Supporting Information

Designable Immune Therapeutical Vaccine System Based on DNA Supramolecular Hydrogels

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Methods

Materials. Amino acids and resins were purchased from GL Biochem(Shanghai)Ltd. (Shanghai, China). All DNA synthesis reagents were purchased from Beijing Zixi Biotechnology Company (Beijing, China). HyClone® phosphate buffer saline (PBS, 1×, 0.0067M PO_4^{2-}, without calcium, magnesium or phenol red) and HyClone® penicillin-streptomycin solution were purchased from Thermo Scientific. Dulbecco's Modified Eagle Medium (DMEM, with phenol red, high glucose, L-glutamine), fetal bovine serum (FBS) and Trypsin-EDTA (0.25%) were all purchased from Gibco. Calcein-AM and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich. Water used in all experiments was Millipore Milli-Q deionized (18.2 MΩ cm). ELISA kits detecting IL-6 and IL-12 were purchased from DAKEWEI Biotech (Beijing, China). All other chemicals were of reagent grade or better.

Apparatus. All DNA sequences (see Supplementary Tab. 1) were synthesized using Mermade 12 DNA synthesizer (BioAutomation, USA) with a standard phosphoramidite DNA synthesis protocol and purified by HPLC (Agilent 1200, USA). ssDNAs were quantified spectrophotometrically at a wavelength of 260 nm and by denaturing polyacrylamide gel electrophoresis (PAGE) followed by staining with stains all (Molecular Probes, USA) and UV transillumination. UV/Vis spectra were recorded on a Varian Cary 100 spectrophotometer equipped with a programmable temperature-control unit. Purification and analysis of peptides were operated on HPLC (SHIMADZU LC-6AD). Absorption signals were measured by the 215 nm UV detector. MALDI-TOF MS spectra were recorded with an Applied Bio-systems 4700 Proteomics Analyzer 283. ESI-MS were detected with a Thermo Fisher Ultimate 3000 Analytical and MSQ Plus. Rheology tests were performed on an ARG2 rheometer manufactured TA Instruments. The staining cells were visualized using a Zeiss LSM 710 Meta Confocal microscope (Germany). FACS analysis was performed on a Calibur flow cytometer (BD Biosciences).

Animals and cell lines. BALB/c female mice (6-8 weeks) and C57BL/6 female mice (4-6 weeks) were fed in Animal Facility of Center of Biomedical Analysis, Tsinghua University. All studies were carried out following the animal ethics guidelines. MCF-7 cell line, B16F0 cell line, and RAW264.7 cell line were purchased from China Infrastructure of Cell Line Resources. B16F0 cell line over-expressing human MUC1 was constructed by ViewSolid Biotech (Beijing). All cells were cultured in DMEM with 10% FBS and 1% antibiotics.

Synthesis and purification of Oligonucleotides. Oligonucleotides were synthesized in 1 μmol scale using a standard phosphoramidite DNA synthesis protocol. Then ssDNAs were purified with HPLC by a C18 column (5 μm, 9.4×30 mm, USA) with a flow rate of 3 mL/min. Finally, the products were analysed by PAGE followed by stains all staining and MALDI-TOF.

Synthesis and purification of peptides. Peptides were synthesized following the Fmoc protocol. For the synthesis of **P1** and **P1-FAM**, Fmoc-Ala-Wang resin (0.13 mmol/g, 0.15 mmol scale) was used. 20% piperidine in DMF was used for the deprotection of Fmoc. Amino acids were coupled with 1-hydroxy-7-azabenzotriazole (HOAt, 4.0 equiv), 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 4.0

equiv), and *N,N*-diisopropylethylamine (DIPEA, 8.0 equiv) in DMF for 1 h. For the synthesis of glycosylated amino acid, HOAt (2.0 equiv), HATU (2.0 equiv), and N-methylmorpholine (NMM, 5.0 equiv) were used for 12 h. **P1** and **P1-FAM** were purified on HPLC by a polar-CN column (5 μm, 20×250 mm, YMC, Japan) with a flow rate of 10 mL/min. The peptides were analyzed on HPLC using the analytic CN column (5 μm, 4.6×250 mm, YMC, Japan) with a flow rate of 0.8 mL/min and identified by ESI-MS and MALDI-TOF (see analytical data).

Fabrication of the DSHV. Stoichiometric amounts of DNA strands of the Y-scaffold (Y1, Y2, Y3, see Supplementary Tab. 1) were lyophilized and then added PBS buffer (pH 7.4) to give a final concentration of 1 mM for each DNA strand. Stoichiometric amounts of linker (L1, L2, L3, see Supplementary Tab. 1) were freeze-dried and added to PBS buffer (pH 7.4) to give a final concentration of 3 mM for each DNA strand. The resulting mixtures were both heated to 95 °C for 5 min, and subsequently cooled to room temperature in 2 h to make the designed structures form. Then equivoluminal linker and **P1** were mixed overnight to give a final concentration of 1.5 mM. Finally, the 1 mM of the Y-scaffold was mixed with 1.5 mM of the linker at the molar ratio of 1:1.5, the solution lost its fluidity within a minute and appeared to be gel-like vaccine.

