A strong CD8$^+$ T cell-stimulating supramolecular hydrogel

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Contents

S1. Synthesis and characterization

S1.1 Chemicals.

O-benzotriazol-1-yl-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU) and Fmoc-amino acids were obtained from GL Biochem (Shanghai, China). 2-Cl-trityl chloride resin (1.1 mmol/g) was obtained from Nankai University Resin Co. Ltd. N,N-Diisopropylethylamine (DIEA) was obtained from Energy Chemical (Shanghai, China). Inc. Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin and fetal bovine serum (FBS) were purchased from Gibco Corporation. The antibodies used in flow cytometry and ELISA Kit were obtained from Biolegend.

S1.2 General Methods.

High performance liquid chromatography (HPLC) was performed on a LUMTECH HPLC (Germany) system using a C18 RP column with MeOH (0.1% of TFA) and water (0.1% of TFA) as the eluents. LC-MS was conducted using an LCMS-2020 (Shimadzu) system. High-resolution mass spectrometers (HR-MS, Agilent 6520 Q-TOF LC/MS) were used to characterize the compounds. Optical imaged were taken by inverted phase contrast microscope (Nikon Eclipse TS100, Japan). Rheology was performed on an ARES 2000ex (TA instrument) system using parallel plates (40 mm) at a gap of 500 μm. The circular dichroism (CD) spectrum was obtained on a BioLogic (MOS-450) system. CLSM images were obtained by a confocal laser scanning microscopy (Leica TSC SP8, Germany). Fluorescence spectrum was acquired on a BioTek SynergyTM 4 Hybrid Microplate Reader.

S1.3 Preparation of tuftsin and peptide derivatives.

Tuftsin (TKPR), Nap-G^{D}F^{D}F^{D}Y and Nap-G^{D}F^{D}F^{D}YTKPR were synthesized by standard solid-phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin and the corresponding N-Fmoc protected amino acids with side chains properly protected by tert-butyl group or t-butyloxycarbonyl group. 20% piperidine in anhydrous N, N'-dimethylformamide (DMF) was used during deprotection of Fmoc group. The experimental procedures for solid phase synthesis were described in our previous work. [1] The crude products were separated by HPLC with MeOH and H_2O containing 0.1% TFA as eluents.

Characterization of TKPR: ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.73 (d, J = 7.3 Hz, 1H), 8.21 (d, J = 7.6 Hz, 1H), 8.10 (s, 1H), 7.80 (s, 1H), 7.60 (d, J = 5.8 Hz, 1H), 4.48 (d, J = 6.9 Hz, 6H), 4.40 – 4.32 (m, 2H), 4.12 (d, J = 6.8 Hz, 1H), 3.76 (q, J = 6.3 Hz, 1H), 3.63 (t, J = 6.3 Hz, 1H), 3.47 (d, J = 0.6 Hz, 1H), 3.44 (d, J = 0.6 Hz, 2H), 3.42 (d, J = 0.6 Hz, 2H), 3.40 (d, J = 0.6 Hz, 1H), 3.10 (d, J = 6.3 Hz, 1H), 2.75 (d, J = 8.2 Hz, 1H), 2.49 (s, 1H), 1.89 (dd, J = 14.2, 7.2 Hz, 1H), 1.63 – 1.48 (m, 3H), 1.17 – 1.13 (m, 1H), 1.08 (d, J = 0.7 Hz, 2H), 1.05 (d, J = 0.6 Hz, 5H), 1.03 (d, J = 0.6 Hz, 3H). MS: calc. M^+ =500.60, obsvd. $(M+H)^+$ =501.3139.

