Unimicellar hyperstars as multi-antigen cancer nanovaccines displaying clustered epitopes of immunostimulating peptides†

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Electronic Supplementary Information (ESI)

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1. EXPERIMENTAL

1.1 Materials

Amino acids, coupling agents and the Wang resin (0.87 mmol/g) were obtained from EMD Millipore USA and used as received. The Rink amide resin (Rink amide chemMatrix, 0.49 mmol/g) was purchased from Biotage. Fmoc-*N*-amido-dPEG$_4$-acid (Quantabiodesign), mannosamine hydrochloride (Aldrich), triethylamine, TEA (Aldrich), dimethylamino pyridine (DMAP) (Aldrich), azido-dPEG$_{12}$-NHS ester (Quantabiodesign), 5/6-carboxyfluorescein succinimidyl ester succinimidyl ester (Thermofisher), *O*-(2-Azidoethyl)-*O*′-methyl-triethylene glycol (Aldrich), 1,2-ethanedithiol, EDT (Aldrich), phenol (Acros Organics), diisopropylethylamine, DIPEA (Aldrich), di-*tert*-butyl decarbonate (Aldrich), trifluoroacetic acid, TFA (Alfa Aesar), thioanisole (Aldrich), triisopropylsilane, TIPS (Aldrich), 5-hexynoic acid (Aldrich), 3-mercaptopropionic acid (Fisher) succinic anhydride (Acros Organics), *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, EDC.HCl (Aldrich), *N*-hydroxybenzotriazole, Oxyma (Novabiochem) and dibenzylcylooctyne (Click Chemistry Tools) were used as received. Methanol (Fisher) and dimethylformamide (Fisher) were dried over molecular sieves (Aldrich), 3 Å and 4 Å respectively. Dry tetrahydrofuran was purchased from Acros Organics and used as received. Azidovaleric acid, azido PEG$_{12}$-mannose and phenyl 6-azidohexanoate were synthesized in accordance with a previously published protocols.[1] Boltorn H40 was purchased from Aldrich and purified as previously reported.[1] All other solvents were purchased from Fisher at the highest purity available and used as received.

For the evaluation of in vivo efficacy of the nanovaccine, flow cytometry was performed with mouse-specific fluorochrome-conjugated monoclonal antibodies (mAbs). Pacific blue (PB)-anti-CD45 (30-F11), fluorescein isothiocyanate (FITC)-anti-F4/80 (BM8), phycoerythrin (PE)-Cy7-anti-CD11c (N418), PE-Cy7-anti-CD3 (145-2C11), allophycocyanin (APC)-Cy7-anti-CD11b (M1/70), Alexa Fluor (AF)-700-anti-Ly6G/Ly6C (Gr-1, RB6-8C5), and AF700-anti-CD8a (53–6.7) were purchased from BioLegend (San Diego, CA). FITC-anti-CD4 (GK1.5) was purchased from BD Biosciences (San Jose, CA). PE-Cy5-anti-MHCII (M5/114.15.2) was purchased from eBioscience (San Diego, CA). Negative fluorescence-minus-one (FMO) control staining was performed with isotype-matched mouse, rat or hamster IgG mAbs, and nonspecific binding was blocked with the FcγIII/II receptor-mediated anti-CD16/CD32 antibody (2.4G2) from BD Biosciences.

B16-F10 cells were purchased from *American Type Culture Collection* (ATCC, #CRL-6475), and B16-OVA cells, genetically modified to express chicken ovalbumin (OVA), were a generous gift from the William Murphy Laboratory (UC Davis, Davis, CA). Cells were cultured in high-glucose DMEM (Gibco, #11995) containing 10% FBS and 1% penicillin-streptomycin in a 37°C humidified chamber containing 5% CO_2. B16-F10 and B16-OVA cells were passaged at a density of 1 to 3 x 10^6 into 175 cm^2 tissue culture flasks (VWR, Radnor PA). Once cells reached 85-90% confluency, approximately 4 days after plating, they were collected and resuspended in

1:1 Matrigel (Corning, #356234): PBS without calcium and magnesium (PBS-/-) for injection into mice at 2.5 x 10^5 cells/ 50 uL.

All animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Davis. To generate the subcutaneous B16-OVA model of melanoma, 7-8-week-old female C57BL/6J mice were used (The Jackson Laboratory, Bar Harbor, ME). B16-OVA cells (William Murphy Laboratory, UCD) were cultured under standard 2D cell culture conditions as described herein. At time of injection, animals were anesthetized with 3% isoflurane (in oxygen, flow rate: 2 L/min) and maintained at 1.5–2.0% and 2.5 x 10^5 cells in 50 µL 1:1 PBS: Matrigel (#356234, BD Biosciences, San Jose, CA) were injected subcutaneously into the right flank regions.

1.2 Characterization

1.2.1 Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS)

MALDI-TOF mass spectrometry experiments were undertaken using a Bruker UltraFlextreme mass spectrometer equipped with a Smartbeam-II laser, (355 nm wavelength). The accelerating voltage was 20 kV. Samples were dissolved in water/acetonitrile/TFA (70/30/0.1) or DMF (PADRE and TRP2) and 10 µL of the solution were taken and mixed with 10 µL of the matrix solution. The matrix was a saturated solution of Sinapic acid in water/acetonitrile/TFA (70/30/0.1). The matrix solution was deposited first on the stainless steel MALDI plate and left to dry followed by deposition of the sample-matrix solution on top of the dry matrix spot. After drying, data collection and analysis was carried out using the Bruker Flex analysis software.