Analysis of electrostatic interaction. 100 μL **P1-FAM** (1 μM) was added to each well on Costar 3915 96-well plate. 100 μL different concentration of linker (0, 50 nM, 100 nM, 200 nM, 500 nM, 1 μM, 2 μM, 5 μM, 10 μM) was separately added to **P1-FAM**. The plate was incubated for overnight at 4 °C and measured.

Rheological characterization of the DSHV. Rheological measurements were carried out using a TA instrument ARG2 equipped with a temperature controller. Samples were allowed to equilibrate for 10 min before starting the measurements. Four types of rheological experiments were performed: (i) time-scan tests were done at a fixed frequency and strain of 1 Hz and 1%, respectively, at 25 °C for 5 min; (ii) frequency sweep tests were carried out on mixtures between 0.1 and 10 Hz at 25 °C at a fixed strain of 1%; (iii) strain sweep tests were carried out on mixtures between 0.1% and 400% strains at 25 °C at a fixed frequency of 1 Hz; (iv) temperature-ramp tests were performed at a fixed frequency and strain of 1 Hz and 1%, respectively, and the changes in the shear-storage modulus (G') and shear-loss modulus (G") were measured from 10 °C to 80 °C at a rate of 1 °C/min.

Distribution of RAW264.7 cells in the DSHV. RAW264.7 cells were cultured in DMEM, 10% (v/v) FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin under sterile conditions and maintained in a 5% CO_2 humidified incubator at 37 °C. Before trapped in the DSHV, cells were digested with 0.25% trypsin-EDTA and stained with 5 μM Calcein-AM and washed with PBS buffer for two times and then adjusted to 4×10^6 cells/mL density with complete medium. Then stoichiometric amounts of the Y-scaffold and DNA linker contained 4 mg/mL **P1** were seeding in 96-well confocal plate with a 1×10^6 cells/mL density of cells. After 6 hours culturing, the cells were visualized using Zeiss LSM 710 Meta Confocal microscope.

Recruitment of RAW264.7 cells by the DSHV. In a typical experiment, a stock solution of the building blocks was prepared, whereby stoichiometric amounts of the Y-scaffold and linker DNA

strands were added in a complete culture medium to obtain a final concentration of 1 mM and 1.5 mM. Then, the cells were stained CM-Dil with 1×10^6 cells/mL solution were added to the confocal cell plate. Finally, the calculated volumes of the Y-scaffold and linker stock solutions were added in 96-transwell plate and were visualized using a rotary scanning confocal microscope for 1 hour.

Activation of RAW264.7 cells by the DSHV. Follow the above method, 40 μL of the DSHV with a density of 5×10^5 RAW264.7 cells/well were seeding on the 96-well plates and cultured for 24 h. Then vaccines with 180 μM or LPS with 5 μg/mL were incubated with cells for 24 h. Then PBS buffer were added to give a final 280 μL solution. The supernatant was measured with the IL-6 and IL-12 ELISA kits.

Vaccine immunizations. BALB/c female mice (6-8 weeks) were intraperitoneally administered with 80 μL DSHV or **P1** (180 μM) every two weeks for five times. Each group included four mice. Seven days after the last immunization, the sera were collected.

Analysis of antibody titers. Costar 3590 96-well ELISA plates were coated with 20 μg/mL MUC1 glycopeptide **P2** (100 μL/well) in $NaHCO_3$ solution (pH=9.6, 0.1 M) for 12 h at 4 °C. 0.25% gelatin-PBS solution was used to block the plates. After washing, the sera of mice were respectively diluted and added to each well (100 μL/well). Then, the plates were incubated for 1.5 h at 37 °C. After washing, IgG-peroxidase antibodies (rabbit anti-mouse, diluted with 1:2000) were added to the plates and shaken for 1 h at 37 °C. Then, the substrate of peroxidase (1.5 μL/mL 30% H_2O_2 and 1 mg/mL *o*-phenylenediamine) was added to the plates and incubated for 15 min at room temperature. The absorption was measured with 450 nm wavelength. Titers are defined as the highest dilution possessing the absorption of 0.1 over that of the blank control. All samples were tested in three repeats.

