Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Multi-component self-assembly anti-tumor nano-vaccines based on MUC1 glycopeptide

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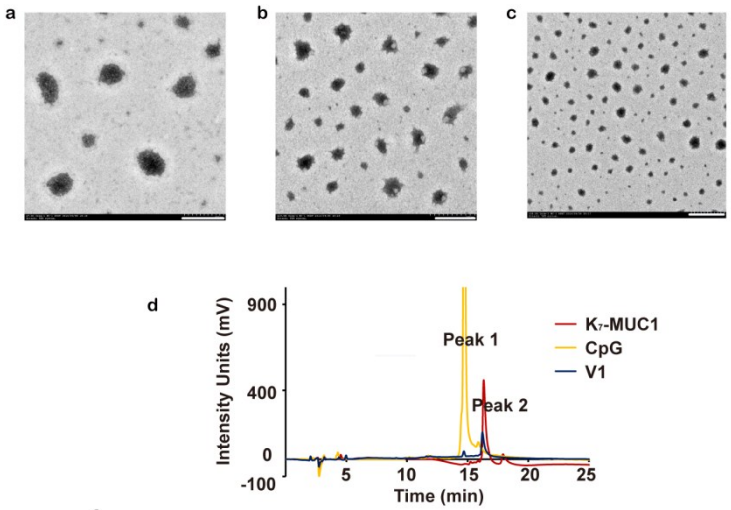

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	Area of CpG	Area of K_7-MUC1	Area of V1	Loading Rate
Peak 1(CpG)	32078268.0		297344.0	99.1%
Peak 2(K_7-MUC1)		8691069.0	2416911.0	72.2%

Fig. S1 (a, b, c) TEM image of **V1** (scale bar: 200 nm), **V2** (scale bar: 100 nm), and **V3** (scale bar: 50 nm). (d) HPLC analysis of the load of CpG and K_7-MUC1 on Pam_3CSK_4-K_5. First, 15 μl 100 μM CpG and 15 μl 100 μM K_7-MUC1 were analyzed by HPLC. Analytic gradient is 5% to 30% of solution A (80% acetonitrile in water with 0.06% trifluoroacetic acid) in solution B (pure water with 0.06% trifluoroacetic acid) in 25 min on the analytic C18 column. Peak 1 represents CpG and Peak 2 represents K_7-MUC1. Then, 15 μl 100 μM **V1** was centrifuged and the supernatant was analyzed under the same conditions. (e) Loading rate of CpG and K_7-MUC1 calculated by areas in HPLC.

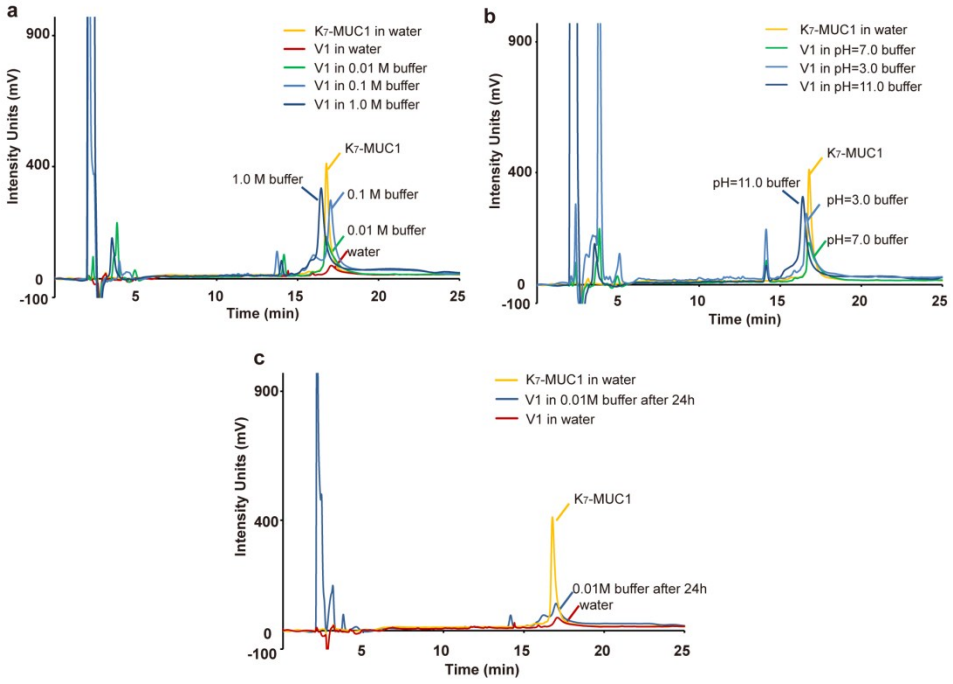


Fig. S2 HPLC analysis of the load of K_7-MUC1 under different conditions. (a) The influence of ionic strength. (b) The influence of pH. (c) The stability of **V1** in buffer solution.