1.2.2 Electrospray ionization mass spectrometry

Mass spectrometry was conducted using a Thermo Electron LTQ-Orbitrap mass spectrometer and XCalibur data processing and instrument control software. Samples of appropriate concentration were made up in water/acetonitrile/TFA (50/50/0.1) v/v/v or DMF/water/TFA (50/50/0.1) v/v/v before injection into the electrospray ionization unit at 0.2 mL min^{-1} using a mobile phase of water with 0.1% formic acid (solvent A) and acetonitrile (solvent B) (50% A and 50% B) . The electrospray voltage was 4.5 kV, the sheath gas was nitrogen at 15 arbitrary units, and the heated capillary was set at 275 °C. High-resolution data was acquired at 100K FWHM in centroided mode with the lockmass feature which typically results in <2ppm mass accuracy.

1.2.3 Liquid chromatography-mass spectrometry (LC-MS)

LC-MS was conducted using a Thermo Ultimate 3000 HPLC system equipped with an autosampler, and a photodiode array detector. and a Sunfire C18(2) 5 µm, 2.1 150 mm column at a flow rate of 0.2 mL min^{-1} coupled to a Thermoquest Finnigan LCQ Deca MS detector. The mobile phase employed was water with 0.1% (v/v) formic acid (Solvent A) and acetonitrile with 0.1% (v/v) formic acid (Solvent B). A gradient of 10% -90% solvent B over 12 minutes was employed.

1.2.4 Reverse Phase High-Performance Liquid Chromatography (RP-HPLC)

Analytical reverse-phase RP-HPLC was performed on a Varian System 2695 separations module with an with a photodiode array detector and employed a Phenomenex Jupiter Proteo C12 column (250 x 4.6 mm column, 4 μm particle size, flow rate of 1 mL min^{-1}).

Semi-Preparative RP-HPLC was performed using Varian ProStar multi-solvent delivery system with a photodiode array detector employing a Phenomenex Jupiter Proteo Semi-Prep C12 column (250 x 10 mm, 10 μm particle size, flow rate 3 mL min^{-1}) at room temperature. Eluent fractions were collected every 30 seconds on a Varian 701 fraction collector. The mobile phase consisted of eluents A (0.1% v/v TFA in water) and B (0.1% v/v TFA in acetonitrile) for all HPLC runs.

Preparative RP-HPLC was performed using Varian ProStar multi-solvent delivery system with a photodiode array detector employing a Phenomenex Jupiter Proteo Prep C18 column (250 x 10 mm, 10 μm particle size, flow rate 7 mL min^{-1}) at room temperature. Eluent fractions were collected every 30 seconds on a Varian 701 fraction collector. The mobile phase consisted of eluents A (0.1% v/v TFA in water) and B (0.1% v/v TFA in acetonitrile) for all HPLC runs.

1.2.5 Nuclear Magnetic Resonance (NMR)

NMR analyses were carried out on Bruker Ultra Shield Avance 500 or 800 spectrometers. NMR analyses, analyses were conducted in either deuterated DMSO (DMSO-d_6) or deuterated chloroform (depending on the solubility of the product).

1.2.6 Fourier transform infra-red (FT-IR)

Solid and liquid samples were analyzed using a Bruker Tensor 27 FT-IR spectrometer equipped with an attenuated total reflectance (ATR) accessory. For pure samples, the number of scans per sample was set at 64.

For samples in solution, 25 μL of the reaction solution was placed on the ATR accessory and the solvent was slowly evaporated to form a thin film before scanning. Spectra were averages of 512 scans, recorded at a resolution of 4 cm^{-1} at room temperature.

1.2.7 Size exclusion chromatography (SEC)

SEC was used to determine the molecular weight of the polymers. SEC analyses were carried out at 60 ^{o}C using an Ultimate 3000 SEC system equipped with a guard column and two Agilent PolarGel-M columns (molecular weight range of 500-2 000 000 g/mol) attached to a differential refractive index (DRI) detector and a variable wavelength UV-vis detector. The flow rate of the system was set at 1 mL/min and the eluent was DMF with 0.25% (w/v) LiBr. The SEC system was calibrated using Agilent narrow molecular weight distribution polystyrene standards.

1.2.8 Amino acid analysis

AAA was conducted using a Hitachi 8800 Amino Acid Analyzer that is equipped with a strong-cation exchange separation via a TransGenomic (cat # AAA-99-6312) column. Amino acids were detected via a secondary post-column reaction with ninhydrin and observed/quantified in the visible spectrum (440 nm and 570 nm). Samples were prepared by transferring a known

volume of sample into a vial which was subsequently placed a centrifugal evaporator to lyophilize the sample. The dry sample was hydrolyzed by treating with 6N HCl/1% phenol at 110 ^{o}C for 24 hours followed by solvent removal. The residue was taken up NorLeu diluent and diluted to a known volume for analysis. NB: The analysis is based on assuming that the contribution of CLEC9A is negligible since only a small amount of it is present and hence doesn't contribute towards the amino acid count and the count also excludes tryptophan, methionine and cysteine units (which requires a different AAA procedure).