Analysis of antibody isotypes. Costar 3590 96-well ELISA plates were coated with 20 μg/mL MUC1 glycopeptide **P2** (100 μL/well) in $NaHCO_3$ solution (pH=9.6, 0.1 M) for 12 h at 4 °C. 0.25% gelatin-PBS solution was used to block the plates. After washing, the sera of mice were respectively diluted and added to each well (100 μL/well). Then, the plates were incubated for 1.5 h at 37 °C. After washing, IgG 1, IgG 2a, IgG 2b, IgG 3, and IgM of isotype antibodies (goat anti-mouse) were diluted to 1:1000 and incubated for 1 h at 37 °C. After washing, IgG-peroxidase antibodies (rabbit anti-goat, diluted with 1:1000) were added to the plates and shaken for 1 h at 37 °C. Then, the substrate of peroxidase (1.5 μL/mL 30% H_2O_2 and 1mg/mL *o*-phenylenediamine) was added to the plates and incubated for 15 min at room temperature. All samples were tested in three repeats.

The binding of sera to MCF-7 cells. MCF-7 cells (8×10^5) were incubated in 100 μL sera induced by the DSHV or **P1** (diluted to 1:25) for 1 h at 0 °C. After washing, MCF-7 cells were incubated with FITC-conjugated IgG antibody (rabbit anti-mouse, diluted to 1:50) for 1 h at 0 °C. After washing, the cells were detected on a BD Calibur flow cytometry.

Analysis of complement dependent cytotoxicity. MCF-7 cells (8000/well) were added to 96-well plate. After cultured for 12 h, sera induced by the DSHV and **P1** (diluted to 1:10) were added (50 μL/well) and incubated for 0.5 h. Then, rabbit complement (50 μL/well, diluted to 1:2) was added and incubated for 8 h. 20 μL 0.5% MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) solution in PBS was added to each well and incubated for 4 h. After removing the solution, 150 μL DMSO was added to each well and the absorption was measured at the wavelength of 490 nm. The survival rate of cells was calculated with the following formula.

Survival rate (%) = (experimental OD/control OD) × 100

Analysis of anti-tumor immune response. B16F0 tumor cells (1×10^5, positive for human MUC1) were administered subcutaneously into the right flank of C57BL/6 female mice (4-6 weeks). Ten days after transplant, mice with 5 mm tumor diameter were used to be given peritumoral injections of 80 μL DSHV or **P1** (180 μM) every four days. PBS was used as the blank control. Each group contained six mice. A total of three injections were injected. The tumor size was detected every two days with a caliper. Mice were euthanized when the tumor length exceeded 15 mm. The tumor volume was calculated by 0.5 × length × width^2.

Name	nt	DNA sequences (5'->3')
Y1	40	CGATTGACTCTCCACGCTGTCCTAACCATGACCGTCGAAG
Y2	40	CGATTGACTCTCCTTCGACGGTCATGTACTAGATCAGAGG
Y3	40	CGATTGACTCTCCCTCTGATCTAGTAGTTAGGACAGCGTG
L1	44	GAGAGTCAATCGTCTATTCGCATGAGAATTCCATTCACCGTAAG
L2	33	TCATGCGAATAGATCCATGACGTTCCTGACGTT
L3	31	GAGAGTCAATCGCTTACGGTGAATGGAATTC

Table S1. Single-stranded DNA sequences for the preparation of DNA hydrogel. Sequences **Y1**, **Y2** and **Y3** form Y-scaffold; sequences **L1**, **L2** and **L3** form linker. Red colored sequences are sticky ends; Green colored bases are CpG motifs.

