Supporting Information

A Stable Gold Nanoparticle-Based-Vaccine for the Targeted Delivery of Tumor-Associated Glycopeptide Antigens

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MATERIALS AND METHODS

General Experimental Procedures: Routine chemicals were purchased from Sigma-Aldrich. Tetrachloroauric acid (HAuCl4) was purchase from Wuhan Golden Wing Industry & Trade Co, Wuhan China. All amino acids and peptide synthesis material were purchased from CEM Corp., (Matthews, NC). Peracetylated TF-Serine glycoamino acid was either prepared as previously described or purchased from Sussex Research, Ottawa, Ontario, Canada. Solvents were dried in a Grubb still percolation system under a nitrogen atmosphere. MALDI mass spectra were collected on a Shimadzu Axima Confidence MALDI-TOF mass spectrometer equipped a high mass CovalX HM4 detector operated in linear positive ion mode. Samples were prepared for MALDI analysis by desalting using a 0.5 mL 30K Amicon Ultra centrifugal filter. Samples were spotted on an Axima 384 well sample plate using the overlayer method with sinapinic acid as the matrix. Dynamic light scattering and zeta potential data were collected on a Malvern Nano-ZS Zetasizer instrument. Proton and carbon NMR data were collected on either a Bruker NanoBay 400 Mhz spectrometer with a Bruker 2-channel SMART probe or on a Bruker AVANCE III 500 MHz spectrometer with a TCI (^{1}H, ^{13}C, ^{15}N) cryoprobe at 25°C. Most data were run in 90%/10% H_2O/D_2O. Water suppression was performed using excitation sculpting (1D pulse sequence zgesgp).

Transmission Electron Microscopy. For TEM imaging, carbon film-coated 200 mesh copper grids were glow-discharged to improve hydrophilicity. One drop (~3.5 µL) of the sample dissolved in water was applied to a grid for 30 seconds to allow adsorption. The excess solution was removed with filter paper, and the grid was air-dried. Digital micrographs were collected using an Hitachi H-7650 electron microscope equipped with a 2k CCD camera at an operating voltage of 80 kV.

Dynamic Light Scattering and Zeta Potential. DLS data were collected on a Malvern Zetasizer model nano ZS in MilliQ water, with samples diluted in 1.5 ml cuvettes. Runs were performed in triplicate at 90 seconds per run. Zeta potential measurements were performed on the same instrument using water as the dispersant in DTS1070 cuvettes, at 25 deg C using the Smoluchowski theoretical model.

ELISA Assays. TF-Ser(5)-MUC4-BSA or MUC4-BSA[1] were coated on Immulon® Microtiter™ 4 HBX 96 well-plates at 1 µg/mL in 0.1 M carbonate buffer (pH 9.2) and incubated for 18 h at 4 °C. Plates were washed three times with 200 µL of washing buffer (1× PBS buffer with 0.05% Tween® 20) and blocked with 200 µL of 3% BSA for 2 h at room temperature. Serum from vaccinated and control mice were initially diluted at 1:1000 and then serially half-log10 diluted and added to wells for a final volume of 100 µL in each well The plates were then incubated for 2 h at 37 °C on an orbital shaker. After incubation, the plates were washed three times with 200 µL of washing buffer. Alkaline phosphatase-linked secondary antibodies (anti-IgM and anti-IgG, Jackson ImmunoResearch) were used to detect primary antibodies bound to either TF-MUC4 or MUC4. The secondary anti-IgM antibodies were diluted 1:1000 while the anti-IgG antibodies were diluted 1:5000. A volume of 100 µL of the respective secondary antibody was placed in each well and incubated for 1 h at 37 °C. The plates

were washed three times with 200 µL of washing buffer followed by addition of p-nitrophenyl phosphate (PNPP, 1 mg mL) in diethanolamine buffer (pH 9.8) at 100 µL per well and further incubated for 30 min. The optical density was read at 405 nm using a BioTek Synergy HTX Multi-Mode Microplate Spectrophotometer. Titers were determined by regression analysis with dilutions plotted against absorbance. The titer cutoff point value was set at 0.3 which was three times the control titer determination for the PBS-innoculated mice anti-sera. Statistical analysis from ELISAs for experimental groups were compared with the controls using the paired t test in GraphPad Prism, version 6.

Ovalbumin Control Assay. P388D1 macrophage cells (2x104 cells/well) were plated and activated by the addition of 10 ng of IFN-γ and 100 ng of LPS per mL of culture for 24 hr. The media was removed and cells were washed carefully to avoid imparting additional stress to the cells. Stimulation of cells with the positive control OVA peptide (17-mer or 21-mer) was performed both at a high (200 nM) and a low concentration (2 nM). The B13G-OVA21-AuNPs and B13G-AuNPs were then used to stimulate the P388D1 macrophage cells at three concentrations (based on an estimated concentration of conjugate on the nanoparticle, see Supporting Information) at 16 nM, 4 nM, and 2 nM. For the unconjugated B13G-AuNP negative control nanoparticle, the cells were stimulated at 16 nM. At 24 hr post-stimulation, the media was removed and 5x104 Do-11.10 T cells were co-cultured with the antigen primed macrophages for 24 hr. Cell supernatants were examined for IL-2 release using the Mouse IL-2 Quantikine ELISA kit following manufacturer instructions

Mouse Immunizations. *In vivo* studies were performed according to the Frederick National Laboratory for Cancer Research (Frederick, MD) Animal Care and Use Committee (ACUC) guidelines. Frederick National Laboratory is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the “Guide for Care and Use of Laboratory Animals” (National Research Council; 2011; National Academies Press; Washington, D.C.). Pathogen-free C57BL/6 female mice age 6–10 weeks were purchased from Charles River Laboratories International, Inc. (Frederick, MD).

Immunizations were performed on sets of 5 mice per group. Animals were injected on days 0, 14, 28 and 42 by intraperitoneal (IP) injection with 30 ug of nanoparticles (B13G-AuNPs, B13G-MUC4-AuNPs or B13G-MUC4-Ser(5)-AuNPs) in 100 µl of buffer solution with addition of either 100 µl of Sigma Adjuvant System (SAS) or TiterMax Gold Adjuvants or 1X PBS for control. On day 52, blood serum was obtained via cardiac puncture and the spleens were harvested. The spleens were pooled and homogenized from each of the experimental and control groups. Mouse CD4+, CD8+, and DC’s were isolated from each of the groups via MojoSortTM isolation kits. The isolated cells were then used in ELISpot assays.

Similar to the protocol above, vaccinations with a TF-MUC4-CRM197 conjugate were performed in a similar manner only this time using 3 µg of the this7 conjugate in 100 µL of 1X PBS (pH 7.4) along with 100 µL of Sigma Adjuvant System (SAS). On day 52 blood sera and spleens were processed as described above.

Cytokine Arrays. Mouse Th1/Th2/Th17 Array Q1 (Raybiotech Cat# QAM-TH17-1) was performed using manufacturers protocols. Pooled neat serum from B13G-AuNPs, B13G-MUC4-AuNPs or B13G-TF-Ser(5)-MUC4-AuNPs and 1X PBS was diluted 2-fold in 1X PBS. These diluted samples (100 µL each) were incubated on the array plates for 1 h at room temperature. The plates were washed 5 times for 5 min at each step and 80 ul of the biotinylated detection antibody cocktail was added and incubated for 1 h. The array plates were further wash 5x for 5 min each. The Cy3 equivalent dye streptavidin-conjugate (80 □L) was added to each well and incubated in the dark for 1 h at room temperature. Plates were read on a Biotek Synergy plate reader.

ELISpot assays. Dual-Color ELISpot Mouse IFN-γ/IL-17 kits (R&D systems catalog #ELD5007) were used and the assays were performed using the manufacturers protocols. A 10% FBS IMDM media, supplemented with 1% penicillin-streptomycin, 1% GlutaMAX and 1% β-mercaptoethanol was used to culture the isolated splenocytes. CD4+ T-cells (100 µL , 2.3 x105 cells per well) and DC's (100 µL , 2.0 x105 cells per well) isolated from experimental mice were incubated with either 20 µg of unglycosylated MUC4 peptide or TF-Ser5-MUC4 glycopeptide antigens to stimulate cytokine release. The plates were incubated for 48 hr at 37°C and 5% CO2. Manufacturers protocols were used to develop the plates and were read on a Immunospot Analyzer.

Total Carbohydrate Determination of B13G AuNPs. For all standards and samples, 50 uL of material was plated on a clear 96 well plate and 150 uL of concentrated H_2SO_4 was added. The plate was placed in a 37° C shaking incubator for 30 minutes. Then, 30 uL of a 5% phenol solution was added to each well and the plate was incubated for 30 minutes at 37 C. Plate were read using an OD of 490 nm. The standard glucose curve was prepared using a two-fold dilution over 7 points ranging from 100-1.56 ug/mL of glucose. For determination of peptide-coated AuNPs, 50 ug/mL of similarly treated B13G AuNP, B13G-OVA21-AuNP and B13G-OVA17-AuNPs were interpolated off of the standard glucose curve.

FITC-PEG-SH 5K loading on B13G-AuNP. One mL of 3 mg/ml solution of FITC-PEG-SH was added to 3 mL of a B13G-AuNP OD=1.0. The reaction was placed in a shaking incubator overnight at 45°C. The solution was concentrated in a spin filter 50K MWC 7 times. A standard curve of FITC-PEG-SH was generated from 100 ul of 0.5 mg solution that was serially diluted. The FITC-B13G-AuNP was examined on a fluorescent intensity plate reader @488 nm. The initial fluorescent intensity was low (due to quenching) and the wells were treated with a solution of DTT (50 mg/mL) and 10 uL was added to the well to displace FITC from gold and fluorescence was read at 488 nm.