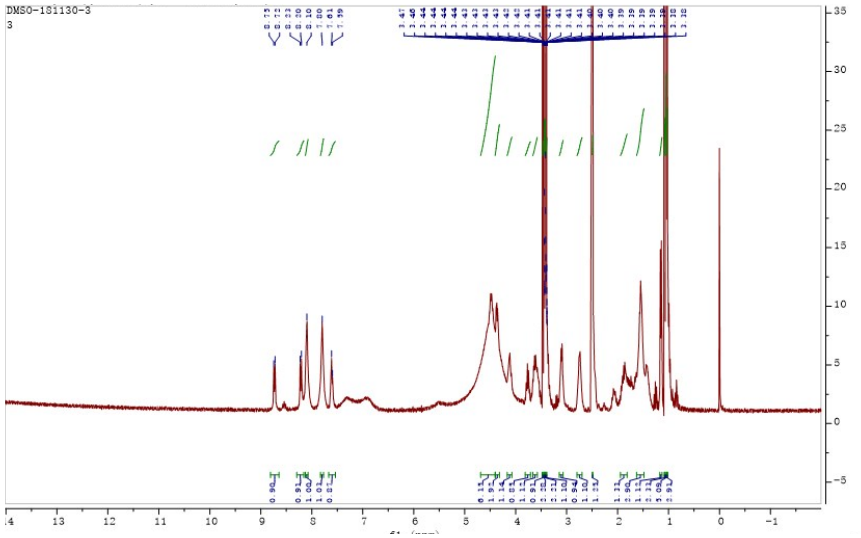

Figure S1. ^{1}H NMR spectrum of ***Comp. 1***

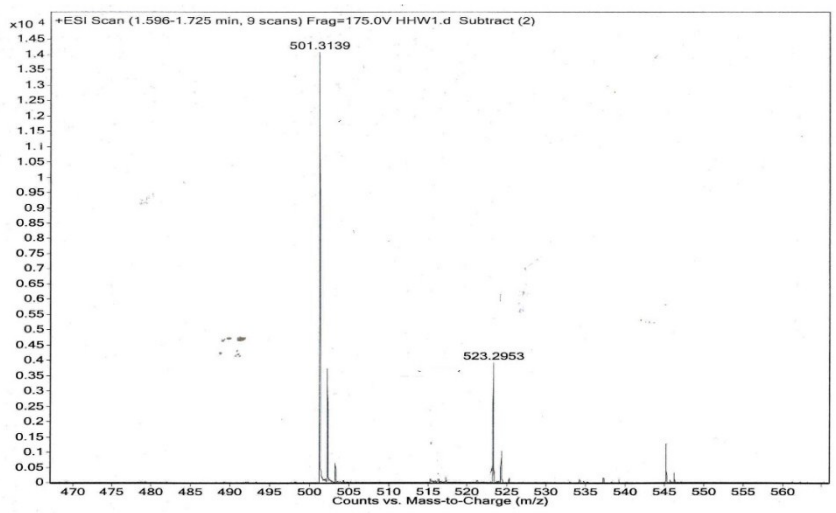

Figure S2. HR-MS spectrum of ***Comp. 1***

Characterization of Nap-G^{D}F^{D}F^{D}Y: 1H NMR (300 MHz, DMSO-d6) δ 12.70 (s, 1H), 9.22 (s, 1H), 8.36 – 8.12 (m, 2H), 8.01 (d, J = 8.4 Hz, 1H), 7.86 – 7.71 (m, 2H), 7.54 – 7.33 (m, 2H), 7.21 (d, J = 4.3 Hz, 2H), 7.14 (s, 2H), 7.02 (d, J = 8.4 Hz, 1H), 6.65 (d, J = 8.3 Hz, 1H), 4.50 (ddd, J = 17.4, 8.7, 4.3 Hz, 2H), 4.37 (q, J = 7.6 Hz, 1H), 3.79 – 3.65 (m, 2H), 3.61 (s, 2H), 3.47 (d, J = 0.5 Hz, 1H), 3.44 (d, J = 0.5 Hz, 2H), 3.42 (d, J = 0.5 Hz, 2H), 3.40 (d, J = 0.5 Hz, 1H), 3.05 – 2.86 (m, 2H), 1.08 (d, J = 0.5 Hz, 2H), 1.06 (d, J = 0.5 Hz, 5H), 1.03 (d, J = 0.5 Hz, 3H). MS: calc. M^{+} =700.79, obsvd. $(M+H)^{+}$ =701.2975.