1.2.9 Transmission electron microscopy (TEM)

TEM images were obtained using JEOL JEM 1011 electron microscope with an acceleration voltage of 100 kV. Samples were prepared by placing a drop of sample onto a carbon coated copper grid followed by negative staining with 2% uranyl acetate. Samples were allowed to dry overnight before imaging.

1.2.10 Dynamic Light Scattering (DLS)

Particle size measurements were carried out by dynamic light scattering (DLS) using a Malvern Instruments Zetasizer Nano series instrument. The system was allowed to equilibrate for 3 minutes before measurements were made and at least three replicate measurements were made for each sample.

1.2.11 Zeta potential

Zeta potential measurements were carried out using a Malvern Instruments Zetasizer Nano series instrument. An equilibration time of 3 minutes was allowed before each measurement and at least five replicate measurements were made for each sample.

1.2.12 Ultra-violet visible (UV-vis) spectrometry

UV-vis spectra were obtained using the NanoDrop 200 (Fisher Scientific).

1.2.13 Flow cytometry

(i) Nanoparticle uptake studies: Fluorescein cellular fluorescence was analyzed with a FACScan flow cytometer (BD Biosciences, San Jose CA) and FlowJo v10 software (Tree Star Inc., Ashland OR).

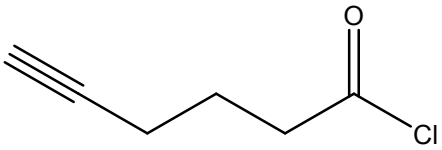
(ii) Immunotherapy studies: Samples were run within 24 hours on either a FACScan or LSRII flow cytometer (BD Biosciences). All data were analysed using Flowjo v10 software (TreeStar).

1.2.14 Statistical Analysis

Statistical analyses were performed using using Prism (Version 7, GraphPad, La Jolla, CA).

1.3 Methods

1.3.1 Synthesis of 5-hexynoic acid chloride



To a dry vial equipped with a stir bar, 5-hexynoic acid (1.00 g, 8.92 mmol, 1 eq.) was added and the vial was sealed with a rubber septum and degassed using nitrogen for 15 minutes. The vial was placed in an ice bath and the contents were left to cool down to 0 °C before oxalyl chloride (4.53 g, 35.67 mmol, 4 eq.) was added dropwise via a syringe. After five minutes, 50 µL of dry *N,N'* dimethylformamide were added using a syringe and the reaction was stired at 0 °C for two hours then overnight at room chloride was confirmed by FTIR. The product was obtained as a brown oil which was used in the next step without further purification. Yield (1.16 g, quantitative)