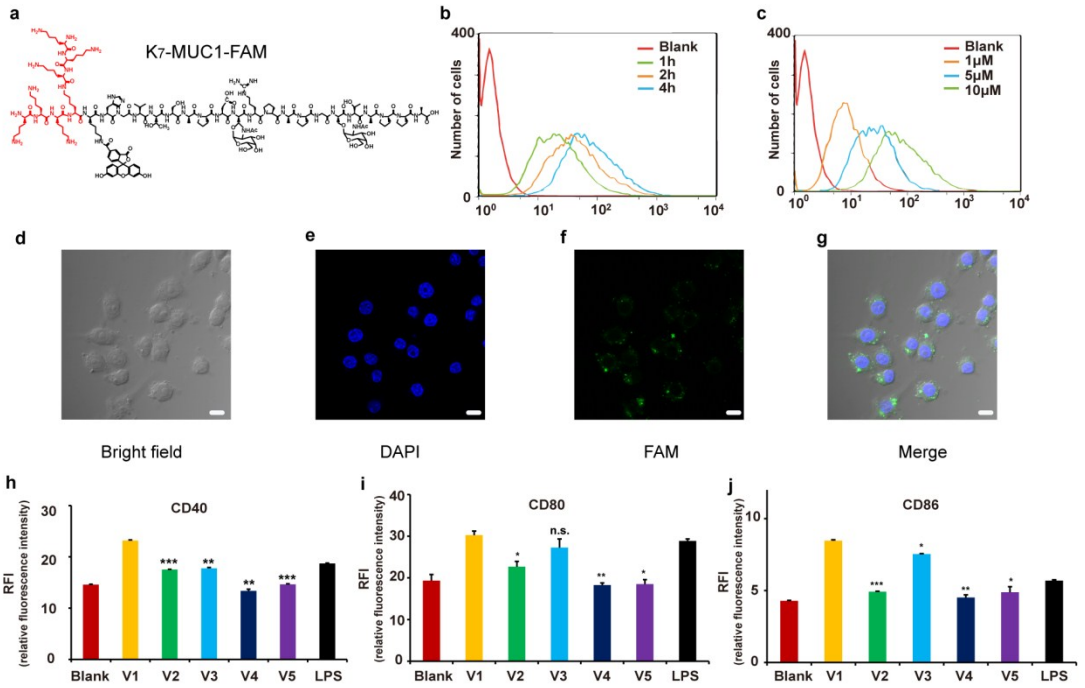


Fig. S3 (a) Structure of K_7-MUC1-FAM. (b, c) Cellular uptake of **V1-FAM**. (d, e, f, g) Fluorescence microscopy images of RAW264.7 cells incubated with **V1-FAM**. The nuclei were stained by DAPI (blue). **V1-FAM** contained FAM green fluorescence. Scale bars=10 μm. (h, i, j) Activation markers CD40, CD80, and CD86 expressed on the surface of RAW264.7 cells were analyzed by FACS based on of the relative fluorescence intensity (RFI). Data are shown as mean ±SD of three separate experiments. ***P < 0.001, **P < 0.01, *P < 0.05, compared with **V1** by student's t test. n.s. = not significant.

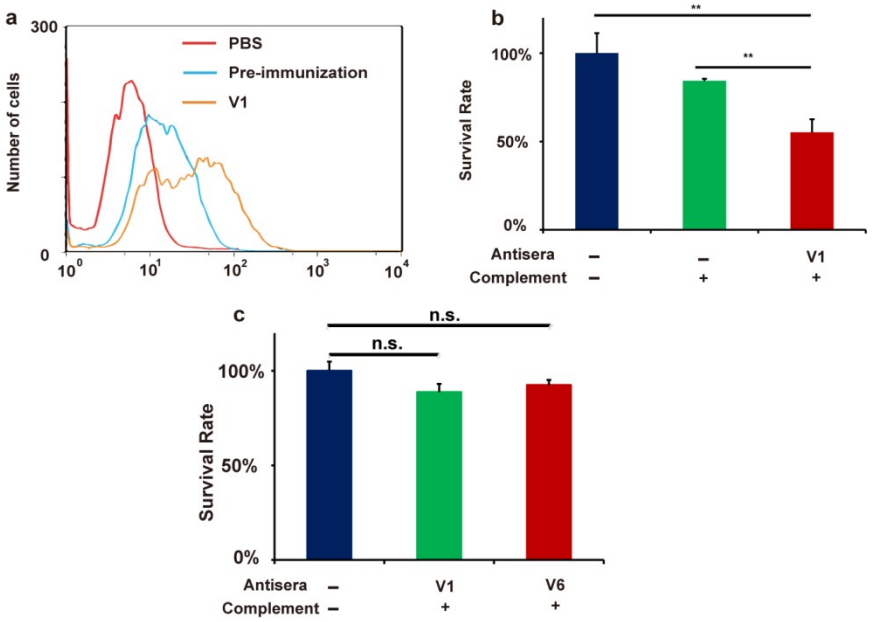


Fig. S4 (a) FACS results of the binding of sera induced by **V1** to MCF-7 tumor cells. (b) CDC results of **V1** in MCF-7 cells. Data are shown as mean ±SD of three separate experiments. **P < 0.01 by student's t test. (c) CDC results of **V1** and **V6** in N2a cells which do not express MUC1. n.s. = not significant by student's t test.

