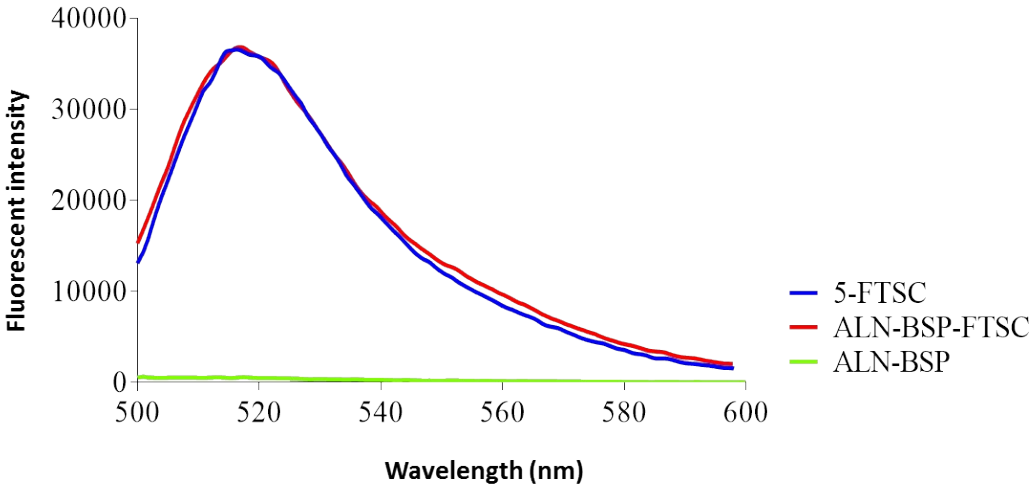
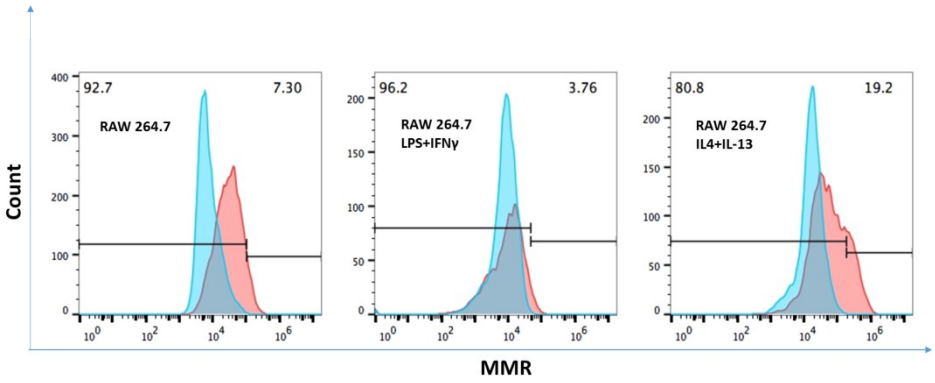

40000
30000
20000
10000
0
Fluorescent intensity
500
520
540
560
580
600
Wavelength (nm)
5-FTSC
ALN-BSP-FTSC
ALN-BSP

**Figure S4**


92.7
7.30
RAW 264.7
96.2
3.76
RAW 264.7
LPS+IFNγ
80.8
19.2
RAW 264.7
IL4+IL-13
Count
MMR