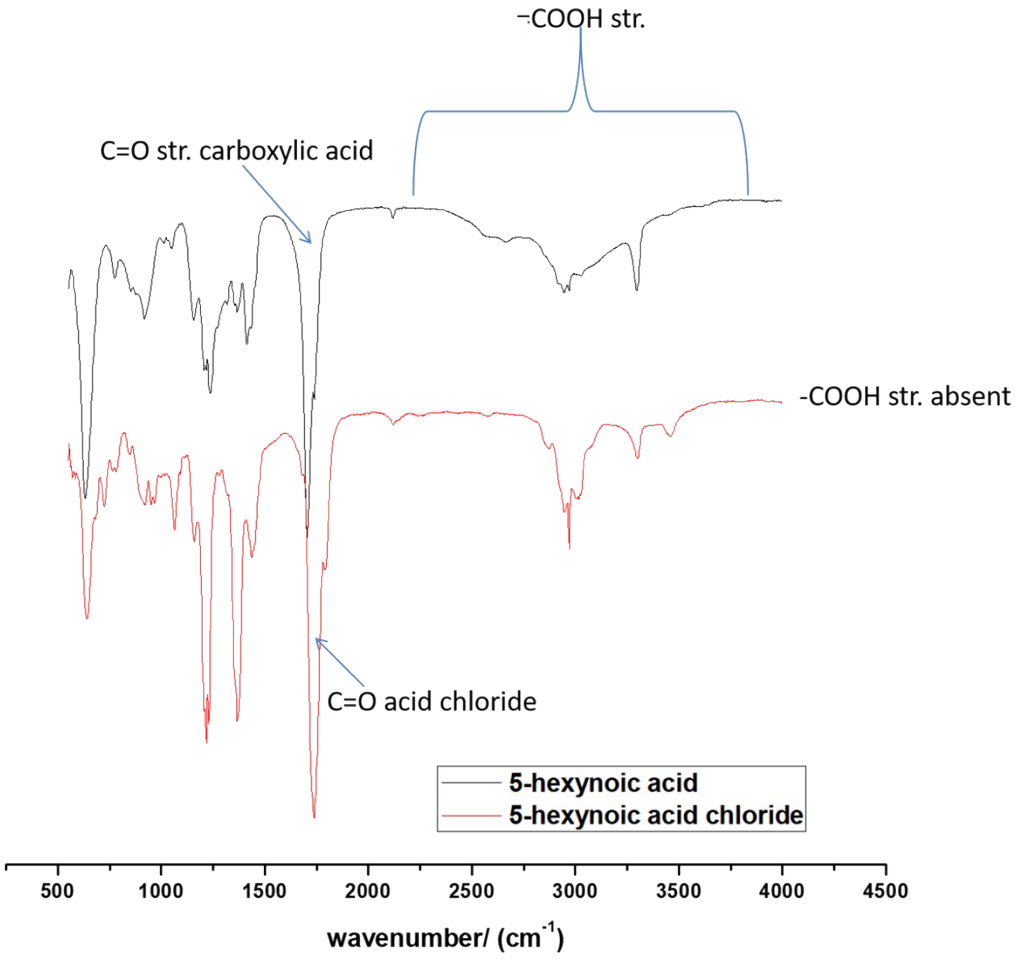


Fig. S1 FTIR spectra of 5-hexynoic acid and 5-hexynoic acid chloride.

1.3.2 Esterification of Boltorn H40 (B64-OH) with hexynoic acid chloride (Synthesis of BH40-Alk64, BH40-Alk128 and BH40-Alk256)

Example, BH40-Alk64: To a solution of B64-OH (0.20 g, 0.1 mmol in dry DMF (1 mL))(molecular weight based on MALDI, 2000 g/mol) in a sealed vial under nitrogen, dry THF (10 mL) and dry triethylamine (1.25 mL, 8.7 mmol) were added and the vial was cooled to 0 ^{o}C in an ice bath. Using a gas tight syringe, freshly prepared 5-hexynoic acid chloride (0.915 g, 7.0 mmol in dry THF (1 mL)) was taken up and added dropwise to the vial at 0 ^{o}C. The reaction was allowed to proceed at 0 ^{o}C for 2 hours then at room temperature for 4 hours. The solvent volume was reduced to near dryness on the rotary evaporator and the remaining residue was taken up in a mixture of ethyl acetate (100 mL) and saturated sodium bicarbonate (100 mL) and placed in a separating flask. The organic layer was washed with saturated sodium bicarbonate (3 × 50 mL), water (3 × 50 mL) and saturated sodium chloride (1 × 100 mL) then dried over $MgSO_4$. After the removal of $MgSO_4$ by filtration, the solvent was removed *in vacuo* and the product was dissolved in a small amount of THF and precipitated thrice in hexane/diethyl ether (1:1). The dark-brown product was dried under vacuum at room temperature and analysed by SEC, ^{1}H NMR and FTIR. Yield (0.6 g).

1.3.3 Synthesis of BH40-128OH and BH40-OH256 (Functionalization of BH40-Alk64, BH40-Alk128 and BH40-Alk256 with hydroxyl groups *via* the radical thiol-yne click reaction)

Example, BH40-OH128: A solution of *BH40-Alk64* (0.5 g, 61.4 μmol) (assuming complete functionalization of 64 groups in the previous step) dissolved in DMF (10 mL) in a sealed quartz glass vial containing a stir bar was degassed for 15 minutes with nitrogen followed by the addition of separately degassed 1.4 mL of 2-mercaptoethanol (1.6 g, 20 mmol) using a gas tight syringe. The solution was degassed for a further 10 minutes followed by the addition of 2,2-dimethoxyacetophenone (1 mL of a 75 mg/mL solution in DMF, 0.1 mmol) which had been degassed separately in an aluminum foil covered glass vial. The solution was degassed for 5 minutes before the vial was placed next to a UV lamp (365 nm) on a magnetic stirrer in a dark compartment and then left to stir for 6 hours under UV irradiation (365 nm). The product was recovered by precipitating (3×) in a cold solution of hexane/diethyl ether (1:3) in an ice bath. The product obtained was characterized by SEC, ^{1}H NMR and FTIR. Yield (1.1 g).

1.3.4 Synthesis of BH40-COOH512 (Functionalization of BH40-Alk256 with carboxylic acid groups)

A solution of *BH40-Alk256* (0.5 g, 6.7 μmol) (assuming complete functionalization of 256 groups in the previous step) dissolved in DMF (10 mL) in a sealed quartz glass vial containing a stir bar was degassed for 15 minutes with nitrogen followed by the addition of separately degassed 3-mercaptopropionic acid (0.8 mL, 1.0 g, 9.4 mmol) using a gas tight syringe. The solution was degassed for a further 10 minutes followed by the addition of 2,2-dimethoxyacetophenone (0.5 mL of a 50 mg/mL solution in DMF, 138.7 μmol) which had been degassed separately in an aluminum foil covered glass vial. The solution was degassed for 5 minutes before the vial was placed next to a UV lamp (365 nm) on a magnetic stirrer in a dark compartment and then left to stir for 6 hours under UV irradiation (365 nm). The product was

recovered by precipitating (4×) in a cold solution of hexane/diethyl ether (1:3) in an ice bath. The product obtained was characterized by ^{1}H NMR and FTIR. Yield (0.8 g).