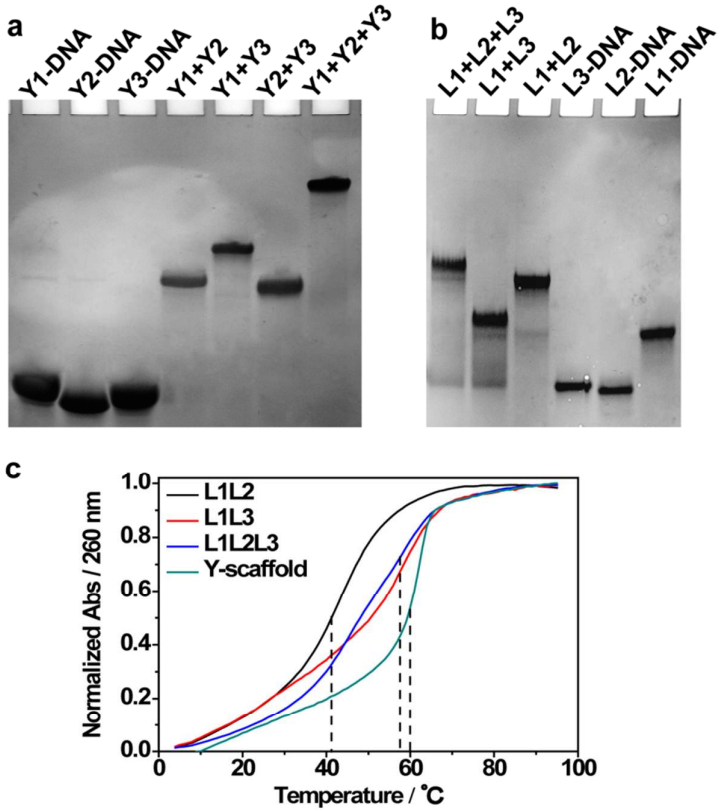

Figure S1. (a, b) Characterization of Y-scaffold and DNA linker by 10% native PAGE. Three ssDNAs Y1, Y2 and Y3 hybridized to form Y scaffold, and three ssDNAs L1, L2 and L3 assembled into DNA linker. (c) Tm measurements of Y-scaffold and Linker. UV melting experiments for UV absorption at 260 nm were carried out from 4 ºC to 95 ºC at a rate of 1 ºC/min. The concentration of Y-scaffold and Linker was 1.5 μM each.

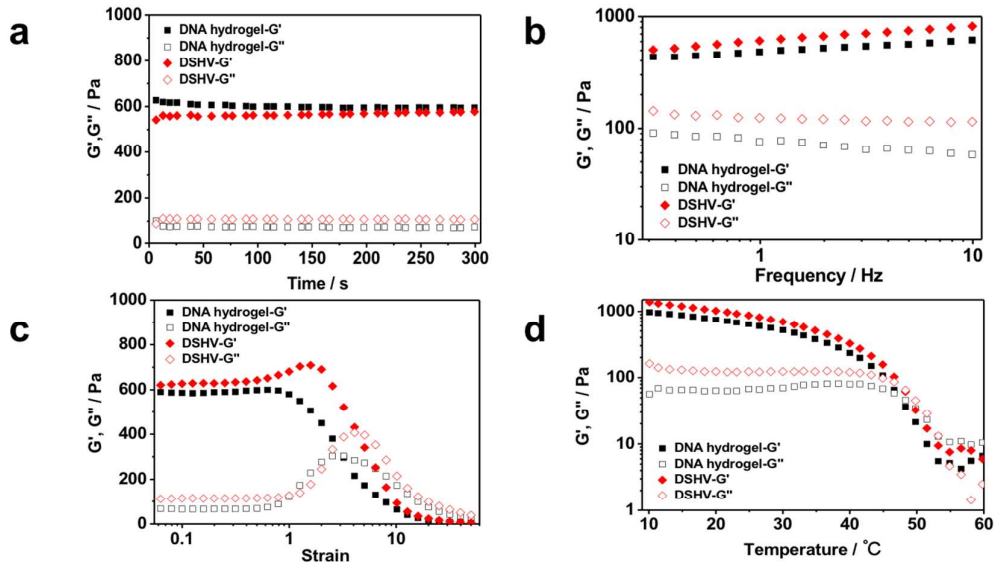

Figure S2. Rheological properties of DNA hydrogel and DSHV with total DNA 4.3 wt%. (a) Time scan tests were done by setting fixed frequency and strain at 1 Hz and 1%, respectively, at 25 ºC for 5 min; (b) Frequency sweep test carried out between 0.05 and 10 Hz at a fixed strain of 1% at 25 ºC; (c) Strain sweep were performed between 0.1% and 400% at 25 ºC with a fixed frequency of 1 Hz; (d) Temperature-ramp rheological tests were carried out from 10 ºC to 60 ºC at a rate of 1 ºC /min at a fixed frequency (1 Hz) and strain (1%).