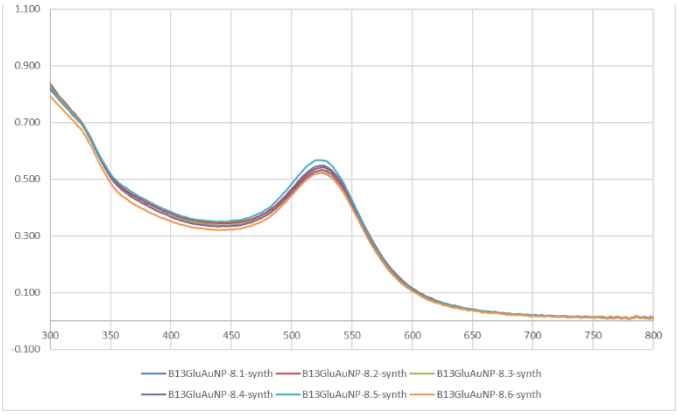
MALDI Mass Spectrometry. MALDI mass spectra were collected on a Shimadzu Axima Confidence MALDI-TOF mass spectrometer equipped a high mass CovalX HM4 detector operated in linear positive ion mode. The samples were prepared for MALDI analysis by desalting using a 0.5 mL 30K Amicon Ultra centrifugal filter. Samples were spotted on an Axima 384 well sample plate using the overlayer method

with sinapinic acid as the matrix. MALDI data for the CRM197 conjugates is shown in Figure S14.

High Resolution Mass Spectrometry. LC/MS analysis was carried out in positive ion mode on an Orbitrap LTQ-XL system that was configured with a UV-visible DAD detector in-line. A 2.0-µl aliquot of the analysis solution (1 mg/ml) was injected onto a 2.1 X 50 mm Phenomenex 2.6 µm XB-C18 Kinetex column for separation using LC/MS-grade CH_3CN and H_2O in a combination isocratic and linear gradient program, as indicated below, at a flow rate of 250 µl /min. With a rapid solvent reset and equilibration, the complete analysis cycle time was 18 min.
Solvent A – 2% CH_3CN/H_2O with 0.1% HCOOH
Solvent B – 90% CH_3CN/H_2O with 0.1% HCOOH
0 – 2 min isocratic at 100% A
2 – 11 min linear gradient from 0% - 100% B
11 – 14 min isocratic at 100% B (90% CH_3CN/H_2O)
14 – 16 min linear reset to 100% A (2% CH_3CN/H_2O)
16 – 18 min equilibrate at 100% A

Dectin-1 Binding. Clear bottom Immulon 4HBX plates were coated with 50 µL of 50 µg/mL solution of B13G-AuNPs, B13G-AuNPs-MUC4, B13G-AuNP-TF-MUC4 or B13Gs. Plates were incubated at 37 C for 4 h to allow for evaporation. Plates were washed with 1X PBS to remove any unbound material. Fc-hDectin-1 (Invivogen, San Diego, Ca; 100 µL) in concentrations of 100 nM, 33 nM, 11 nM, 3.7 nM, and 1.2 nM was added in triplicate to the aforementioned plate coatings and incubated for 2 hr at 37°C. Plates were washed 3 times with washing buffer. Goat Anti-Human IgG, (Fc fragment specific) HRP conjugate was diluted to 1:5000, (100 µL) of this solution was added to the wells and incubated for 1 h at 37°C. Plates were washed with washing buffer 3 times and 100 uL of TMB liquid substrate system for ELISA was added to each well. Plates were incubated for 10 minutes and the reaction was stopped by adding 50 uL of H_2SO_4. Plates were read at 450 nm.

Analytical HPLC. Analytical HPLC chromatograms were collected using an Agilent 1100 series analytical HPLC system with an Agilent Eclipse plus C18 (3.5 µm, 4.6X100 mm) column. Lyophilized peptides were reconstituted in LC-MS grade water to 5 mg/mL. 5 µL of sample was injected into the system at a draw speed of 100 µL/min and a flow rate of 1.0 mL/min. A linear gradient was used over the course of 30 minutes beginning at a ratio of 100% water:0% acetonitrile to 20% water:80% acetonitrile.



Z-average and PDI

8.1 – 38.72 nm (0.208)
8.2 – 36.86 nm (0.220)
8.3 – 38.56 nm (0.242)
8.4 – 37.67 nm (0.213)
8.5 – 38.47 nm (0.198)
8.6 – 41.63 nm (0.212)

Figure S1. UV/SPR spectra of six different syntheses (labeled 8.1 – 8.6) of B13G AuNPs. The table to the right depicts the Z-average sizes and polydispersity indices of the six synthetic AuNPs.

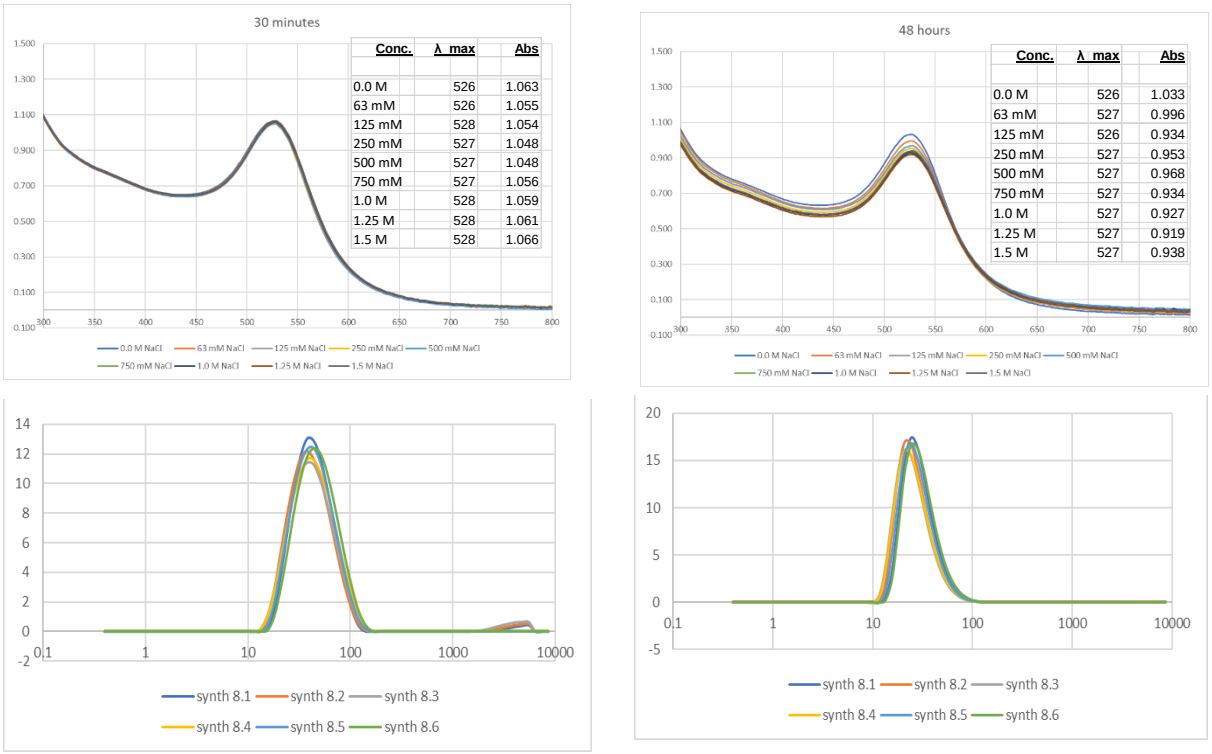
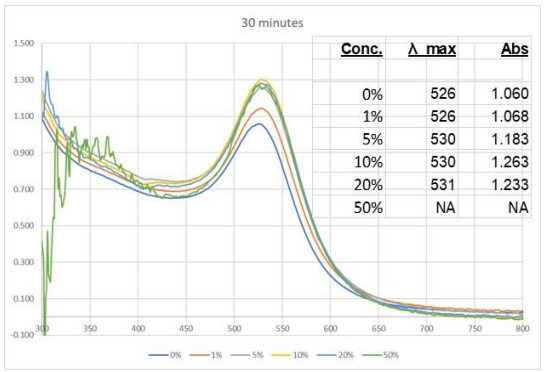


Figure S2. UV/SPR and DLS spectra of the same six different syntheses of B13G AuNPs. Top figures show an overlay of UV spectra of B13G AuNPs incubated with increasing concentrations of NaCl (0 – 1.5 M) after 30 min (upper left) and 48 hours (upper right). The tables show the salt concentration, absorbance maxima and raw absorbance units for each experiment. Lower figures: Overlays of DLS intensity data (lower left) and volume data (lower right) from the six synthetic B13G AuNPs.



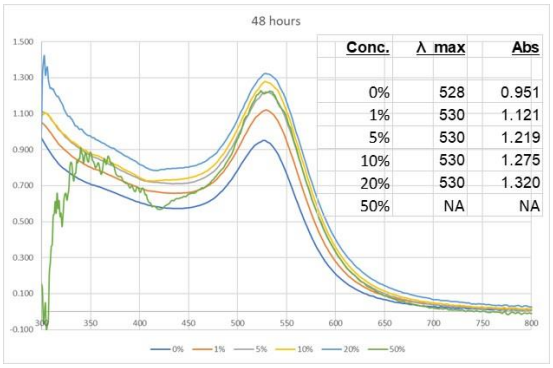


Figure S3. UV/SPR of a select synthesis (8.4) of B13G AuNPs after treatment with increasing percentages of human serum (0% - 50%, by volume). The figure on the left shows an overlay of UV spectra after 30 min and the one on the right is after 48 h. The noise in the spectrum of highest percentage serum is due to the increasing turbidity of the solution as serum is increased and not due to any particle aggregation.