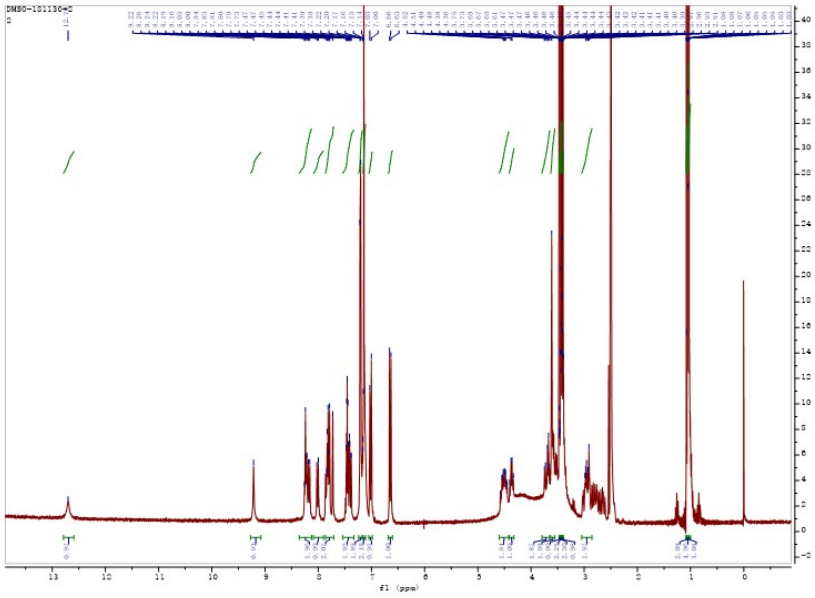

Figure S3. ^{1}H NMR spectrum of ***Comp. 2***

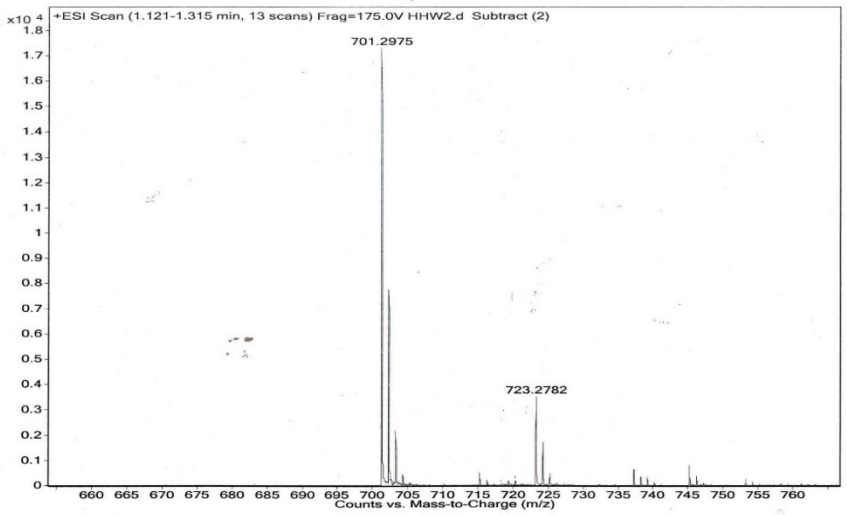

Figure S4. HR-MS spectrum of ***Comp. 2***

Characterization of Nap-G^{D}F^{D}F^{D}YTKPR: ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.21 (d, J = 7.6 Hz, 1H), 7.80 (d, J = 8.5 Hz, 2H), 7.72 (s, 1H), 7.48 – 7.43 (m, 1H), 7.40 (dd, J = 8.4, 1.7 Hz, 1H), 7.19 (d, J = 3.0 Hz, 2H), 7.14 (s, 2H), 7.05 (d, J = 8.4 Hz, 1H), 6.63 (d, J = 8.4 Hz, 1H), 4.47 (s, 2H), 4.08 (s, 18H), 3.62 (s, 1H), 3.47 (s, 2H), 3.44 (s, 5H), 3.42 (s, 5H), 3.40 (s, 2H), 3.08 (s, 1H), 3.02 – 2.84 (m, 2H), 2.71 (s, 2H), 1.82 (s, 2H), 1.53 (s, 3H), 1.08 (s, 5H), 1.05 (s, 9H), 1.03 (s, 6H), 0.93 – 0.88 (m, 1H). MS: calc. M^{+} =1183.36, obsvd. $(M+H)^{+}/2$ =592.3005.

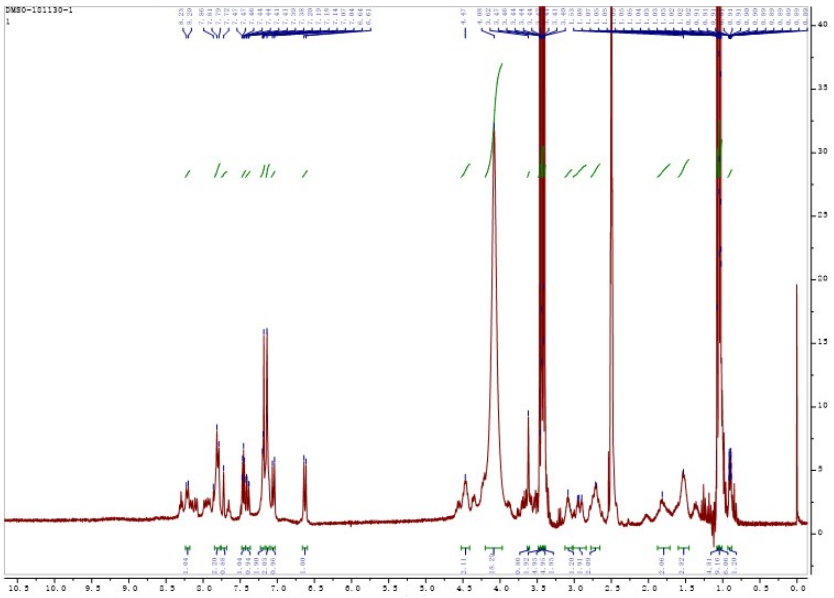

Figure S5. ^{1}H NMR spectrum of ***Comp. 3***

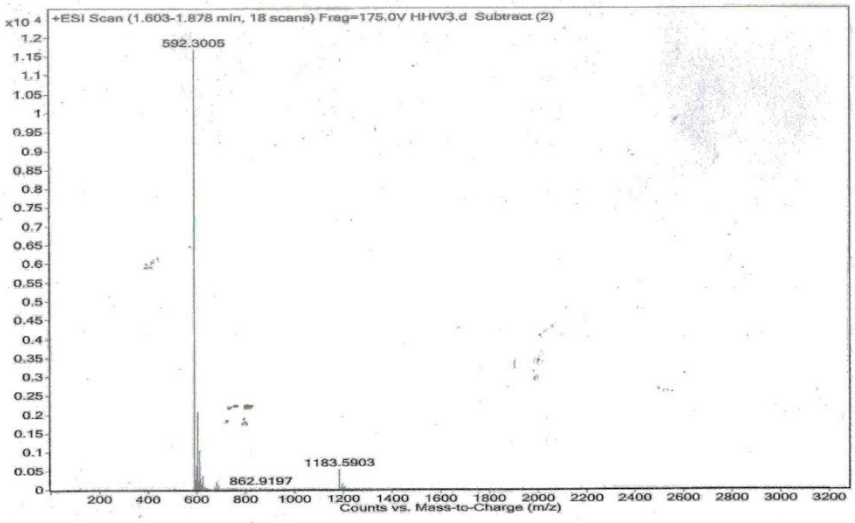

Figure S6. HR-MS spectrum of ***Comp. 3***

S1.4 Preparation of hydrogel

5 mg of the peptide powder was added to a glass vial containing 1 mL of PBS. The Na_2CO_3 solution (1M) was used to adjust the final pH value to be 7.4 (about 2 equiv. to the peptide). The suspension was heated by a burner with gentle shaking to obtain a clear solution. After cooling back to room temperature, a stable hydrogel was obtained.