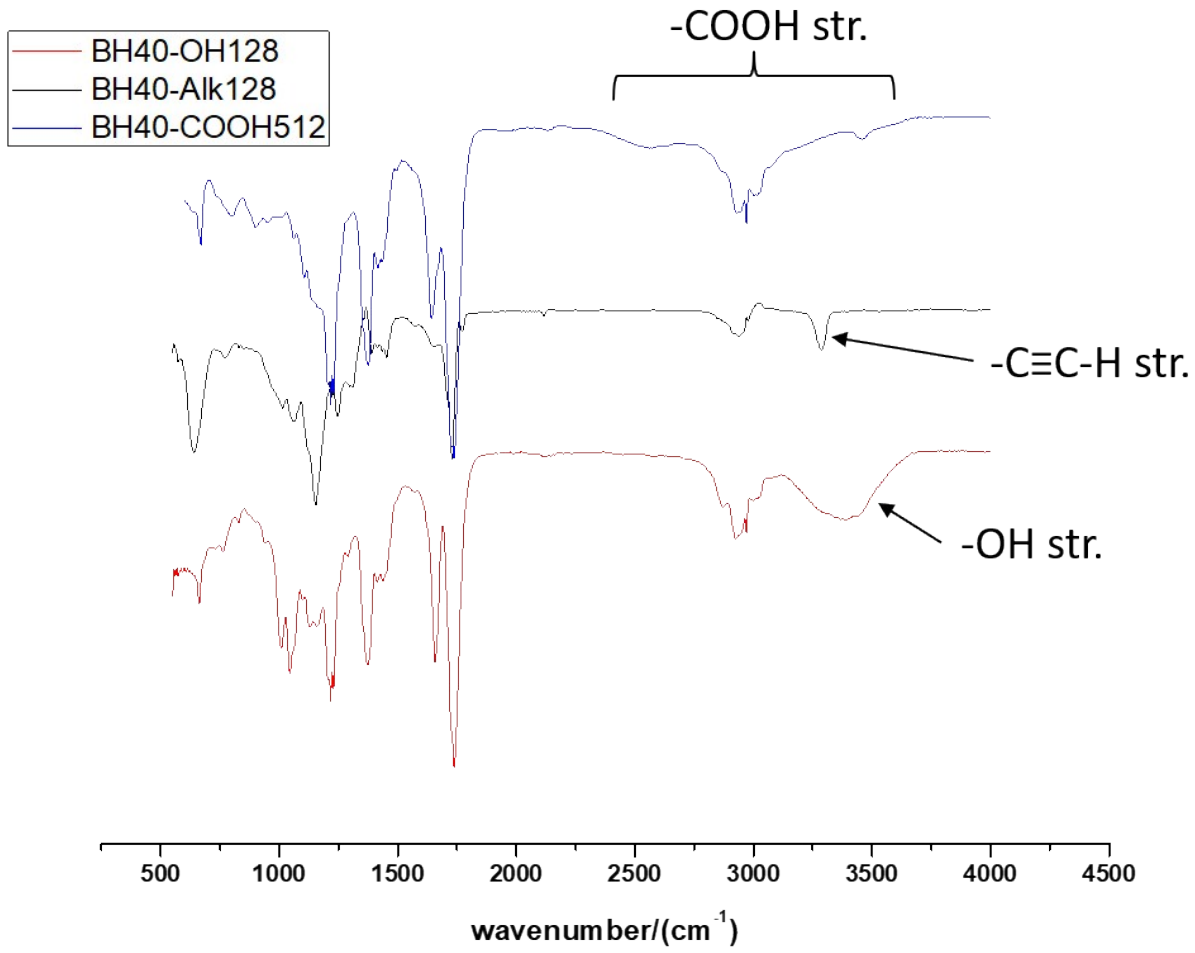


Fig. S2 Example FTIR spectra showing changes in the nature of the end group after esterification and radical thio-yne reactions.

1.3.5 Modification of BH40-COOH512 end groups with dibenzylcyclooctyne (DBCO)

B512-COOH (0.055 g, 0.39 μmol) was dissolved in dry DMF (15 mL) under nitrogen before dibenzylcyclooctyne amine (DBCO) (0.045 g, 181 μmol) was added. The temperature of the solution was lowered to 0 °C using an ice bath and EDC.HCl (0.052 g, 271.5 μmol), DIPEA (0.070 g, 543 μmol, 96 μL) and Oxyma (0.039 g, 271.5 μmol) were added. The reaction was allowed to gradually get to room temperature and left to stir for 48 hours. From the reaction solution, 5 μL were obtained and spotted on a TLC plate against a solution of DBCOA in DMF of the same concentration as that at the start of the reaction. The TLC was carried out using a solvent comprising DCM/MeOH (4:1) and the plate was treated with ninhydrin staining solution

to determine the presence of free amines (R_f DBCOA=0.6). A very faint orange-greenish spot was observed on the TLC plate for the reaction solution compared to the DBCOA solution. Removal of the DMF was done on a rotary evaporator and the resulting residue was suspended in dichloromethane (50 mL) followed by washing with 0.001N HCl (3 × 50 mL), water (3 × 50 mL) and brine (1 × 50 mL). The organic layer was dried over sodium sulfate and the solvent volume was lowered *in vacuo* followed by precipitation in diethyl ether. The precipitate obtained was dissolved in dichloromethane and the product was again precipitated in diethyl ether. Precipitation was conducted one more time and the solvent was removed *in vacuo* to obtain the desired product as a pale brown amorphous solid. Yield 80 mg.