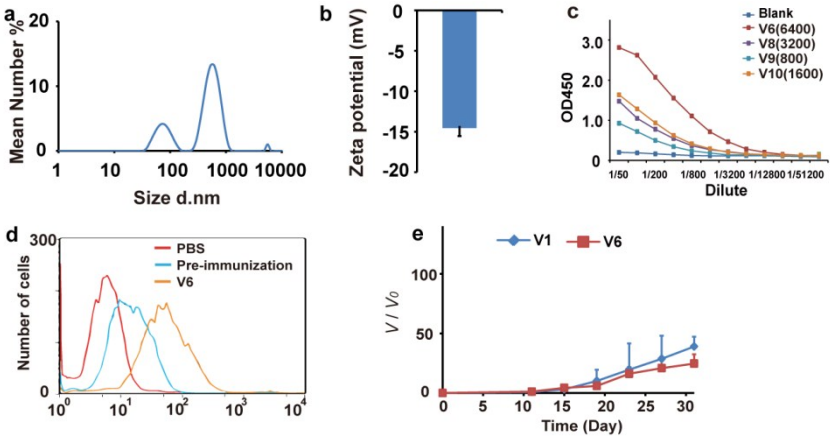


Fig. S5 (a) DLS of **V6**. (b) Zeta potential of **V6**. (c) ELISA results of different vaccines. Antibody titers were labeled in parentheses. Titers are defined as the highest dilution possessing the absorption of 0.1 over that of the blank control. (d) FACS results of the binding of sera induced by **V6** to tumor cells. (e) The tumor volume growth *V* versus the initial volume V_0 (V/V_0) of mice immunized with **V1** and **V6**.

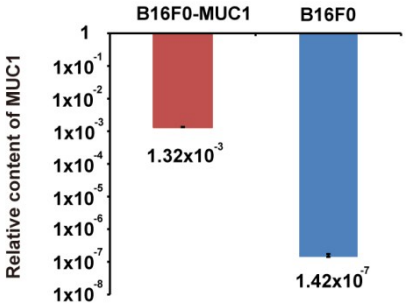


Fig. S6 Relative content of MUC1 gene measured by quantitative real-time PCR. Relative content of MUC1 gene in MUC1-expressing B16F0-MUC1 cells and wild-type B16F0 cells was respectively 1.32 x 10^{-3} and 1.42 x 10^{-7}. MUC1 expressed in B16F0-MUC1 cells was 9296 fold higher than MUC1 expressed in B16F0 cells.

Materials and Methods

Materials.

All commercial reagents were purchased from Aldrich, Sigma, Acros, and GL Biochem(Shanghai, China)Ltd. CpG ODNs 1826 (5'-TCCATGACGTTCCTGACGTT-3') was purchased from SBS(Beijing, China) Genetech Co., Ltd. IL-6 and IL-12 ELISA kits were purchased from DAKEWEI Biotech (Beijing).

Apparatus.

Peptides were synthesized by SPPS on microwave peptide synthesizer (Liberty, CEM). Analysis of peptides was performed on a SHIMADZU LC-6AD HPLC with the UV detector of 215 nm using solution A (80% acetonitrile in water with 0.06% trifluoroacetic acid) and solution B (pure water with 0.06% trifluoroacetic acid). ESI-MS spectra were detected on a ThermoFisher Ultimate 3000 Analytical and MSQ Plus. TEM was recorded on a Hitachi H-7650B transmission electron microscopy. DLS and zeta potential were carried out on a Malvern ZEN3690 Zetasizer apparatus. FACS was analyzed on a Calibur (BD Biosciences) flow cytometer. Fluorescence microscopy images were captured on the Zeiss LSM 780.

Cell lines and animals.

MCF-7 cells, B16F0 cells, RAW264.7 cells, and N2a cells were purchased from China Infrastructure of Cell Line Resources. B16F0 cells over-expressing human MUC1 were constructed by ViewSolid Biotech (Beijing) and the results of over-expression were showed in Fig.S6. Cells were cultured in Dulbecco minimum essential medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics. 6-8-week-old BALB/c female mice and 4-6-week-old C57BL/6 female mice were purchased from Animal Facility of Center of Biomedical Analysis (Tsinghua University) and performed following the animal ethics guideline.

Synthesis and purification of peptides.

Peptides were synthesized by SPPS using the Fmoc protocol. Fmoc group was cleaved by 20% piperidine in dimethylformamide (DMF). Natural amino acids were coupled with N-hydroxybenzotriazole (HOBt, 4.0 equiv), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylhexafluorophosphate (HBTU, 4.0 equiv), and N,N-Diisopropylethylamine (DIEA, 8.0 equiv). Glycosylated amino acids synthesized as our previous works[1] were coupled with 1-Hydroxy-7-azabenzotriazole (HOAt, 2.0 equiv), 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3 -tetramethyluronium hexafluorophosphate (HATU, 2.0 equiv), and N-methylmorpholine (NMM, 5.0 equiv). For the synthesis of Pam_3CSK_4-K_5 and Pam_3CSK_4, Pam_3Cys-OPfp synthesized as our previous works[1] was used and coupled to the peptides with HATU (4.0 equiv), HOAt (4.0 equiv), and NMM (8.0 equiv) for 8 h. Peptides were cleaved from the resin using TFA/H_2O/TIS (20/1.2/1.2, v/v/v) for 3h.