S1.5 Critical aggregation concentration (CAC)

The CAC values of ***Comp. 2*** and ***Comp. 3*** were determined by dynamic light scattering (DLS). Solutions containing different concentrations of compound were tested and the light scattering intensity was recorded for each concentration analyzed.

The lower CAC values represent better assembly capacity.

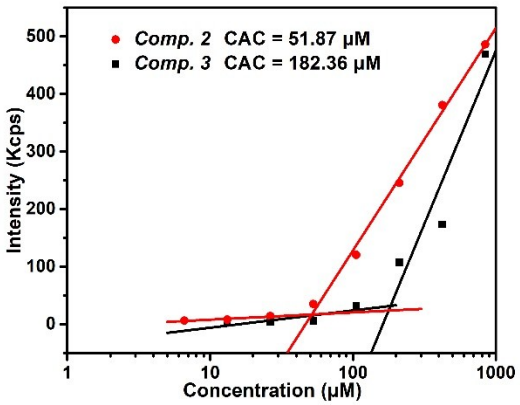

Figure S7. Critical aggregation concentration values of ***Comp. 2*** and ***Comp. 3.***

S1.6 Fluorescence.

Fluorescence spectra of PBS solutions of ***Comp. 3*** and gel of ***3***. The sample was diluted to be 1 mM using PBS, and excitation wavelength was 272 nm.

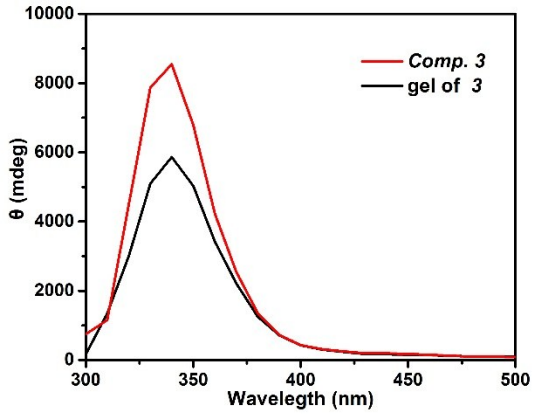

Figure S8. Fluorescence spectra of PBS solutions of ***Comp. 3*** and gel of ***3.***

S1.7 Rheology

A hydrogel at the concentration of 0.3 wt% was subjected to a rheological test on an AR 2000ex (TA Instruments) system. The parallel plates used for the test were 25 mm and the gap during the experiment was 500 μm.

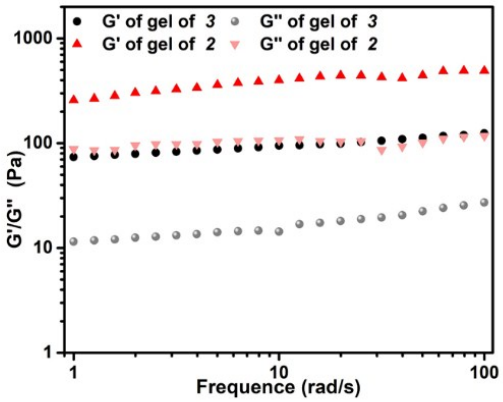

Figure S9. Dynamic frequency sweep of gel of ***2*** and gel of ***3.***

S1.8 Transmission electron microscopy

10 μL of material was dropped on a carbon-coated copper grid for 1 minute and the excess material was removed from the edges with a filter paper. 8 μL of uranyl acetate was added to the carbon coated copper grid and the uranyl acetate was removed by a filter paper. The sample was placed in a desiccator overnight and then imaged at 200 kV using a Tecnai G2 F20 system.

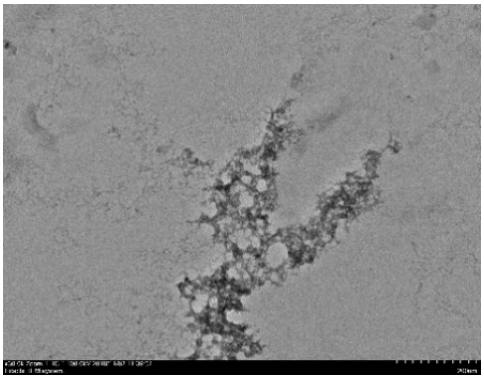

Figure S10. TEM image of tuftsin in PBS

S1.9 Circular Dichroism Spectrum.

Circular dichroism spectrum of gel of ***3*** at the concentration of 3 mM was acquired on a MOS-450 (Biologic).