1.3.6 Determination of BH40-COOH512DBCO alkyne loading

Alkyne loading was determined by reacting a known amount of the cyclooctyne terminated polymer with a slight excess of phenyl 6-azidohexanoate over a 72 hour period. The amount of azide consumed (alkyne loading) was calculated by difference based on quantification by HPLC.[1]

1.3.7 Synthesis of peptides

Solid phase peptide synthesis was performed on a Biotage Initiator Alstra automated microwave peptide synthesizer using the Fmoc SPPS. For coupling, HATU/DIPEA was employed and for Fmoc deprotection 20% piperidine in DMF was used while Ivdde removal was effected using 5% hydrazine/DMF (v/v). The Wang resin was pre-loaded with the first amino acid via DIC/DMAP coupling prior to automated synthesis as described below.

1.3.7.1 Pre-loading of the Wang resin

The Wang resin was swollen in DMF for one hour before use. In a separate vial, Fmoc-Leu-OH (8 eq.) was dissolved in anhydrous DMF (final concentration 0.1 M) and cooled to 0 ^{o}C. *N,N*-Diisopropylcarbodiimide (4 eq.) was added to the amino acid and the vial was placed on a shaker for 30 min at room temperature. The amino acid solution was added to the resin and cooled to 0 ^{o}C followed by the addition of DMAP (0.1 eq.). The reaction was placed on a rotary shaker overnight. The resin was washed with DMF (× 5), DCM (× 5) and DMF (× 5) before capping with acetic anhydride/DIPEA solution 1:9 v/v followed by washing with DMF (× 5), DCM (× 5) and DMF (× 5). Resin loading was shown to be quantitative, as determined by deprotecting with 20% piperidine in DMF (2 × 5 min) and measuring the absorbance of piperidine-fulvene adduct at λ = 301 nm.

1.3.7.2 Iterative peptide assembly

(i) Deprotection (Fmoc removal): The resin was treated with 20% piperidine/DMF (1 × 3 min) followed by a second deprotection 20% piperidine/DMF (1 × 10 min) at room temperature without microwave irradiation. After deprotection, the resin was washed with DMF (× 5), MeOH (× 5) and DMF (× 5). (ii) Deprotection (Ivdde removal): The resin was treated with 5% hydrazine/DMF (2 × 3 min) at room temperature. After deprotection, the resin was washed with DMF (× 5), MeOH (× 5) and DMF (× 5).

(ii) Coupling: Coupling of amino acids and Fmoc-amido-d-PEG_4 were done under microwave irradiation for 5 min at 20 W power at a maximum temperature of 75°C. Coupling was done

using 4 equivalents of Fmoc protected amino acid dissolved in DMF (0.2 M), 4 equivalents of HBTU in DMF (0.45 M) and 8 equivalents of DIPEA in DMF (2 M). The resin was washed with DMF (× 5), MeOH (× 5) and DMF (× 5) without microwave irradiation after each coupling step.

(iii) Azidovaleric acid coupling (azido peptides): The coupling was done twice (2 × 1 hour) at room temperature without microwave irradiation using 4 equivalents of azidovaleric acid dissolved in DMF (0.2 M), 4 equivalents of HATU in DMF (0.45 M) and 8 equivalents of DIPEA in DMF (2 M). The resin was washed with DMF (× 5), MeOH (× 5) and DMF (× 5) without microwave irradiation after this step.

(iv) Acetic anhydride capping: Capping was conducted with acetic anhydride/DIPEA solution 1:9 v/v followed by washing with DMF (× 5), MeOH (× 5) and DMF (× 5).

(v) Cleavage and amino acid side chain deprotection: The resin was washed several times with methanol and dried under vaccum in a dessicator at room temperature. Cleavage and deprotection was effected using a mixture of TFA/TIPS/Thioanisole/H_2O/EDT/phenol (81.5/1/5/5/2.5/5), (v/v/v/v/w) at room temperature overnight. The peptide containing solution was collected and the remaining resin was washed with TFA (3 × 2 mL)

(vi) Work-up: The combined cleavage solution and TFA washings were concentrated by evaporating the volatiles under a stream of nitrogen gas to near dryness. The remaining solution was added to cold diethyl ether to precipitate the peptide which was recovered after centrifugation as a white solid. The peptide was dissolved in a small amount of DMF, precipitated into cold diethyl ether and recovered by centrifugation (× 5). The precipitate was washed with ether (× 5) and allowed to dry under a stream of nitrogen gas before it was dissolved in DMF/water and purified by semi-preparative HPLC.

(vii) Azido CLEC9A peptide synthesis (CLEC9A): The rink-amide resin was first loaded with Fmoc-Lys (ivdde) followed by preparation of the CLEC9A sequence via standard Fmoc SPPS. The Fmoc on the last amino acid was removed and the terminal amine (1 eq) was boc protected using di-*tert*-butyl decarbonate (10 eq.) in the presence of TEA (1 eq) at room temperature in THF. After reagent removal and washing (× 5), the ivdde was removed via the ivdde removal protocol detailed above. The resin was swollen in DMF before coupling azido-PEG_{12} to the pendant amine of the lysine group using coupling conditions detailed above but without microwave irradiation.