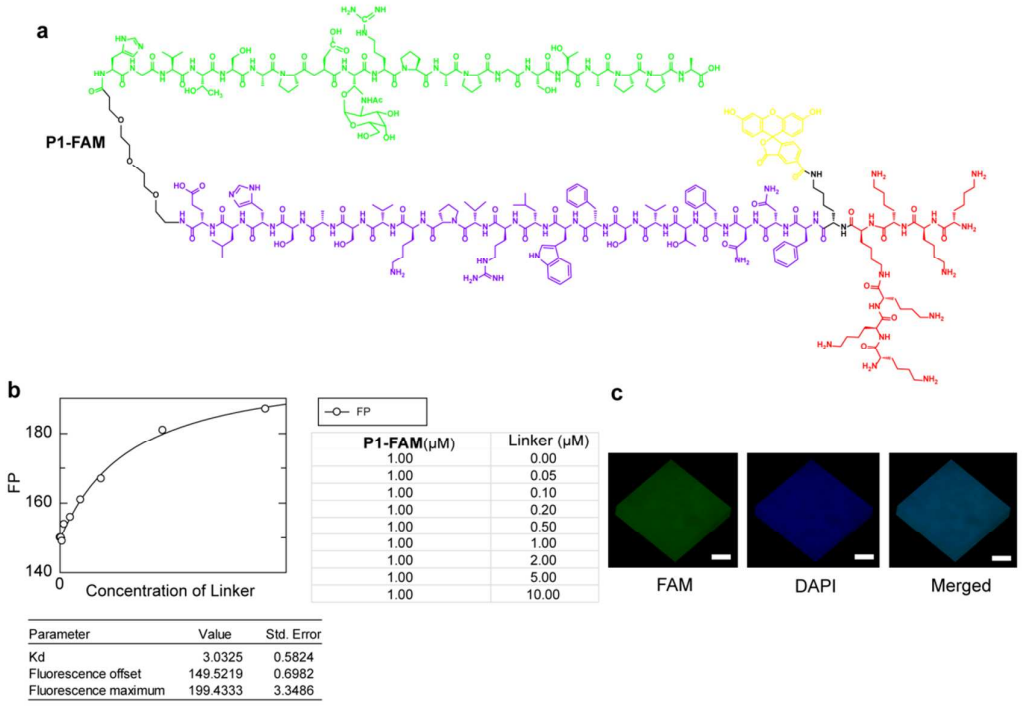

Figure S3. (a) The structure of **P1-FAM**, which contained a B cell epitope (green portion), a helper T cell epitope (purple portion), seven lysine residues (red portion), and carboxyfluorescein

(orange portion). (b) Fluorescence polarization results of the binding between **P1-FAM** and linker. (c) Fluorescence images of the distribution of **P1-FAM** in the DSHV.

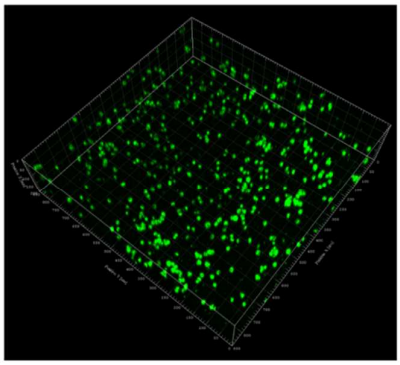

Figure S4. Fluorescence image of the distribution of RAW264.7 cells in the DSHV. RAW264.7 cells were stained by the 5 μM Calcein-AM.

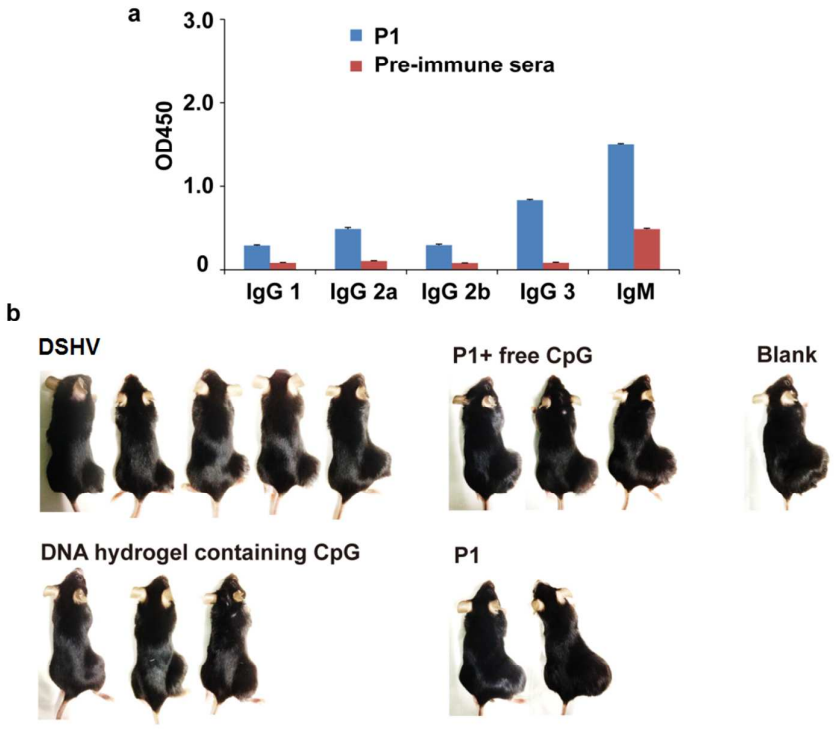

Figure S5. (a) Antibody isotypes of **P1**. Pre-immune sera were the blank control. Data are shown as mean ± SD of three separate experiments. (b) Images of mice at day 30.

Analytical data.