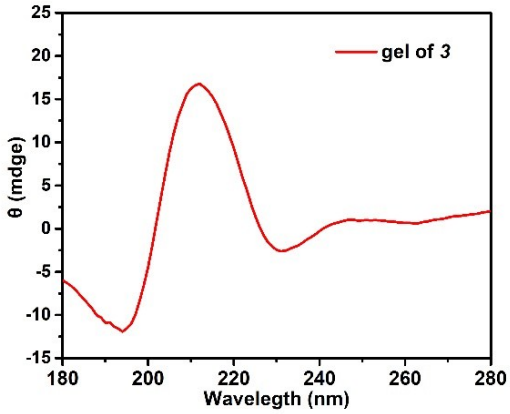

Figure S11. Circular dichroism spectrum of gel of ***3*** at the concentrations of 3 mM

S1.10 Stability

The stability of tufsin and its derivatives were analyzed by treating with leucine aminopeptidase. Equal amounts of enzyme were added to the solution of tuftsin in PBS and gel of ***3*** (pH=7.4) at 37°C, and 50 μL samples were removed into the new ep tube at 0, 5, 10, 20, 30, 60, 120, 240, 480minutes time point, respectively. The samples were added with 450 μL of methanol to denature the enzyme. The mixture was centrifuged for 10 minutes and filtered through 0.45 nylon membrane and analyzed on LC-MS. The peak area at 0 min was used as the 100% control.

S2. In vitro experiments

S2.1 The effect of hydrogel on macrophages and BMDCs

Peritoneal macrophages were collected as described by Yingying Xu. et al. [2] Firstly, the abdominal cavity of BALB/c mice (6-8 weeks) was opened to expose the peritoneum, and 4ml of cold PBS was injected into the abdominal cavity, then gently massaged for 3 minutes and collected. Repeat the previous procedure and collect the peritoneal lavage fluid into a 15ml centrifuge tube. After centrifugation at 1500rpm for 5 min, cells were resuspended with DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. Then, Peritoneal macrophages were treated Rho-OVA ***Comp. 1***, the gel of ***2*** and the gel of ***3***.

Bone marrow cells were isolated from BALB/c mouse femur and tibia, and then cultured in X-vivo 15 medium (Lanza, MD, USA) containing IL-4 (20 ng mL^{-1}) and GM-CSF (40 ng mL^{-1}) at 37 °C for 6 days to acquire immature DCs. [3] The immature DCs were stimulated with ***Comp.1,*** gel of ***2*** and gel of ***3*** for 24h. Then the DCs were collected and incubated with PE-antimouse-CD80 or FITC-antimouse CD40 (1:200) antibodies in the dark on ice for 30 min. The expressions of CD80 and CD40 on BMDCs were detected by flow cytometry.

S2.2 Confocal Fluorescence Laser Scanning Microscopy (CLSM)

Effects of nanomaterials on the phagocytosis of mouse peritoneal macrophages

was determined by CLSM. Primary peritoneal macrophages were seeded into a 24-well plate at a density of 3×10^5 cells/well. After incubating for 2 hours in a 37°C incubator, the medium was discarded and washed 3 times with PBS to remove other cells and then fresh DMEM medium containing ***Comp.1,*** gel of ***2*** and gel of ***3*** (100μM) and Rho-OVA (10μg/mL) was added. The medium was removed after 1 hour of incubation and each well was washed three times with PBS. After fixation for 20 minutes by adding 1 ml of paraformaldehyde, the nuclei were stained with DAPI. The prepared cell samples were observed under CLSM and the difference in red fluorescence intensity in the cells was used to reflect the amount of antigens taken by macrophages after stimulation with different materials.

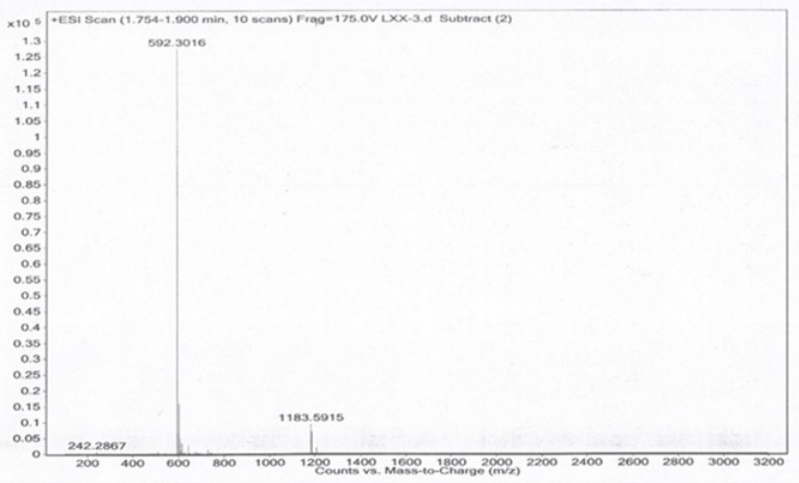

Figure S12. HR-MS spectrum of Nap-G^{D}F^{D}F^{D}YKTR

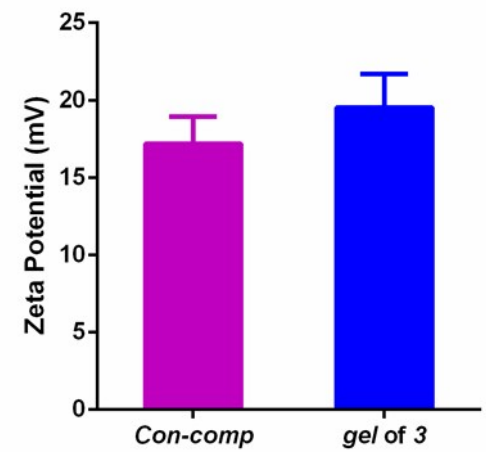

Figure S13. Zeta-potential of ***Con-comp*** and the gel of ***3***. (mean ±SEM, n =3)