1.3.7.3 Characterization and purification data

(i) Azido TRP2 (TRP2): $C_{77}H_{106}N_{14}O_{20}$, Expected mass =1546.77 Da. Found, LC-MS = 1547.7869 Da $[M+H]^+$, MALDI-TOF = 1570.263 Da $[M+Na]^+$, 1592.251 Da $[M+2Na-H]^+$ and 1614.232 Da $[M+3Na-2H]^+$. Purification gradient, 45% to 75% solvent B over 60 minutes.

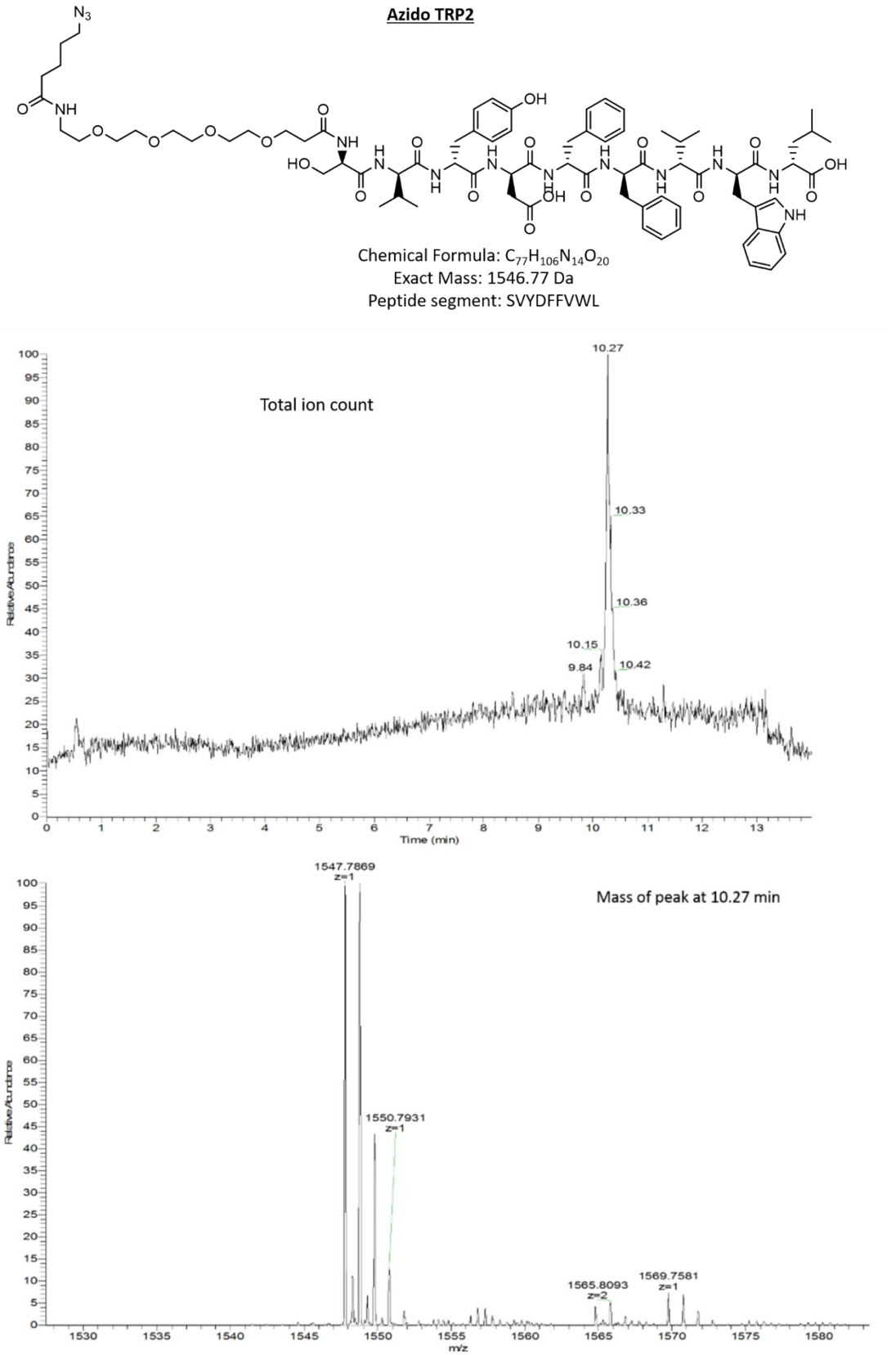


Fig. S3 Full structure and characterization of the TRP2 peptide by LCMS.

(ii) Azido GP100 (GP100): $C_{68}H_{110}N_{20}O_{20}$, expected mass =1526.82 Da. Found, (LC-MS) = 764.4235 Da $[M+2H]^{2+}$, (MALDI-TOF) = 1572.985 Da $[M+H]^{+}$, 1549.985 Da $[M+Na]^{+}$ and 1565.954 Da $[M+K]^{+}$. Purification gradient, 45% to 75% solvent B over 60 minutes.