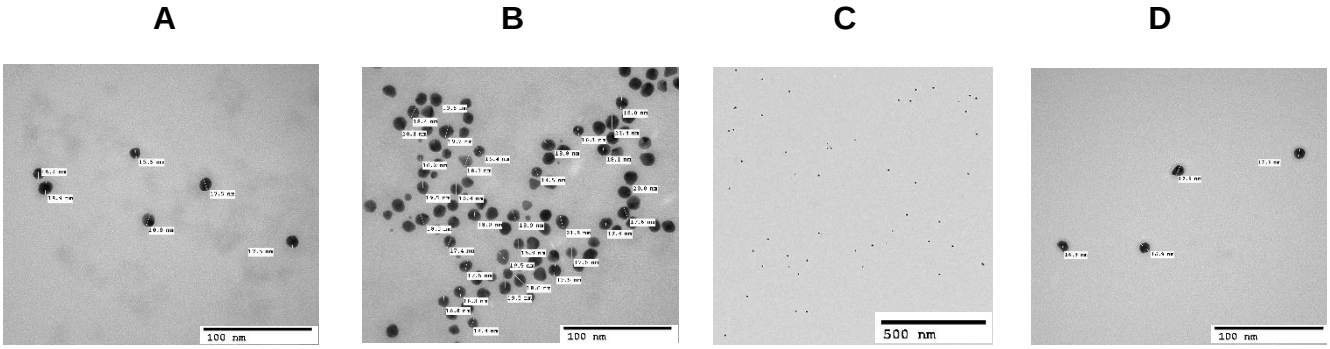


Figure S4. Representative TEM images of B13G-AuNP-MUC4 (A, B) and B13G-AuNP-TF-MUC4 (C, D) with size measurements. The sample in **C** is more dilute but particles are very uniform. AuNPs in D are an expansion of the upper right portion of C

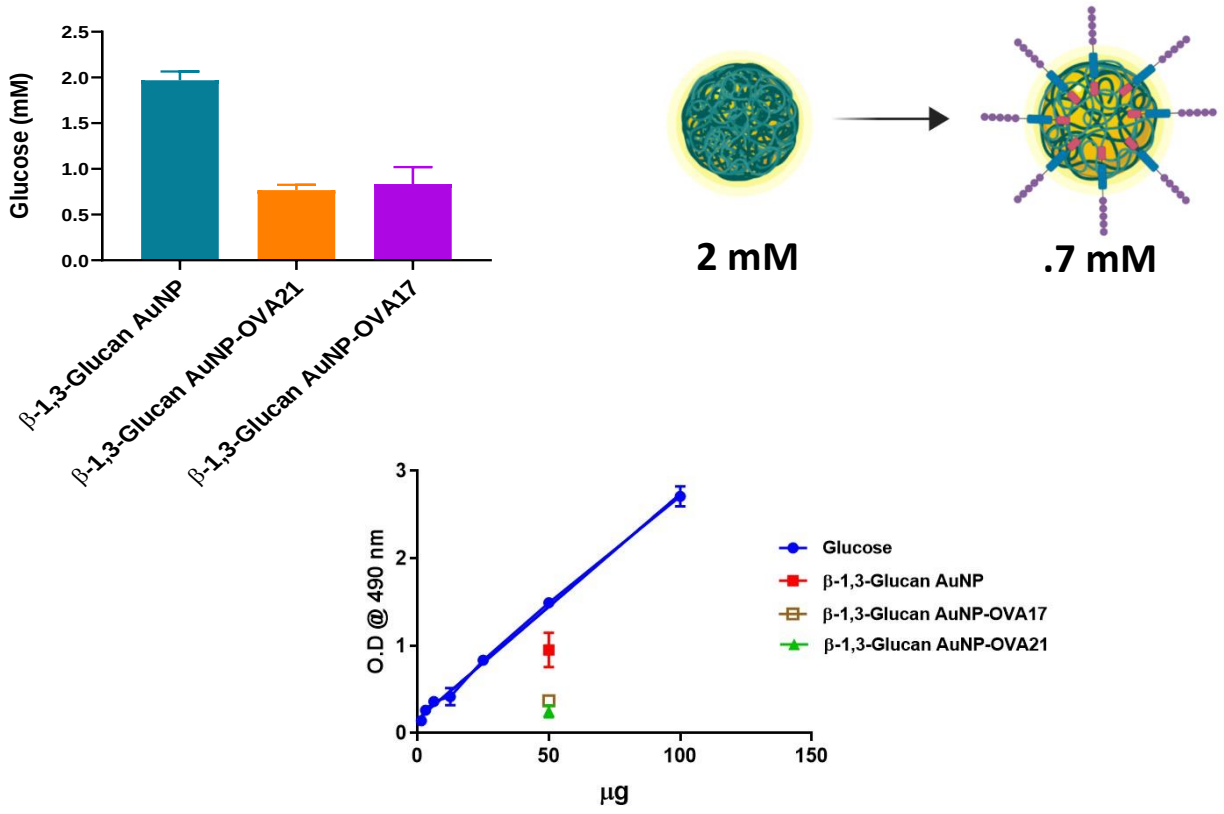


Figure S5. Displacement of B13G from particle after place exchange with OVA peptides. Concentration of sugar from the glycopolymer goes from 2 mM to 0.7 mM upon coating. Standard curve of glucose concentration is also shown below

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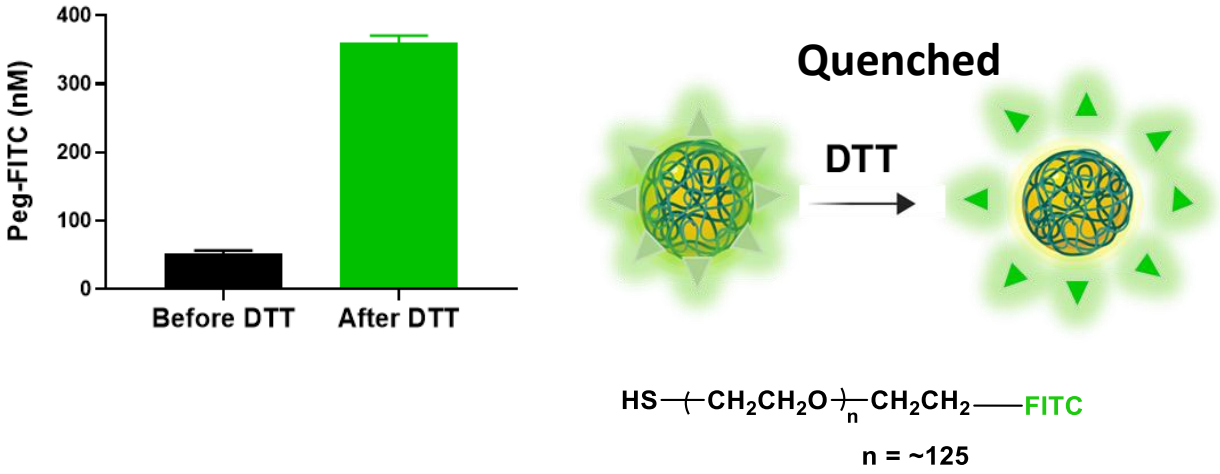


Figure S6. Graph of concentration of PEG-FITC-Thiol (structure on lower right) before and after Dithiothreitol treatment. Coupling to AuNPs will quench fluorescence; displacement restores fluorescence and signal is enhanced. Structure of FITC Thiol is shown in lower right.

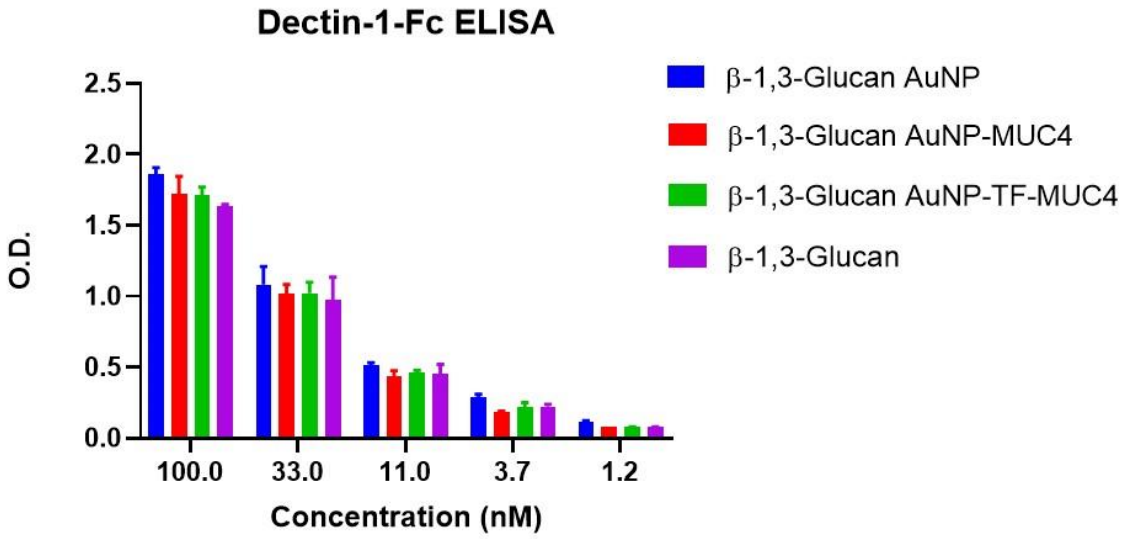


Figure S7. Dectin-1 ELISA assay showing binding to various AuNPs used in this study. The C-terminal domain of human Dectin-1 was used conjugated to a human IgG Fc region through a 10 amino acid linker. This allowed for better detection with a Fc-specific human anti-IgG antibody. Dectin1 binding remains even after subsequent coating with antigen peptide/glycopeptide and is detectable down to single digit nanomolar concentrations.