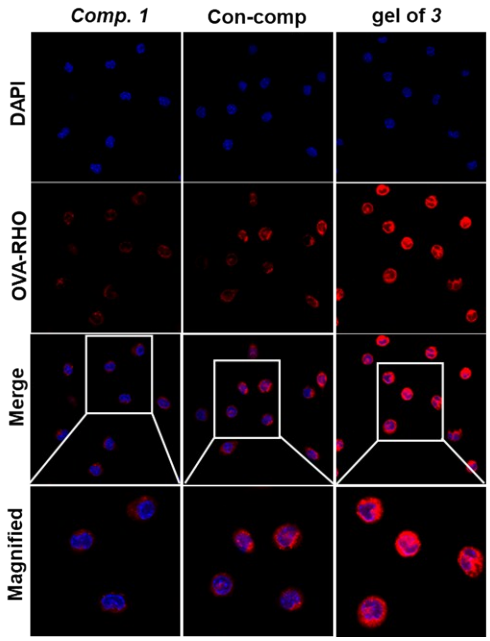

Figure S14. Confocal laser scanning microscopy images of primary peritoneal macrophages incubated with Rho-OVA under the stimulation of Comp. 1, Con-comp, or the gel of 3.

S2.3 Flow cytometry

The effect of hydrogels and tuftsin on the expression of CD80 and CD86 on the surface of Raw264.7 cells was investigated by fluorescence activated cell separation (FACS).

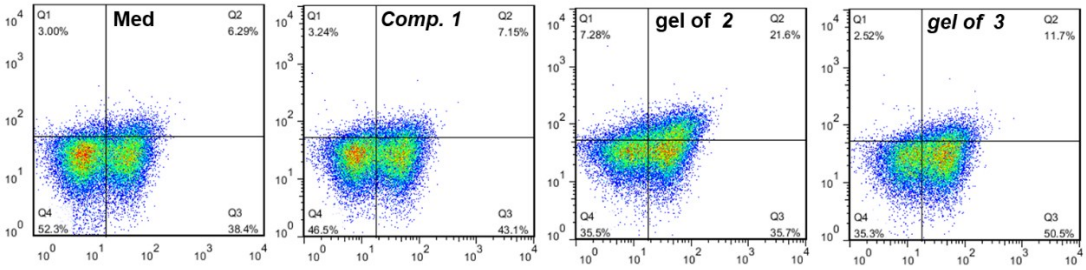

Figure S15. The expression of CD80 and CD86 on Raw264.7 cells stimulated by ***Comp. 1,*** gel of ***2*** and gel of ***3*** (100 μM) for 24 hours.

S2.4 Cytokine

BMDCs and T lymphocytes were extracted according to the method of Luo et al. Cells were seeded into 24-well plates and then cells were incubated for 24 hours in a medium containing ***Comp.1,*** gel of ***2*** and gel of ***3*** (100μM). The cell culture medium was collected and centrifuged to obtain the supernatant. Production of cytokines was measured by ELISA kit.

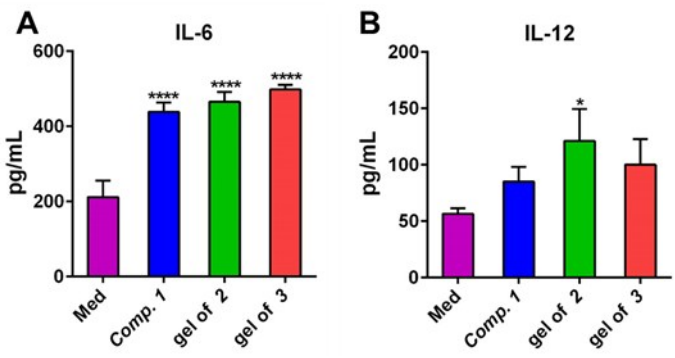

Figure S16. The productions of IL-6(A), IL-12(B) in culture supernatants of BMDCs treated with ***Comp. 1,*** gel of ***2*** and gel of ***3*** (100 μM) for 24 hours.

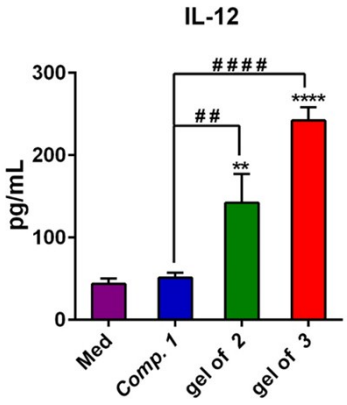

Figure S17. Raw264.7 cells expression of IL-12 were stimulated by ***Comp. 1,*** gel of ***2*** and gel of ***3*** (100 μM) for 24 hours.