P1:

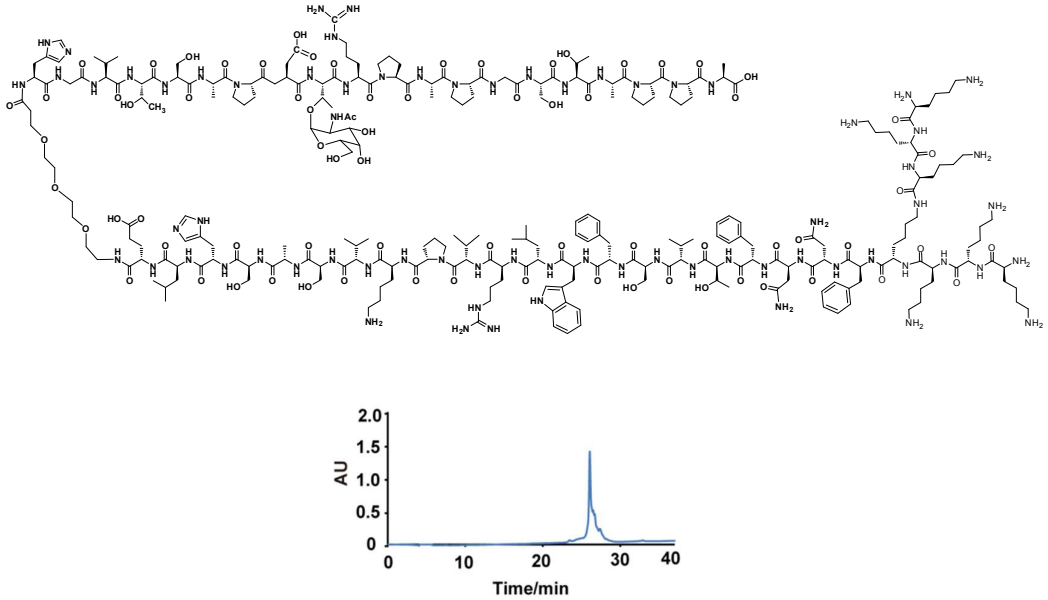

Figure S6. Analytic HPLC trace of **P1**. Analytic gradient is 10% to 50% of solution A (80% acetonitrile/water with 0.06% trifluoroacetic acid) in solution B (water with 0.06% trifluoroacetic acid) in 40 min on the analytic CN column. Retention time is 26.7 min.

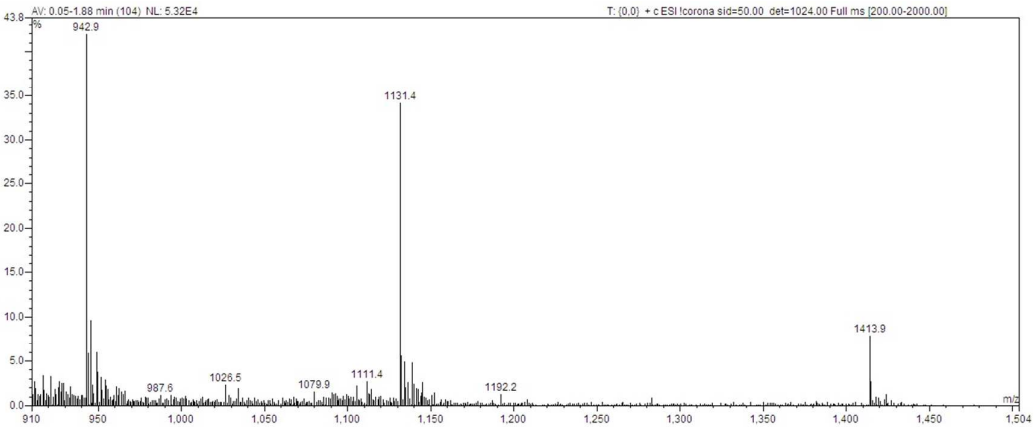

Figure S7. MS (ESI) of **P1**. $C_{257}H_{413}N_{70}O_{73}$ m/z: 5651.5, $[M+H]^+$. Found: 942.9, $[M+6H]^{6+}$; 1131.4, $[M+5H]^{5+}$; 1413.9, $[M+4H]^{4+}$.