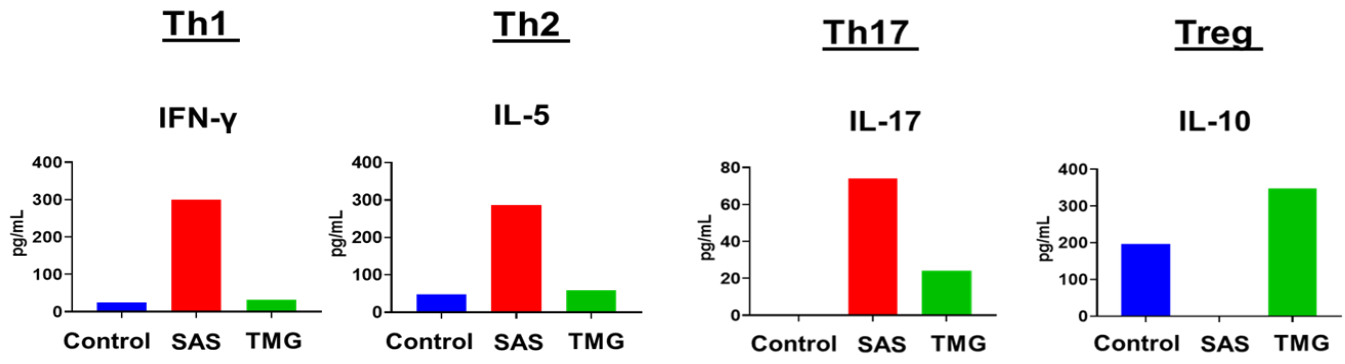


Figure S8. Expression of IFN-γ, IL-5, IL-17 and IL-10 after vaccination with MUC4-B13G-AuNP, with a comparison between amounts (pg/ml) generated using SAS or TMG adjuvants. Controls are uncoated B13G AuNPs.

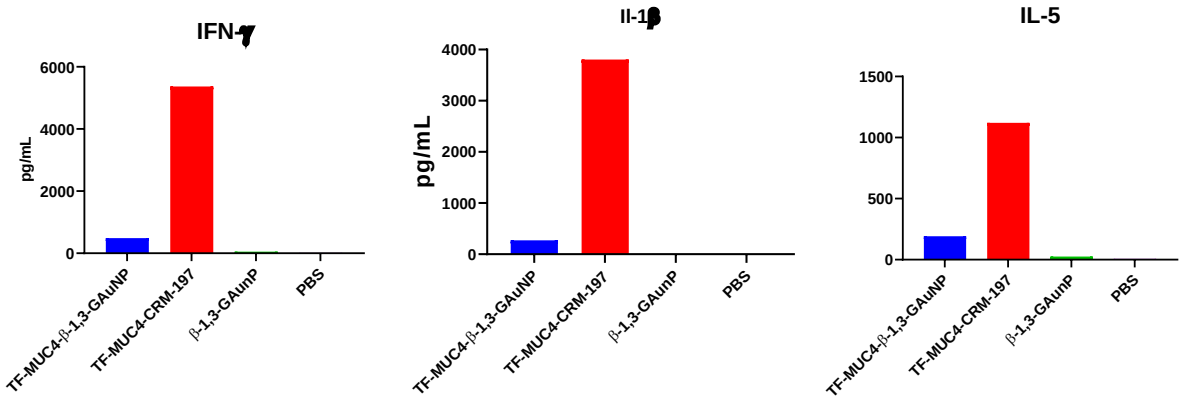


Figure S9. Comparison between amounts (pg/ml) of IFN-γ, IL-1β and IL-5 after vaccination with TF-MUC4-B13G-AuNP and TF-MUC4-CRM197 conjugates, both with the SAS adjuvant. Controls are unconjugated B13G AuNPs and PBS.

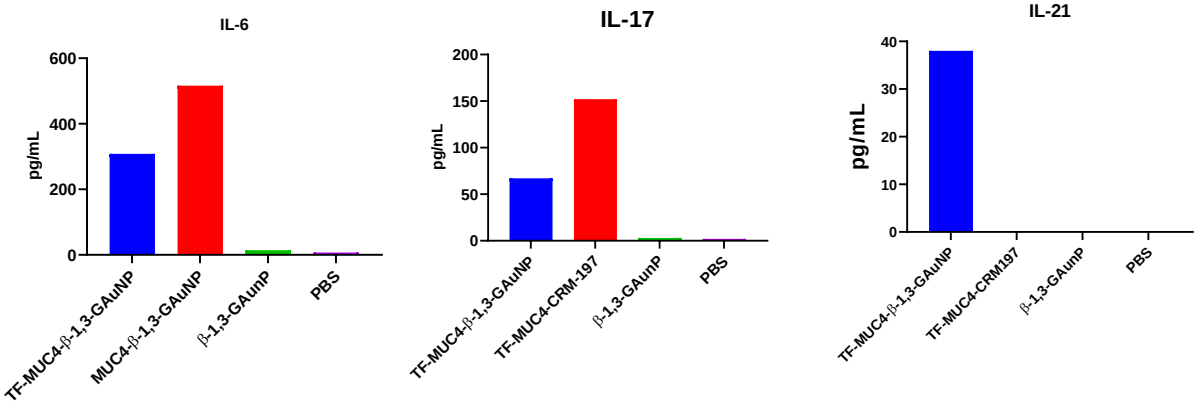


Figure S10. Comparison between amounts (pg/ml) of IL-6, IL-17 and IL-21 after vaccination with TF-MUC4-B13G-AuNP and TF-MUC4-CRM197 conjugates, both with the SAS adjuvant. Controls are unconjugated B13G AuNPs and PBS.

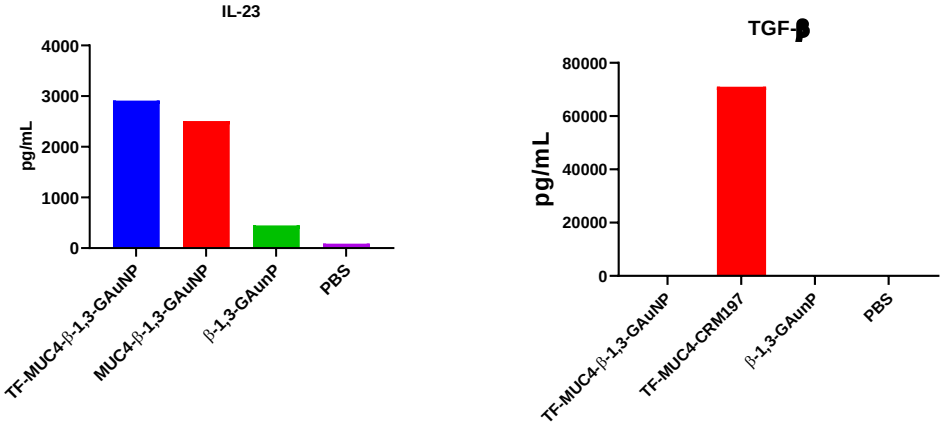
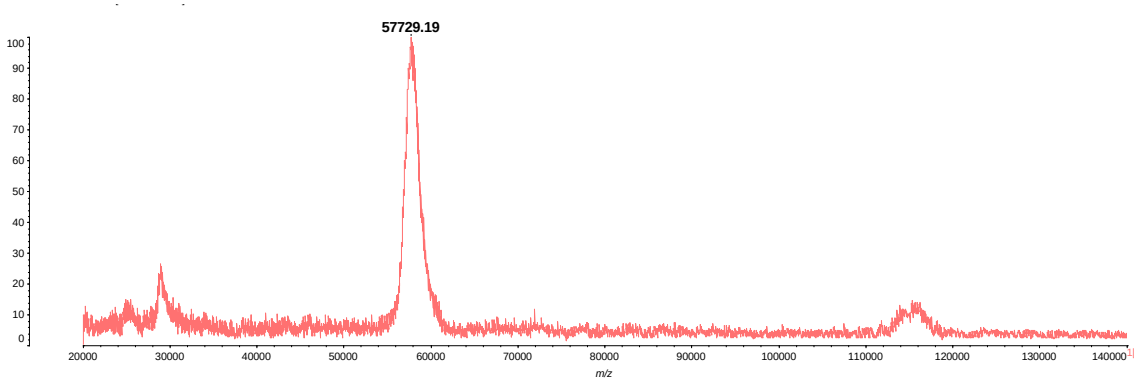
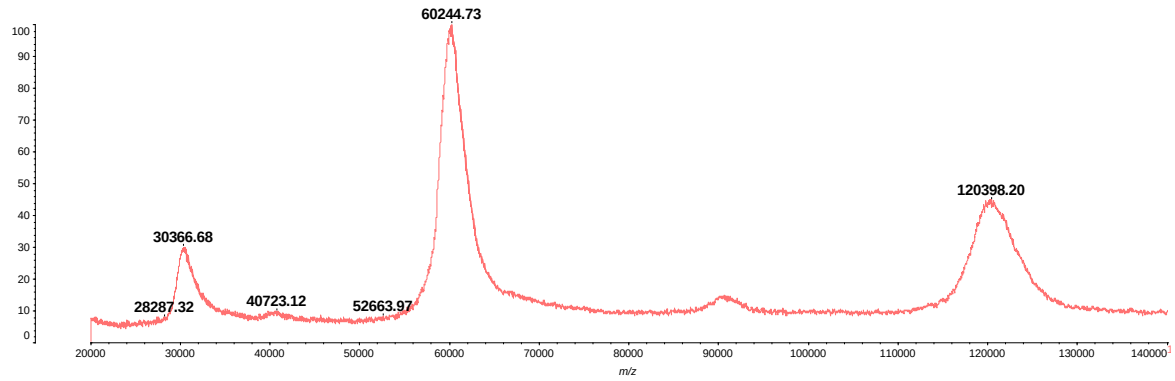


Figure S11. Comparison between amounts (pg/ml) of IL-23 and TGF-β after vaccination with TF-MUC4-B13G-AuNP and TF-MUC4-CRM197 conjugates, both with the SAS adjuvant. Controls are unconjugated B13G AuNPs and PBS.