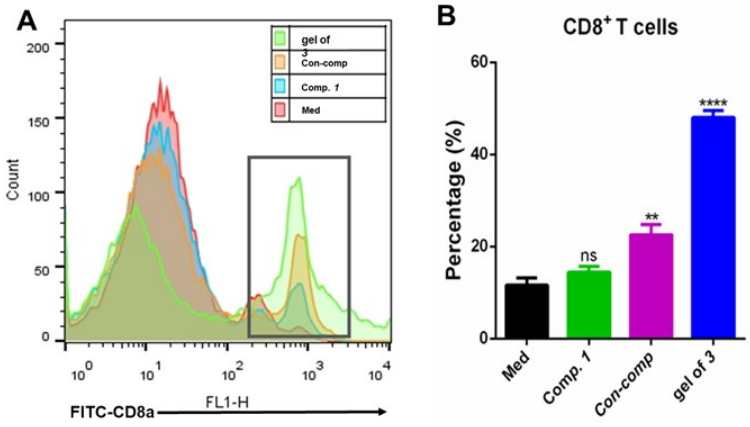

Figure S18. (A) Representative results of the proportion of CD8+ T cells after 24 h stimulation with different compounds, (B) The percentage of CD8+ T-cell responses after incubation with different materials was detected by flow cytometry.

S3. Animals experiments

All animal studies were performed in compliance with the guidelines set by the

Tianjin Committee of Use and Care of Laboratory Animals, and the overall project protocols were approved by the Animal Ethics Committee of Nankai University. 6-8 weeks old BALB/c mice were bought from Beijing Vital River Laboratory Animal Technology Co., Ltd and fed in SPF animal center of college of life sciences, Nankai University.

S3.1 Immunotherapy of tumors

We established a tumor model of 4T1 to investigate the anticancer activity of tuftsin and hydrogels. After the tumor reached a certain size, three doses were administered and the changes of tumor size and body weight were recorded. After 10 measurements, the tumor was taken out and weighed.

The compounds were subcutaneously injected, and the tumor weight in the group of gel of ***3*** was about 1.18 g and that in the group of PBS was about 2.5 -fold than that of the gel of ***3*** group.

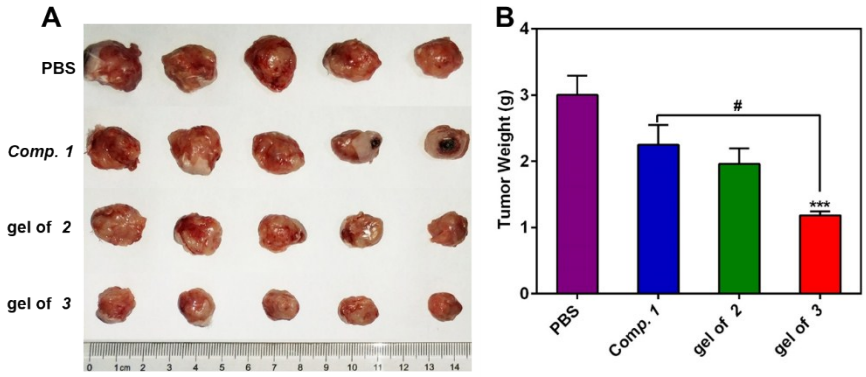

Figure S19. Digital photograph and weight of tumors in PBS, C***omp.1,*** gel of ***2*** and gel of ***3*** group.

4T1 cells were seeded in 96-well plates at 8×10^3 cells per well for 24 h followed by culture medium removal and subsequently addition of culture medium containing our hydrogels at different concentrations. After 48 hours, 10 μL of MTT solution (5 mg/mL) was added to wells and incubated at 37 °C for 4 h, and then the optical density was measured at 490 nm using a microplate reader. (Bio-RAD iMarkTM, America).

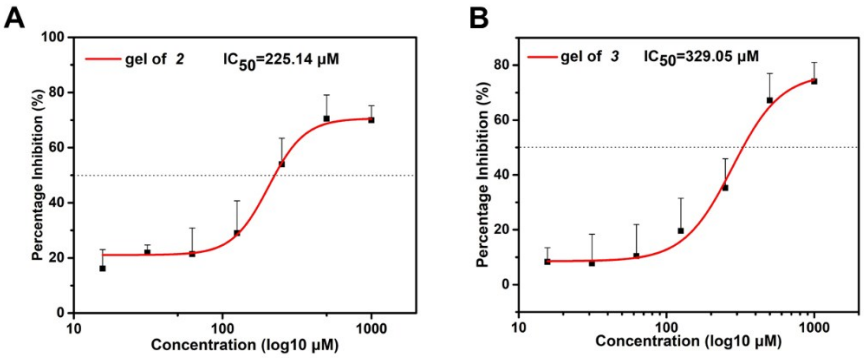

Figure S20. IC_{50} profiles of different drugs against 4T1 cancer cells, each result

obtained by average nine separate cell viability experiments. (mean ±SEM, n = 5)

Next, the anticancer activity of gel of ***3*** was investigated by intraperitoneal injection, subcutaneous injection and tail vein injection. As shown in the figure below, subcutaneous injection and caudal vein administration have similar anticancer effects and are more potent than intraperitoneal administration.

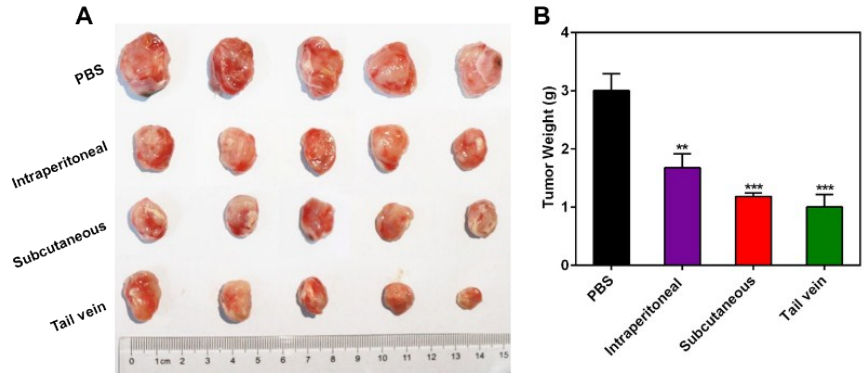

Figure S21. The anti-tumor effect of gel of ***3*** through different drug delivery methods and the tumor weight of mice in different groups

S3.2 Biocompatibility

Tumor models were established and changes in body weight were detected after administration.