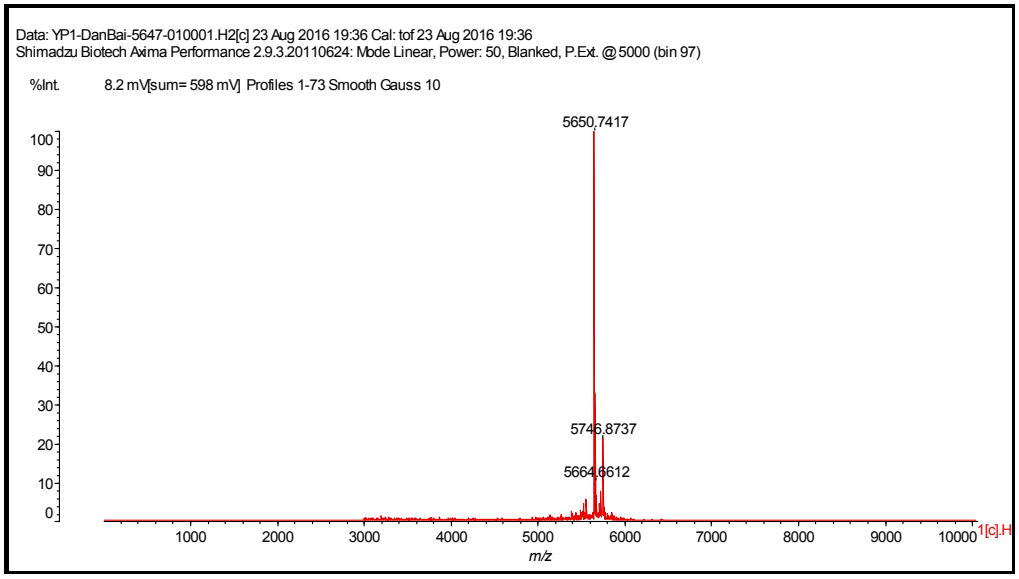

Figure S8. MS (MALDI-TOF) of **P1**. $C_{257}H_{413}N_{70}O_{73}$ m/z: 5651.4543, $[M+H]^+$. Found: 5650.7417, $[M+H]^+$.

P1-FAM:

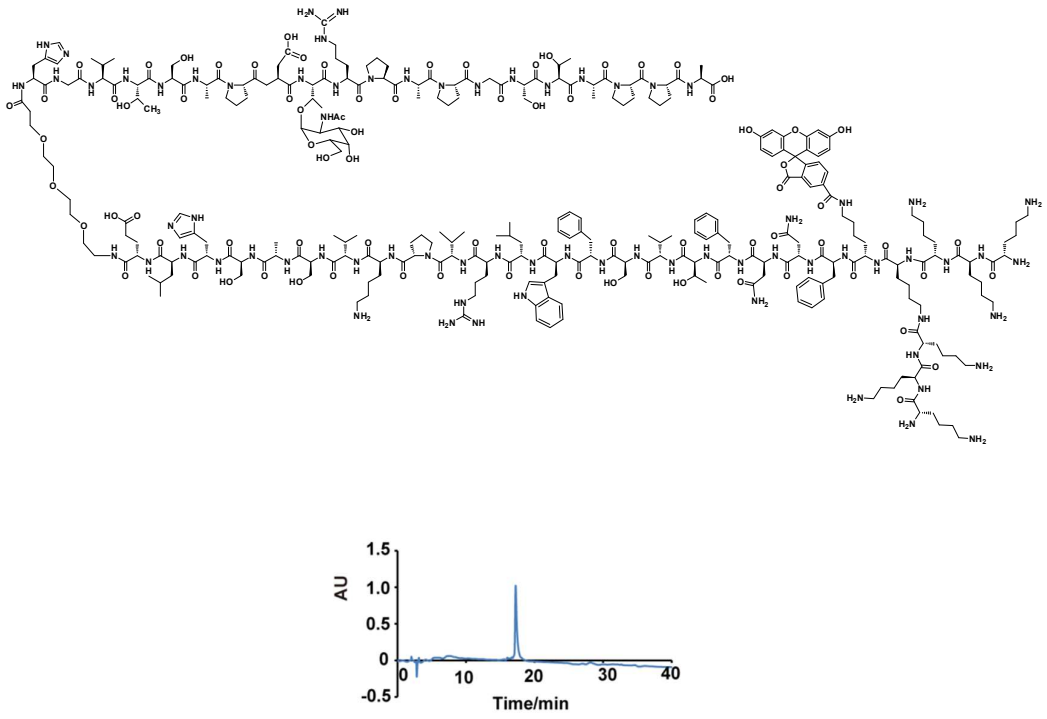

Figure S9. Analytic HPLC trace of **P1-FAM**. Analytic gradient is 20% to 70% of solution A (80% acetonitrile/water with 0.06% trifluoroacetic acid) in solution B (water with 0.06% trifluoroacetic acid) in 40 min on the analytic CN column. Retention time is 17.2 min.