A



B



C

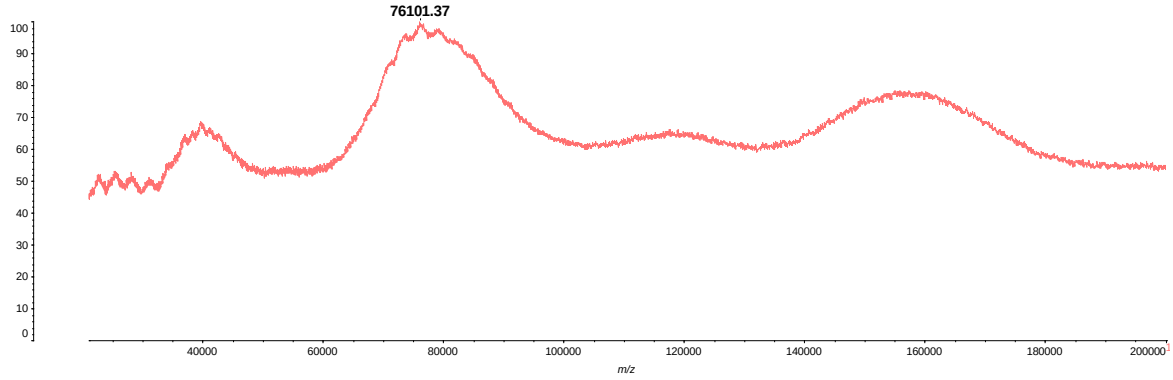


Figure S12. MALDI Mass spec spectra of (A) CRM197, (B) CRM197-Maleimide and (C) CRM197-Maleimide-TF-MUC4 conjugate, indicating approximately 8-10 glycopeptides were conjugated to the protein.

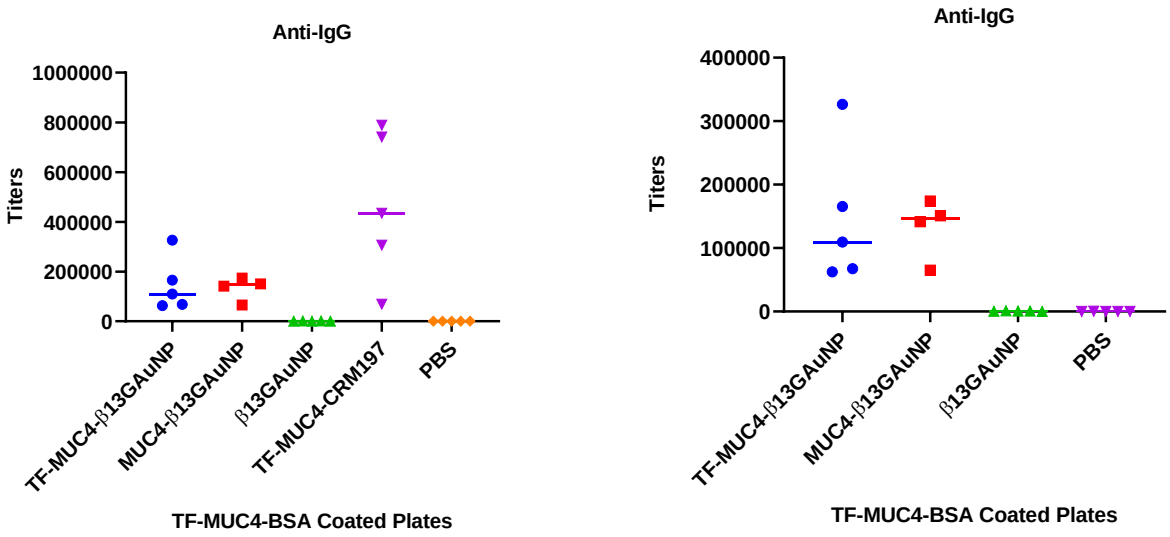


Figure S13. IgG Antibody titers generated to vaccinations with glycopeptide-coated B13G AuNPs and to the same glycopeptide conjugated to CRM197. Graph on the right is an expansion of lanes 1, 2, 3 and 5 from the graph on the left

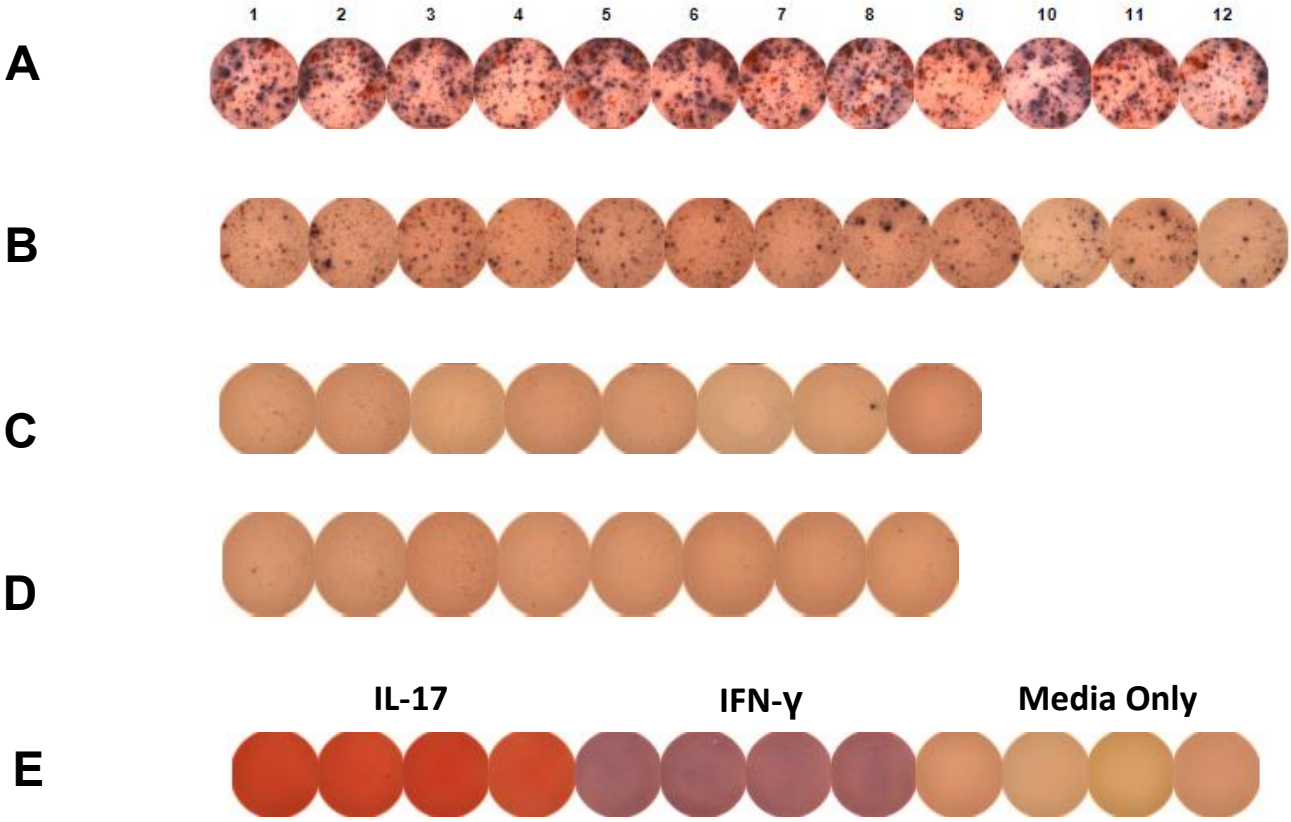
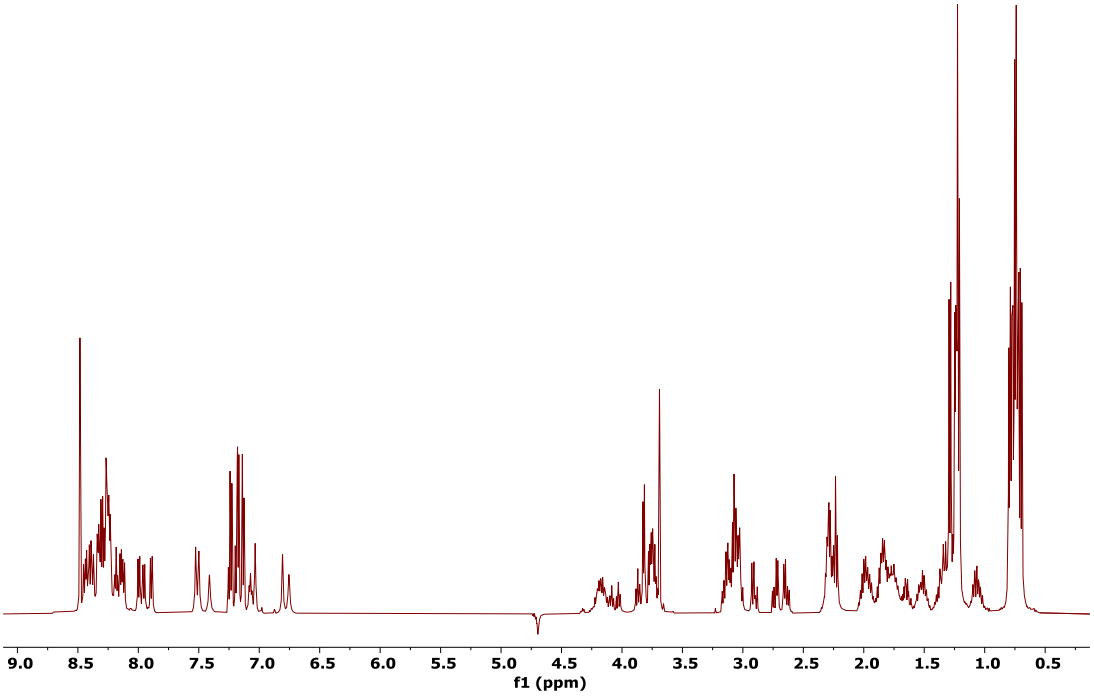
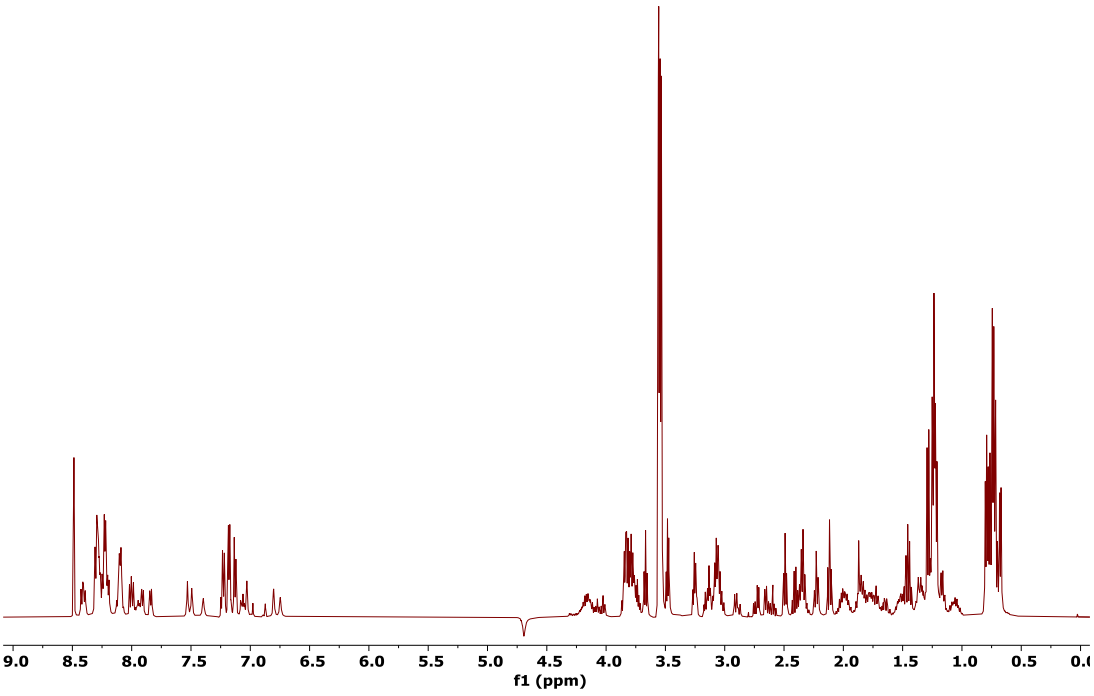