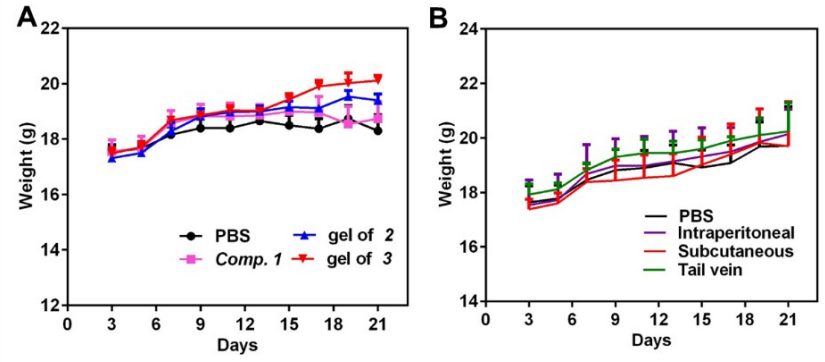

Figure S22. In vivo toxicity performance of different drugs compared with PBS

S3.3 Cytotoxicity evaluations

The CCK-8 assay was used to assess the cytotoxicity of hydrogels. After incubation of immune cells with hydrogels of different concentrations for 24 hours, the cell survival rate was determined by CCK8 kit. LO2 cells after different concentrations of nanomaterials were stimulated for 48h.

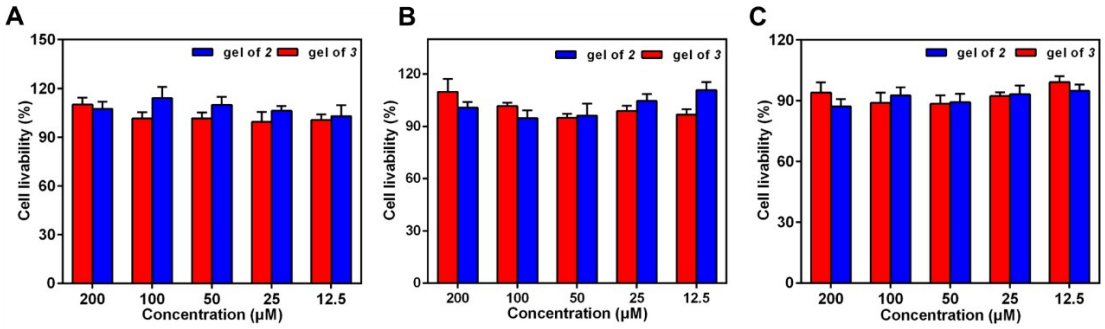

Figure S23. Cell livability of different types of immune cells (Primary peritoneal macrophages(A), Splenocytes(B) and Raw264.7 cells(C)) stimulated by hydrogels.

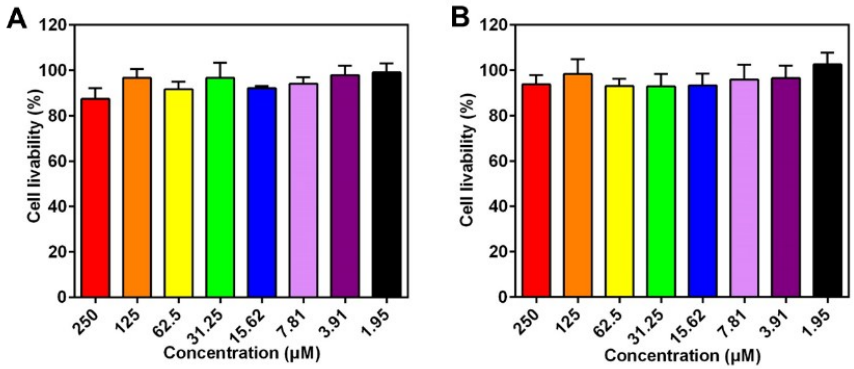

Figure S24. Cell livability of LO2 cells stimulated by gel of ***2***(A) and gel of ***3***(B) for 24 hours.

S4. References

[1] Y. Cai, H. Shen, J. Zhan, M. Lin, L. Dai, C. Ren, Y. Shi, J. Liu, J. Gao and Z. Yang, *J. Am. Chem. Soc.*, 2017, 139, 2876-2879.

[2] Y. Xu, J. Zhu, K. Xiang, Y. Li, R. Sun, J. Ma, H. Sun and Y. Liu, *Biomaterials*, 2011, 32, 9940-9949

[3] Z. Luo, Q. Wu, C. Yang, H. Wang, T. He, Y. Wang, Z. Wang, H. Chen, X. Li, C. Gong and Z. Yang, *Adv. Mater.*, 2017, 29, 1601776.