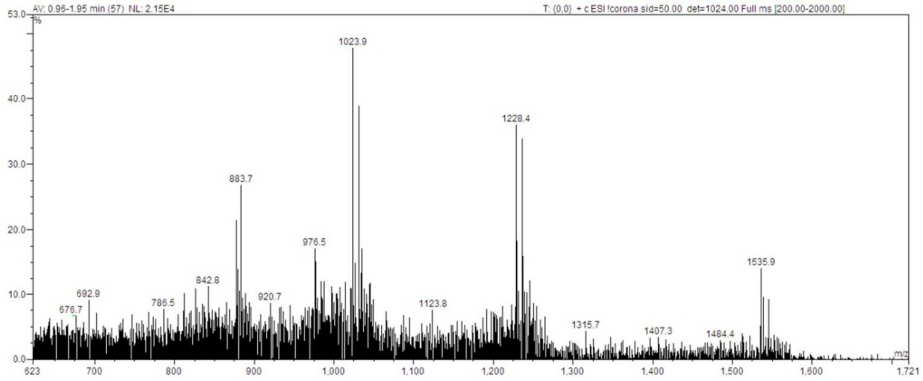

Figure S10. MS (ESI) of **P1-FAM**. $C_{284}H_{435}N_{72}O_{80}$ m/z: 6137.9, $[M+H]^{+}$. Found: 883.7, $[M+6H+K]^{7+}$, 1023.9, $[M+6H]^{6+}$; 1228.4, $[M+5H]^{5+}$; 1535.9, $[M+4H]^{4+}$.

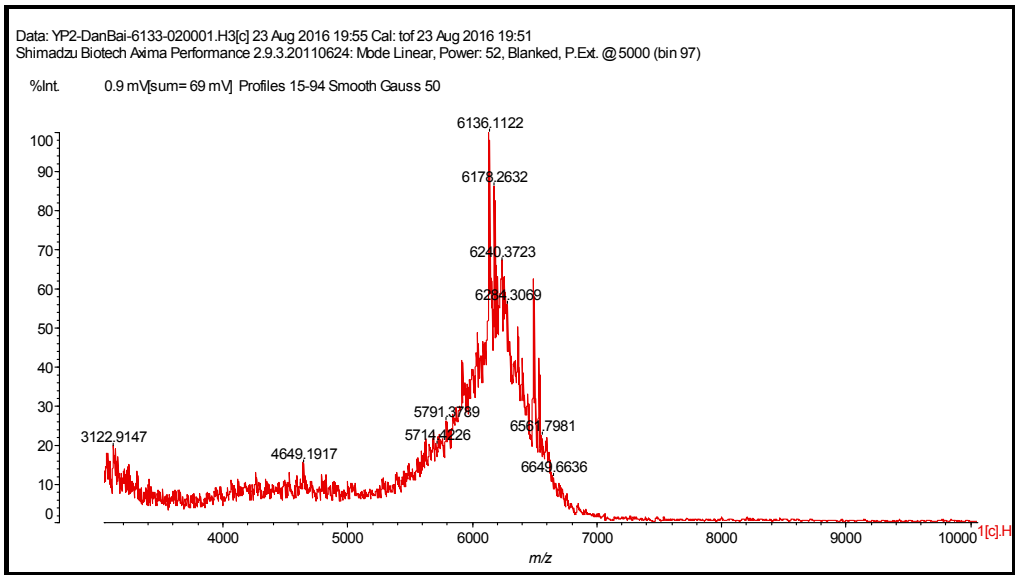

Figure S11. MS (MALDI-TOF) of **P1-FAM**. $C_{284}H_{435}N_{72}O_{80}$ m/z: 6137.9271, $[M+H]^{+}$. Found: 6136.1122, $[M+H]^{+}$.

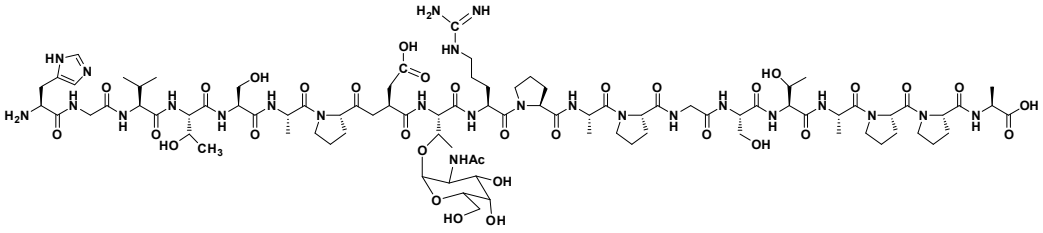

Figure S12. P2 (MUC1 glycopeptide): Analytical data was reported.[1]

References

(1) Cai, H.; Huang, Z. H.; Shi, L.; Zou, P.; Zhao, Y. F.; Kunz, H.; Li, Y. M. Synthesis of Tn/T Antigen MUC1 Glycopeptide BSA Conjugates and Their Evaluation as Vaccines. *Eur. J. Org. Chem.* **2011,** 3685−3689.