The purification of Pam_3CSK_4-K_5 and Pam_3CSK_4 was carried out on HPLC with a polar-CN column (YMC, 5 μm, 20×250 mm, Japan). Other peptides were purified on HPLC with a C-18 column (YMC, 5 μm, 20×250 mm, Japan). The acetyl on glycopeptides was deprotected with 1% MeONa/MeOH (pH=9.5). After purification, peptides were lyophilized and analyzed on HPLC with the analytic CN or C-18 column. Then, peptides were identified by ESI-MS (see analytical data).

Preparations of vaccines.

For the preparation of **V1**, three components Pam_3CSK_4-K_5, CpG, and K_7-MUC1 were respectively dissolved in water and 3 mM solutions of Pam_3CSK_4-K_5, CpG, and K_7-MUC1 were formed. The solutions of CpG and K_7-MUC1 were successively added to the Pam_3CSK_4-K_5 solution (1/1/1, v/v/v). The solution was diluted 1:10 with water and the 100 μM **V1** was prepared. Analogously, vaccines **V2**-**V10** were prepared using the respective components.

Analysis of transmission electron microscopy.

8 μl 100 μM **V1**, **V2**, and **V3** were respectively added on the copper grids of carbon support films for 1.5 min. 8 μl phosphotungstic acid solution (15 mg/ml, pH=7.0) was used for the negative-stain for 1.5 min. After drying, the copper grids were imaged on a Hitachi H-7650B transmission electron microscopy.

Analysis of dynamic light scattering and zeta potential.

DLS and zeta potential of 100 μM vaccines were measured on a Malvern ZEN3690 Zetasizer apparatus.

HPLC analysis of the load of K_7-MUC1 under different conditions.

To analyze the influence of ionic strength, 3 mM Pam_3CSK_4-K_5, 3 mM CpG, and 3 mM K_7-MUC1 respectively dissolved in water, 0.01 M citric acid/sodium citrate buffer solution (pH 7.0), 0.1 M citric acid/sodium citrate buffer solution (pH 7.0), and 1.0 M citric acid/sodium citrate buffer solution (pH 7.0) were prepared. The solutions of CpG and K_7-MUC1 were successively added to the Pam_3CSK_4-K_5 solution (1/1/1, v/v/v). The solution was diluted 1:10 with the corresponding buffer solution and 100 μM **V1**, 100 μM **V1 (0.01 M)**, 100 μM **V1 (0.1 M)**, and 100 μM **V1 (1.0 M)** were prepared. Then, **V1**, **V1 (0.01 M)**, **V1 (0.1 M)**, and **V1 (1.0 M)** were centrifuged

(10000rpm, 10min) and 15 μl supernatants were respectively analyzed by HPLC. Analytic gradient is 5% to 30% of solution A (80% acetonitrile in water with 0.06% trifluoroacetic acid) in solution B (pure water with 0.06% trifluoroacetic acid) in 25 min on the analytic C18 column. In addition, 15 μl 100 μM K_7-MUC1 dissolved in water was analyzed by HPLC under the same conditions.

To analyze the influence of pH, 3 mM Pam_3CSK_4-K_5, 3 mM CpG, and 3 mM K_7-MUC1 respectively dissolved in 0.01 M citric acid/sodium citrate buffer solution (pH 3.0), 0.01 M citric acid/sodium citrate buffer solution (pH 7.0), and 0.01 M Na_2CO_3/$NaHCO_3$ buffer solution (pH 11.0) were prepared. Following the above method, 100 μM **V1 (pH 3.0)**, 100 μM **V1 (pH 7.0)**, and 100 μM **V1 (pH 11.0)** were prepared. Then, **V1 (pH 3.0)**, **V1 (pH 7.0)**, and **V1 (pH 11.0)** were centrifuged and 15 μl supernatants were respectively analyzed by HPLC under the same conditions.

To analyze the stability of **V1** in buffer solution, 1.0 mM **V1** was diluted 1:10 with 0.01 M citric acid/sodium citrate buffer solution (pH 7.0). After 24h, 100 μM **V1** was centrifuged and 15 μl supernatant was analyzed under the same conditions.

Cellular uptake of V1.

6×10^5 RAW264.7 cells were added to each well on 24-well culture plates and cultured for 12 h. **V1-FAM** with different concentrations (10 μM, 5 μM, and 1 μM) were incubated for 4 h. In addition, 10 μM **V1-FAM** was separately incubated for 1 h, 2 h, and 4 h. The cells were collected and washed four times with PBS buffer and analyzed on a BD Calibur flow cytometry.

For the fluorescence microscopy images, 2×10^5 RAW264.7 cells were planted on glass slides in 6-well culture plates and cultured for 12 h. Then, **V1-FAM** (10 μM) was incubated for 24 h. The cells were washed four times with PBS buffer and incubated with 4% (w/v, in PBS) paraformaldehyde for 30 min. After washing, 0.2% (w/v, in PBS) Triton X-100 was added and incubated for 10 min. 10 μL DAPI (4',6-diamidino-2-phenylindole) was added to stain the cells for 1 min. The fluorescence microscopy images were captured on the Zeiss LSM 780.

Analysis of the cell activation based on cytokines and cell markers.