Figure S14. ELISPOT membranes from analysis of the TF-MUC4-B13G and TF-MUC4-CRM197 vaccinations. All wells contained $2.3x10^5$ $CD4^+$ and $2.0x10^5$ Dendritic Cells. Rows are from vaccinations with (A) TF-MUC4-β-1,3-Glucan AuNP, (B) TF-MUC4-CRM197 conjugate, (C) B13G AuNP and (D) PBS. Row (E) is a control row where $1x10^5$ $CD4^+$ cells were stimulated with Phytohemagglutinin and stained for IL-17, IFN-γ (positive controls) and media only (negative control)

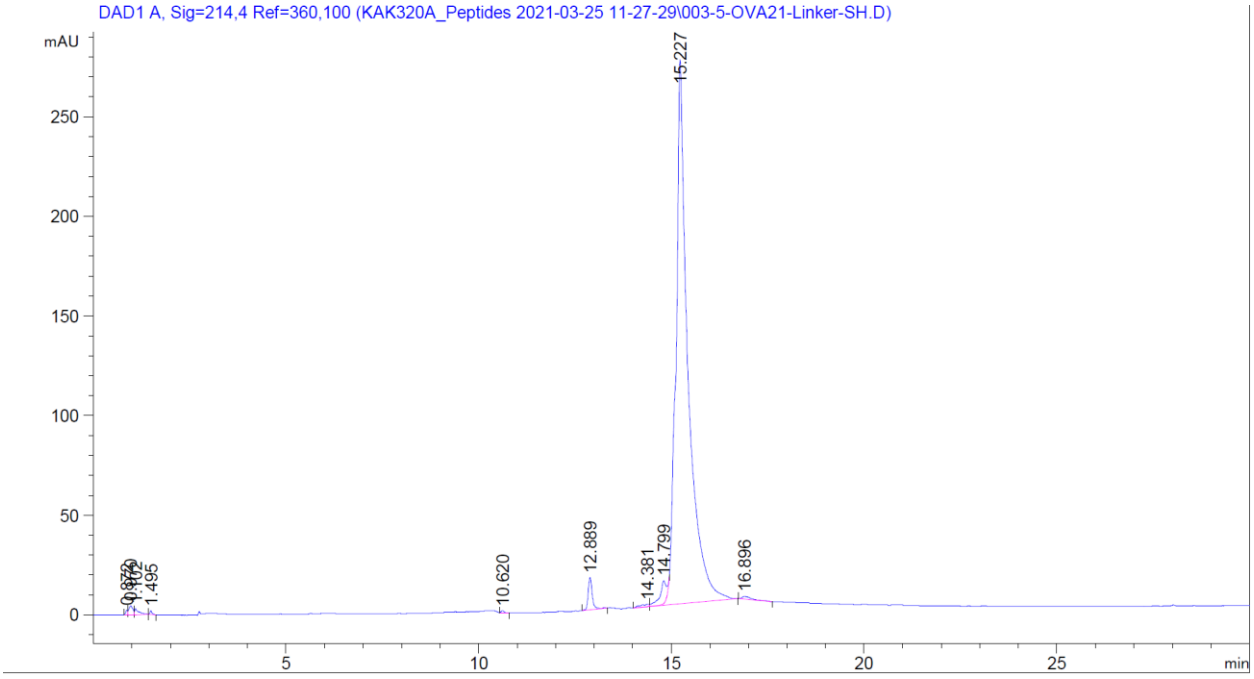
^{1}H NMR spectrum of OVA 21-mer peptide (without N-terminal PEG linker)