6×10^5 RAW264.7 cells were added to each well on 24-well culture plates and cultured for 24 h. Then vaccines with 10 μM and LPS with 5 μg/ml were incubated with cells for 24 h. The supernatant was analyzed with IL-6 and IL-12 ELISA kits. Cells were harvested and incubated with the mouse cell marker antibodies (anti-CD40-phycoerythrin, anti-CD80-phycoerythrin, and anti-CD86-phycoerythrin, dilution to 1:100) at 0 ^oC for 1 h. After washed, the cells were analyzed on a BD Calibur flow cytometry.

Immunizations of vaccines.

6-8-week-old BALB/c female mice were breeded in Animal Facility of Center of Biomedical Analysis in Tsinghua Universiy. Four mice each group were separately intraperitoneally administered with 100μL 10 nmol vaccines every two weeks. One week after the fifth immunization, antisera were collected and preimmune sera were used as the blank control. All mice were treated according to the animal ethics guidelines.

Analysis of antibody titers.

20 μg/ml MUC1 (100 μl/well) in $NaHCO_3$ solution (0.1 M, pH=9.6) was added to each well on high-binding 96-Well ELISA plate for 12 h at 4^oC. After washed by 0.05% Tween-PBS solution, the plates were blocked by 0.25% gelatin-PBS solution for 3 h. After washing, the diluted antisera were added to the plates (100 μl/well) and incubated for 1.5 h at 37 ^oC. After washing, rabbit anti-mouse IgG-Peroxidase antibodies with 1:2000 dilution were added to the plates (100 μl/well) and incubated for 1 h at 37 ^oC. After washing, 100 μl OPD substrate (1 mg/ml 1,2-

phenylenediamine and 1.5 μl/ml 30% H_2O_2) was added to each well. After 20 minutes, the absorbance was detected at 450 nm wavelength. Titer is defined as a highest dilution possessing an absorption of 0.1 over that of the blank control.[2]

Analysis of antibody isotypes.

MUC1 was coated on the plate as the above methods. The antisera of **V1** were diluted to 1:50 and the antisera of **V6** were diluted to 1:200. Then 100 μl antisera were added to each well and incubated for 1.5 h at 37 °C. After washing, goat anti-mouse isotype antibodies (IgM, IgG1, IgG2a, IgG2b, and IgG3 diluted to 1:1000) were added to the plates (100 μl/well) and incubated for 1 h at 37 °C. After washing, 100 μl rabbit anti-goat IgG-Peroxidase antibodies (diluted to 1:1000) were added to each well and incubated for 1h at 37 °C. After washing, 100 μl OPD substrate was added to each well. After 20 minutes, the absorbance was also detected at 450 nm wavelength.

The binding of antisera to tumor cells.

6×10^5 MCF-7 cells were incubated with 100 μl **V1** and **V6** antisera diluted to 1:25 for 1 h at 0 °C. After washed with PBS buffer, the cells were incubated with FITC-conjugated rabbit anti-mouse IgG antibody diluted to 1:50 for 1 h at 0 °C. After washing, the cells were analyzed on a BD Calibur flow cytometry.

Analysis of complement dependent cytotoxicity.

8000 MCF-7 cells were added to each well on 96-well culture plate and cultured for 12 h. **V1** and **V6** antisera diluted to 1:10 were added (50 μl/well) and incubated for 0.5 h. 50 μl rabbit complement diluted to 1:2 was added to each well and incubated for 8 h. 0.5% MTT solution in PBS (MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) was added (20 μl/well) and incubated for 4 h. After removing the medium, DMSO was added (150 μl/well) and the absorption was analyzed at 490 nm wavelength. The survival rate of cells was measured with the following formula.

$$\text{Survival rate (\%)} = (\text{experimental OD}/\text{control OD}) \times 100$$

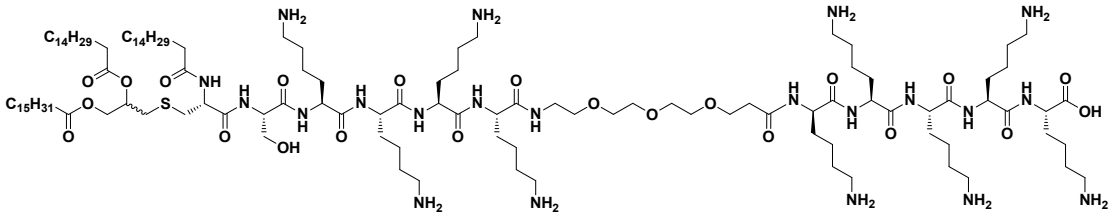
The CDC results of **V1** and **V6** with N2a cells were performed according to the above methods.

Analysis of anti-tumor immune responses

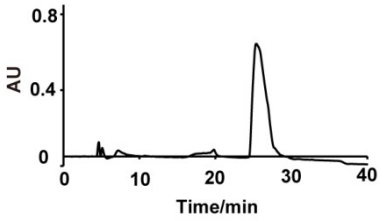
1×10^5 B16F0 tumor cells (positive for human MUC1) were administered subcutaneously into the right flank of 4-6-week-old C57BL/6 female mice. Nine days after injection, the tumor volume reached about 60 mm^3 and 100 μl 10 nmol vaccines were separately given three peritumoral injections every three days. PBS buffer was used as the blank control. Each group contained five mice. The length and width of tumor were measured with a caliper every four days. The volume of tumor was calculated with $0.5 \times \text{length} \times \text{width}^2$.