^{1}H NMR spectrum of OVA 21-mer peptide with N-terminal PEG linker



Analytical HPLC of OVA 21-mer peptide with N-terminal PEG linker

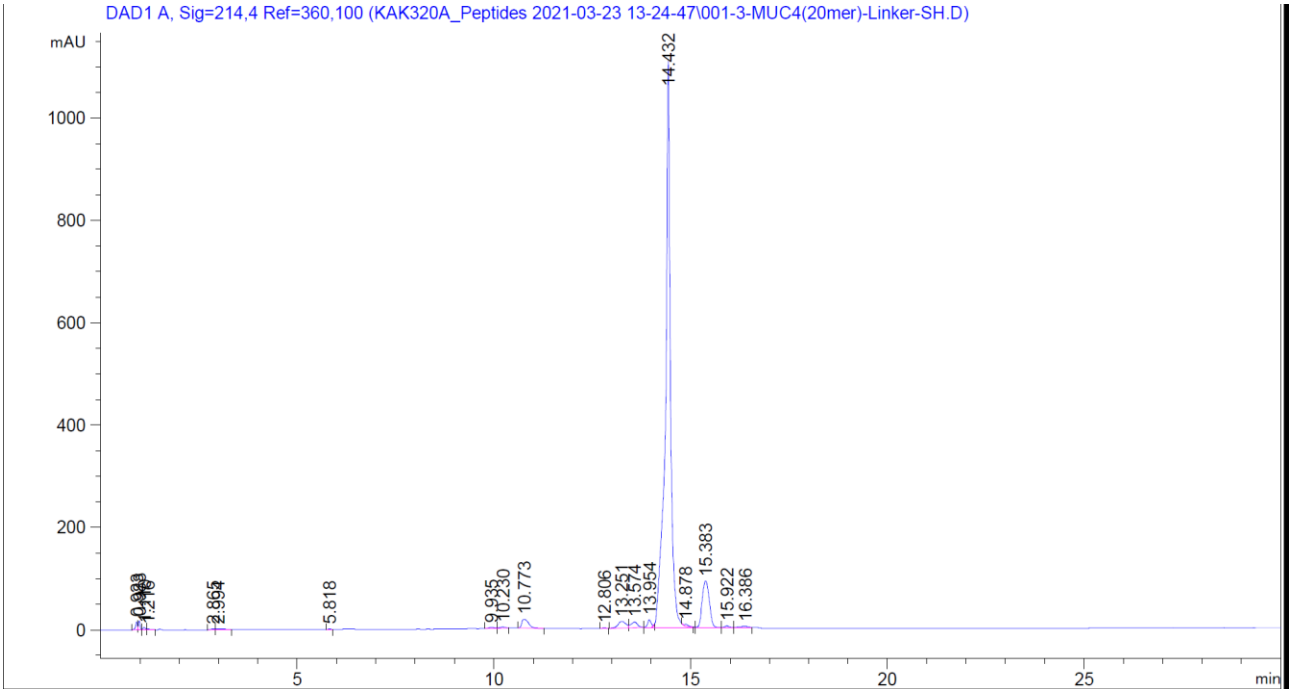


High Resolution MS: OVA 21-mer peptide with N-terminal PEG linker

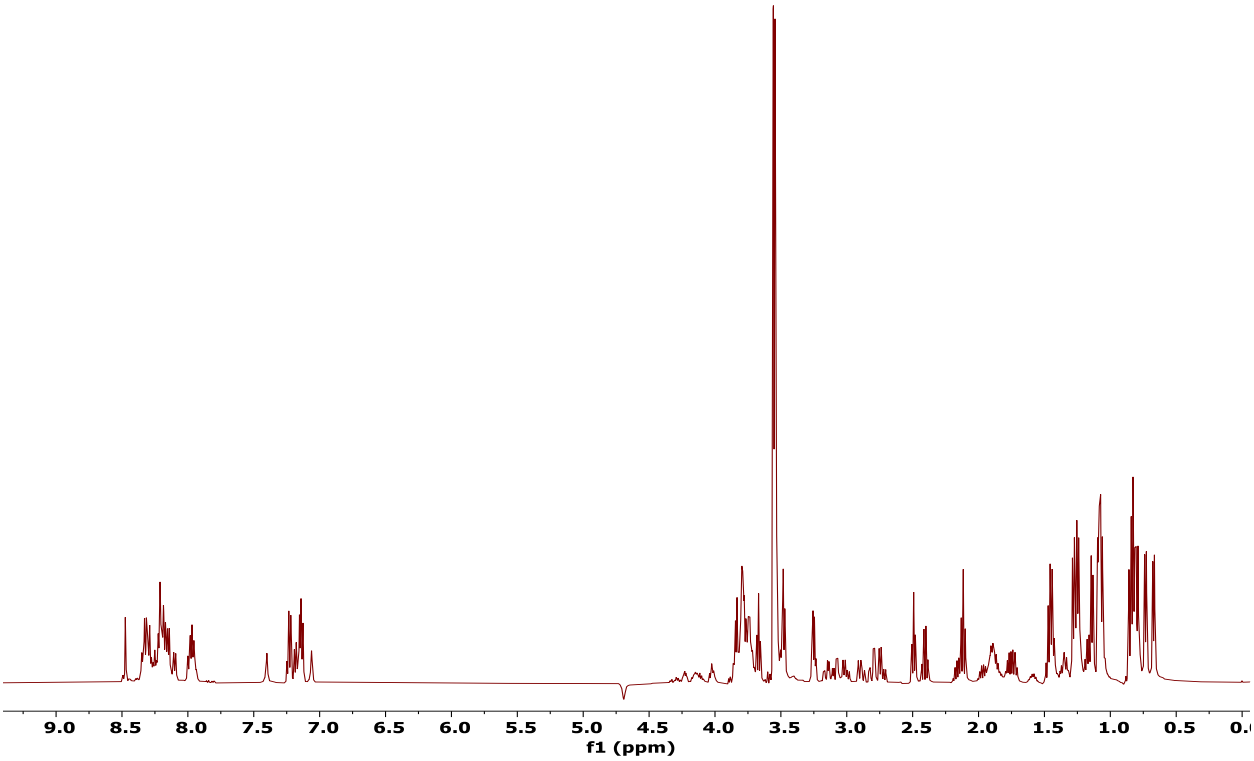
Measured [M+ 3H]3+ = m/z 895.1364 (Δ = 2.8 ppm)
Calculated [M+ 3H]3+ = m/z 985.1339 (for C117H194N33O37S; z = 3)
Measured [M+ 2H]2+ = m/z 1342.2013 (Δ = 3.1 ppm)
Calculated [M+ 2H]2+ = m/z 1342.1972 (for C117H193N33O37S; z = 2)

Xtract - Deconvoluted MW = m/z 2682.3875 (Δ = 2.8 ppm)
Calculated MW = m/z 2682.3799 (for C117H191N33O37S)

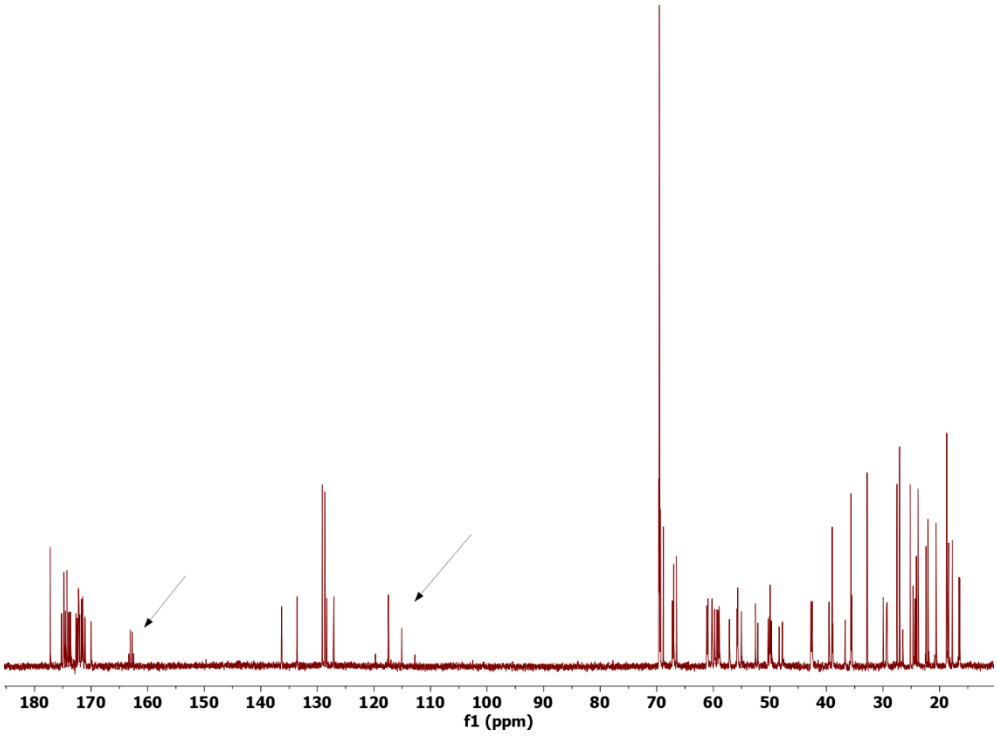
Analytical HPLC of PEG-(GFLG)-MUC4 20 mer



^{1}H NMR of PEG-(GFLG)-MUC4 20 mer



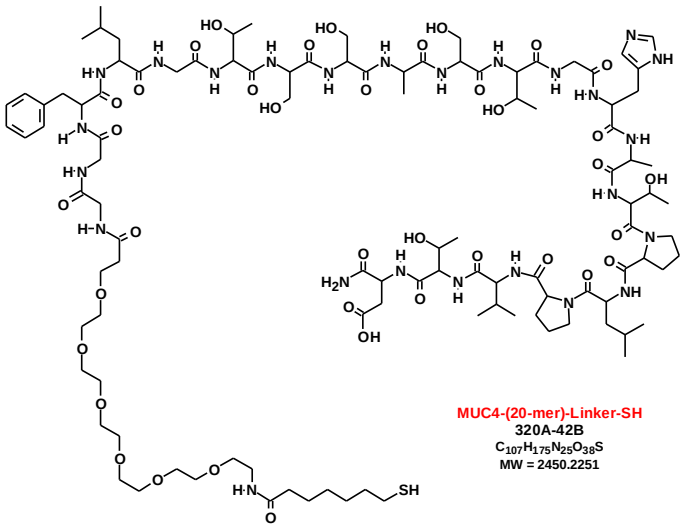
^{13}C NMR of PEG-(GFLG)-MUC4 20 mer (Arrows indicate **C**F_3**C**OOH carbons)



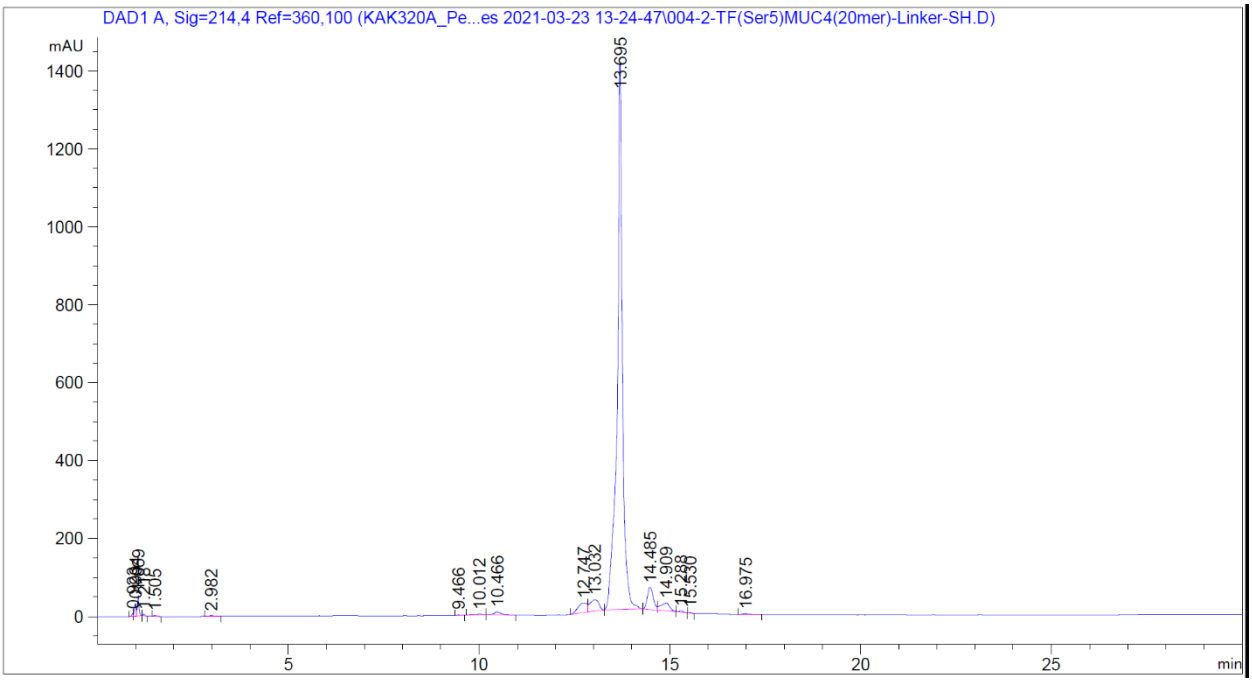
High Resolution MS: PEG-(GFLG)-MUC4 20 mer