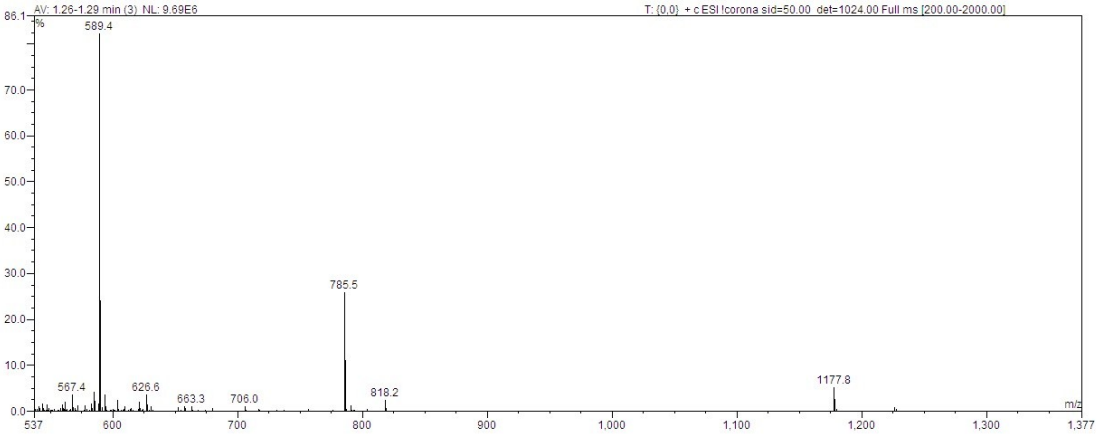
Analytical data.

Pam_3CSK_4-K_5:



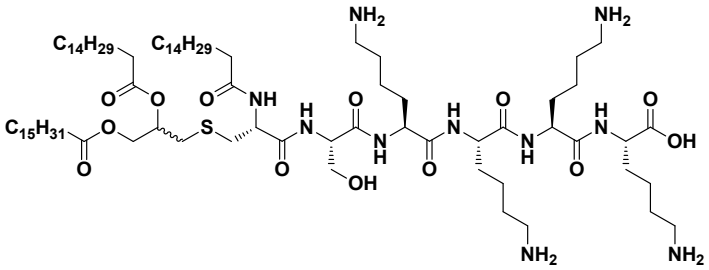
Analytic HPLC trace of Pam_3CSK_4-K_5. Analytic gradient is 20% to 60% of solution A (80% acetonitrile/water with 0.06% trifluoroacetic acid) in solution B (pure water with 0.06% trifluoroacetic acid) in 40 min on the analytic CN column. Retention time is 25.4 min.



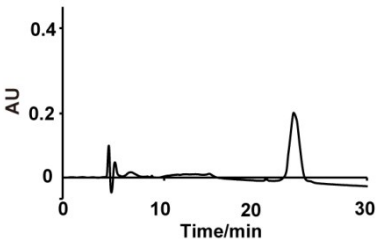


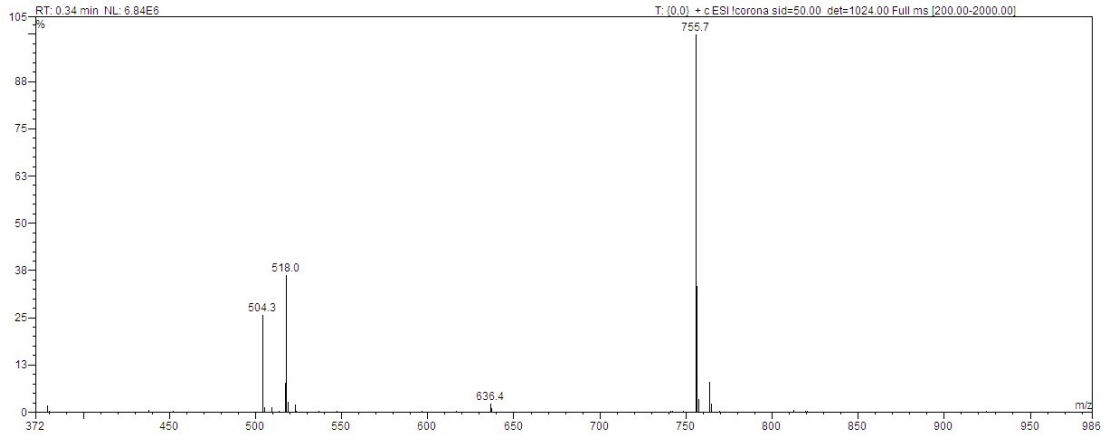
MS (ESI) of Pam_3CSK_4-K_5. $C_{120}H_{233}N_{21}O_{22}S$ m/z: 2353.8, $[M+H]^+$. Found: 589.4, $[M+4H]^{4+}$; 785.5, $[M+3H]^{3+}$; 1177.8, $[M+2H]^{2+}$.

Pam_3CSK_4:



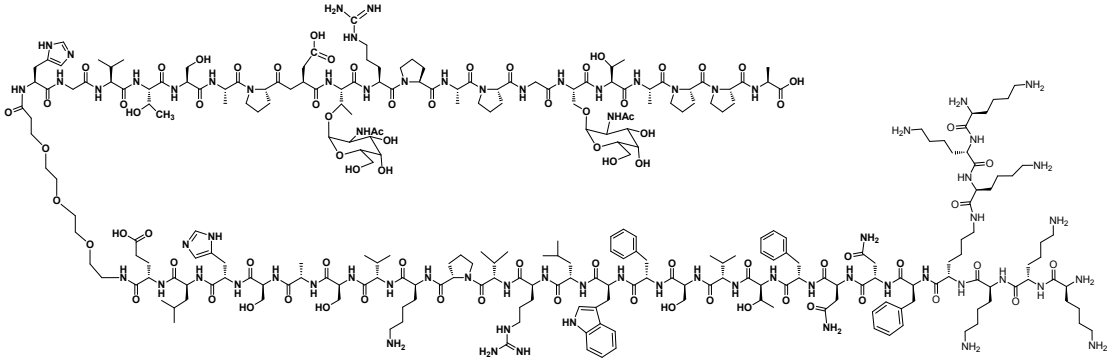
Analytic HPLC trace of Pam_3CSK_4. Analytic gradient is 50% to 90% of solution A (80% acetonitrile/water with 0.06% trifluoroacetic acid) in solution B (pure water with 0.06% trifluoroacetic acid) in 30 min on the analytic CN column. Retention time is 22.8 min.



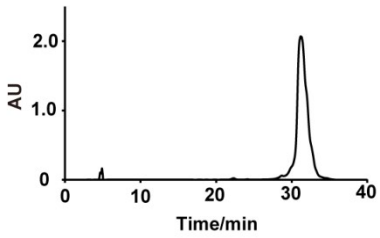


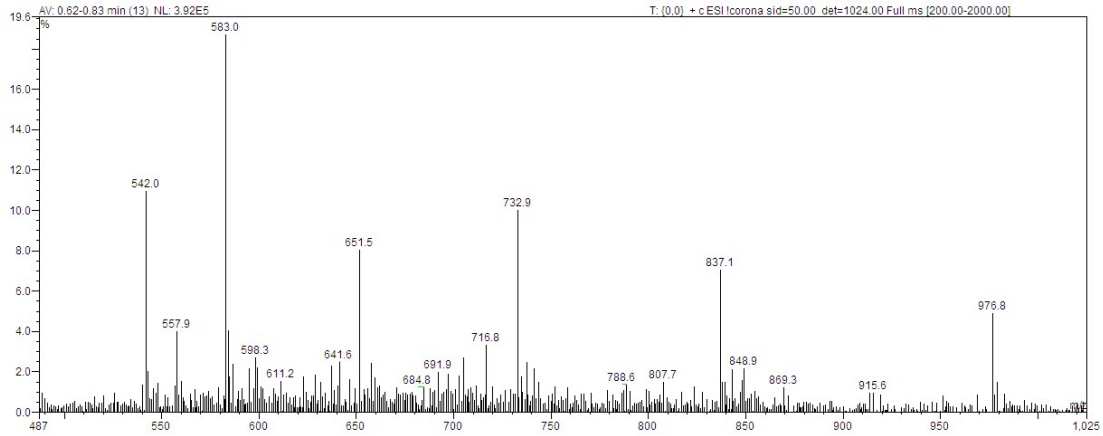
MS (ESI) of Pam_3CSK_4. $C_{81}H_{156}N_{10}O_{13}S$ m/z: 1510.2, $[M+H]^+$. Found: 504.3, $[M+3H]^{3+}$; 518.0, $[M+2H+K]^{3+}$; 755.7, $[M+2H]^{2+}$.

K_7-T^{21}-MUC1:



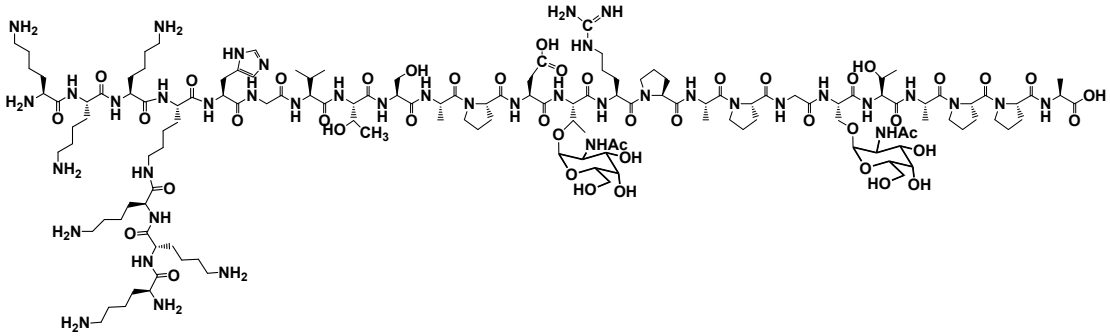
Analytic HPLC trace of K_7-T^{21}-MUC1. Analytic gradient is 10% to 40% of solution A (80% acetonitrile/water with 0.06% trifluoroacetic acid) in solution B (pure water with 0.06% trifluoroacetic acid) in 40 min on the analytic CN column. Retention time is 31.2 min.