Measured [M+ 3H]3+ = m/z 817.7506 (Δ = 2.0 ppm)
Calculated [M+ 3H]3+ = m/z 817.7490 (for $C_{107}H_{178}N_{25}O_{38}S$; z = 3)
Measured [M+ 2H]2+ = m/z 1226.1223 (Δ = 2.0 ppm)
Calculated [M+ 2H]2+ = m/z 1226.1198 (for $C_{107}H_{177}N_{25}O_{38}S$; z = 2)

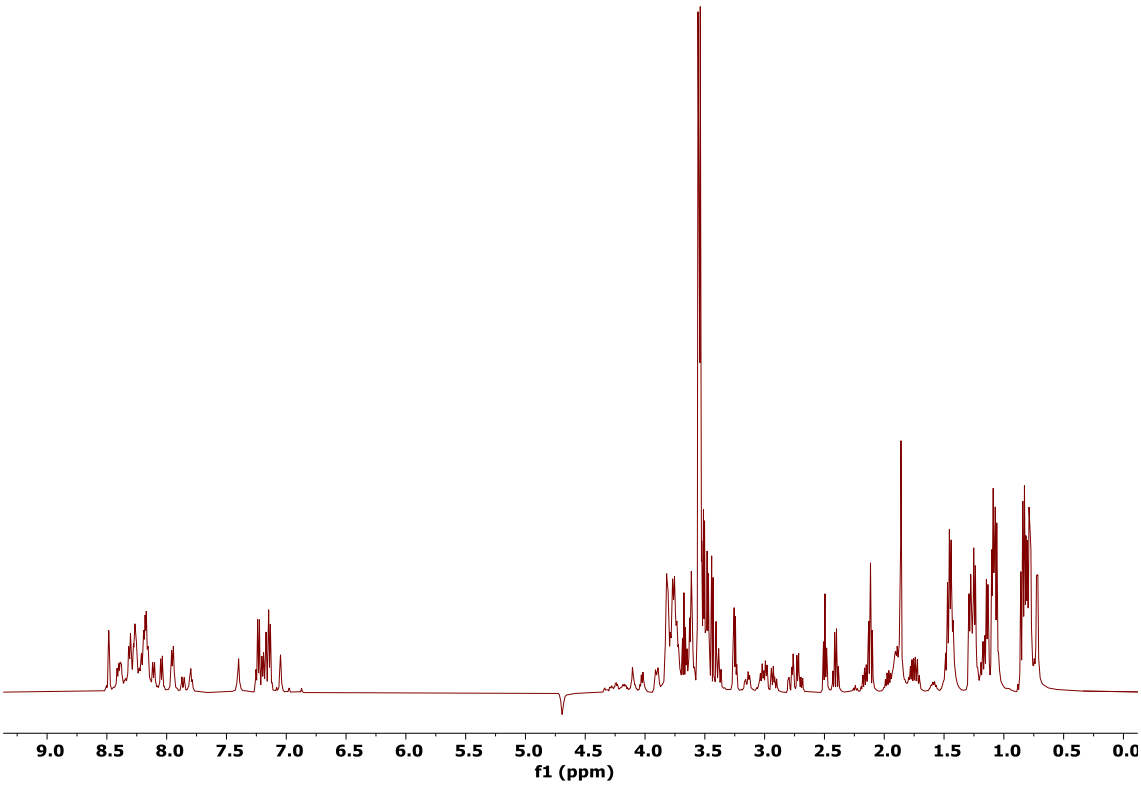
Xtract - Deconvoluted MW = *m/z* 2450.2303 (Δ = 2.2 ppm)
Calculated MW = *m/z* 2450.2250 (for $C_{107}H_{175}N_{25}O_{38}S$)



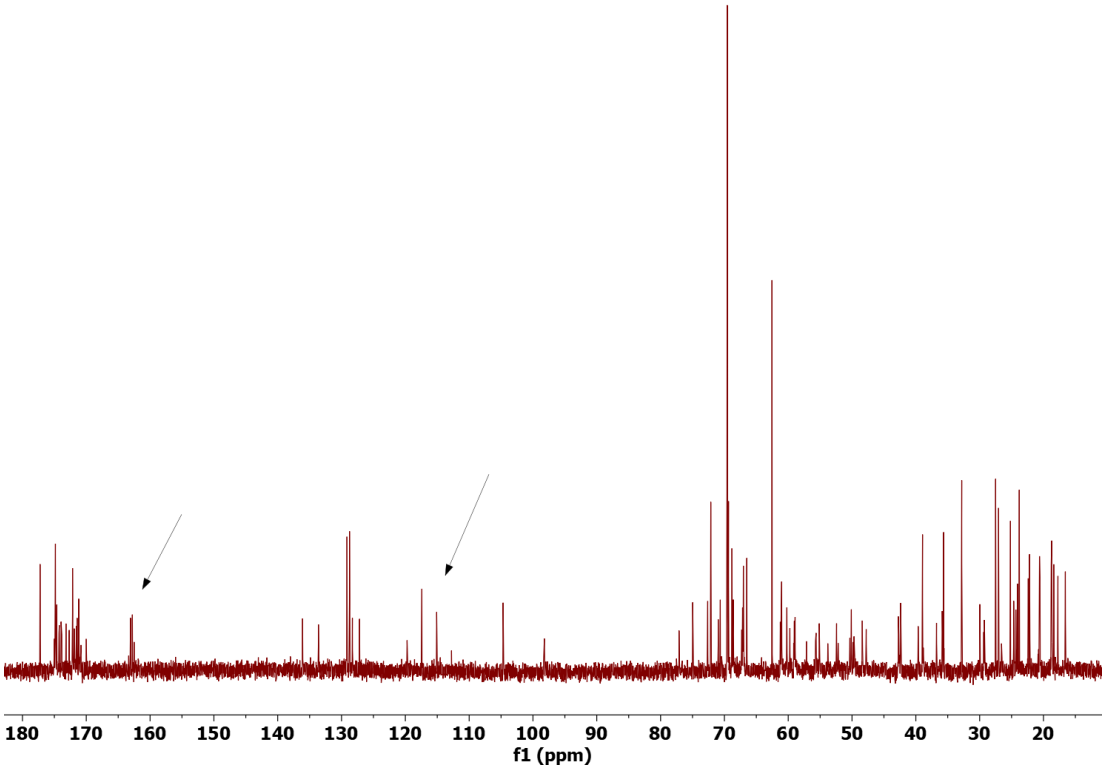
Analytical HPLC of PEG-GFLG-TF-MUC4 20-mer



^{1}H NMR of PEG-(GFLG)-TF-MUC4 20 mer



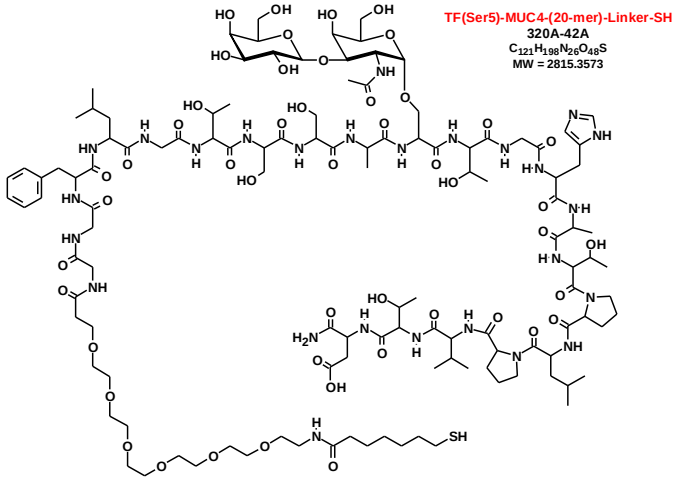
^{13}C NMR of PEG-(GFLG)-TF-MUC4 20 mer (Arrows indicate **C**F_3**C**OOH carbons)



High Res MS: PEG-GFLG-TF-MUC4 20-mer

Measured $[M+ 3H]^{3+}$ = *m/z* 939.4627 (Δ = 3.2 ppm)
Calculated $[M+ 3H]^{3+}$ = *m/z* 939.4597 (for $C_{121}H_{201}N_{26}O_{48}S$; *z* = 3)
Measured $[M+ 2H]^{2+}$ = *m/z* 1408.6905 (Δ = 3.3 ppm)
Calculated $[M+ 2H]^{2+}$ = *m/z* 1408.6859 (for $C_{121}H_{200}N_{26}O_{48}S$; *z* = 2)

Xtract - Deconvoluted MW = *m/z* 2815.3664 (Δ = 3.3 ppm)
Calculated MW = *m/z* 2815.3572 (for $C_{121}H_{198}N_{26}O_{48}S$)



1. Trabbic, K. R.; Whalen, K.; Abarca-Heideman, K.; Xia, L.; Temme, J. S.; Edmondson, E. F.; Gildersleeve, J. C.; Barchi, J. J., A Tumor-Selective Monoclonal Antibody from Immunization with a Tumor-Associated Mucin Glycopeptide. *Sci Rep-Uk* **2019,** *9*.