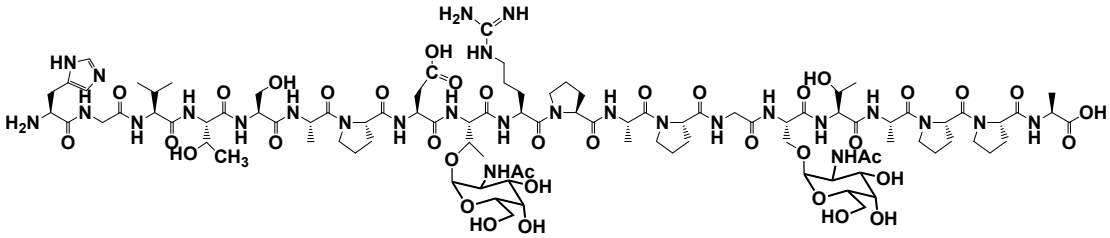


MS (ESI) of K_7-T^{21}-MUC1. $C_{265}H_{424}N_{71}O_{78}$ m/z: 5851.2, $[M+H]^+$. Found: 542.0, $[M+11H]^{11+}$; 583.0, $[M+10H]^{10+}$; 651.5, $[M+9H]^{9+}$; 732.9, $[M+8H]^{8+}$; 837.1, $[M+7H]^{7+}$; 976.8, $[M+6H]^{6+}$.

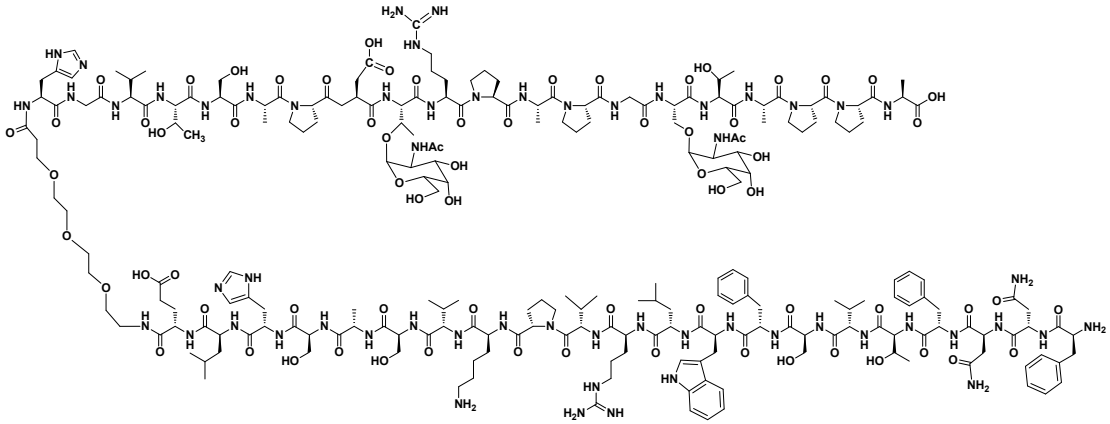
K_7-MUC1: Analytical data was reported.[3]



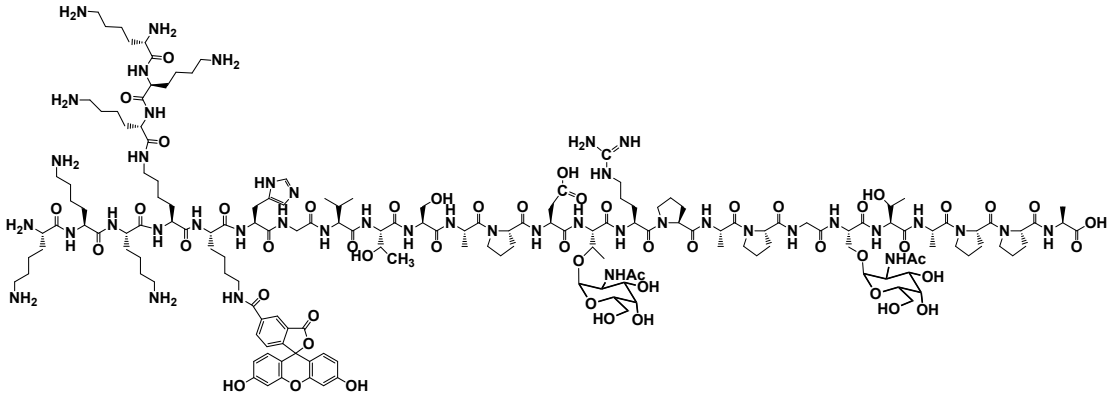
MUC1: Analytical data was reported.[3]



T^{21}-MUC1: Analytical data was reported.[3]



K_7-MUC1-FAM: Analytical data was reported.[3]



Reference

1 H. Cai, Z. Y. Sun, Z. H. Huang, L. Shi, Y. F. Zhao, H. Kunz and Y. M. Li, *Chem.-Eur. J.*, 2013, **19**, 1962.

2 S. Ingale, M. A. Wolfert, J. Gaekwad, T. Buskas and G. J. Boons, *Nat. Chem. Biol.*, 2007, **3**, 663.

3 Y. F. Liu, Z. Y. Sun, P. G. Chen, Z. H. Huang, Y. Gao, L. Shi, Y. F. Zhao, Y. X. Chen and Y. M. Li, *Bioconjugate Chem.*, 2015, **26**, 1439.