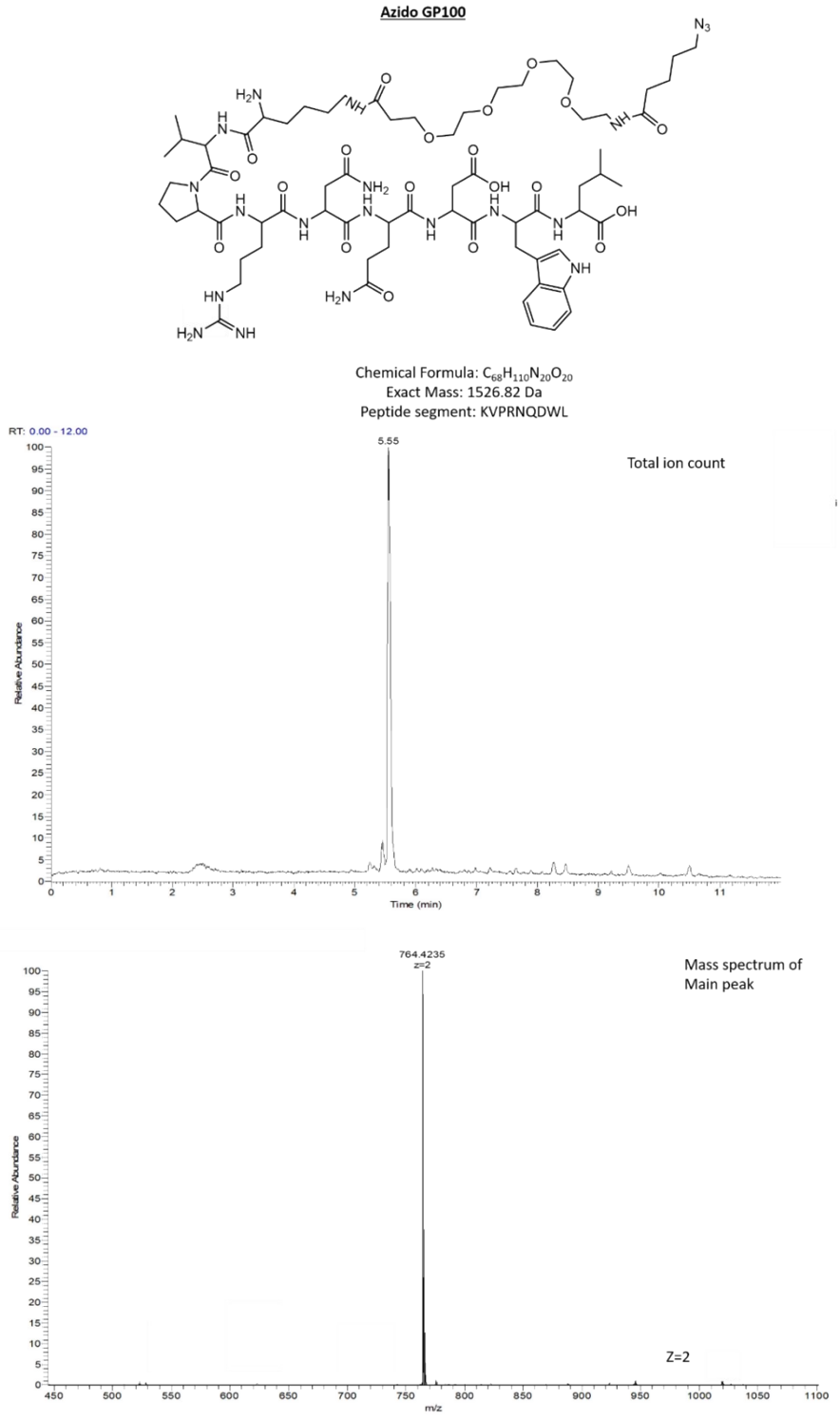
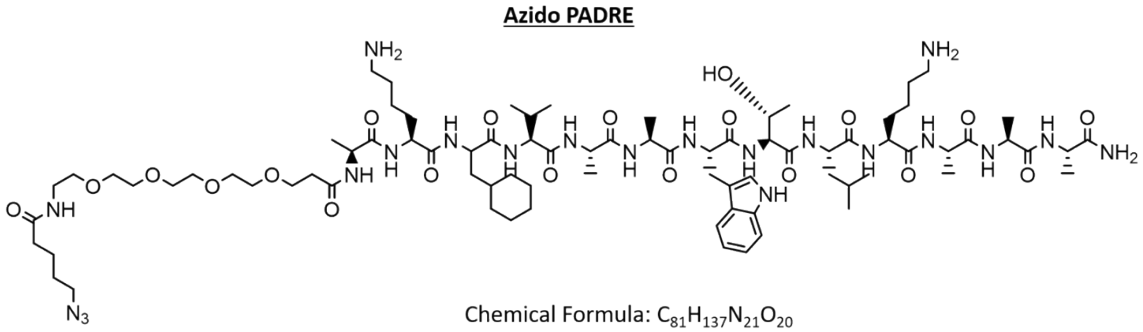


Fig. S4 Full structure and characterization of the GP100 peptide by LCMS.

(iii) Azido PADRE: $C_{81}H_{137}N_{21}O_{20}$, expected mass = 1724.03 Da. Found, (LC-MS) = 863.0276 Da $[M+2H]^{2+}$, (MALDI-TOF) = 1725.119 Da $[M+H]^{+}$, 1747.096 Da $[M+Na]^{+}$ and 1763.119 Da $[M+K]^{+}$. Purification gradient, 10% to 80% solvent B over 60 minutes.



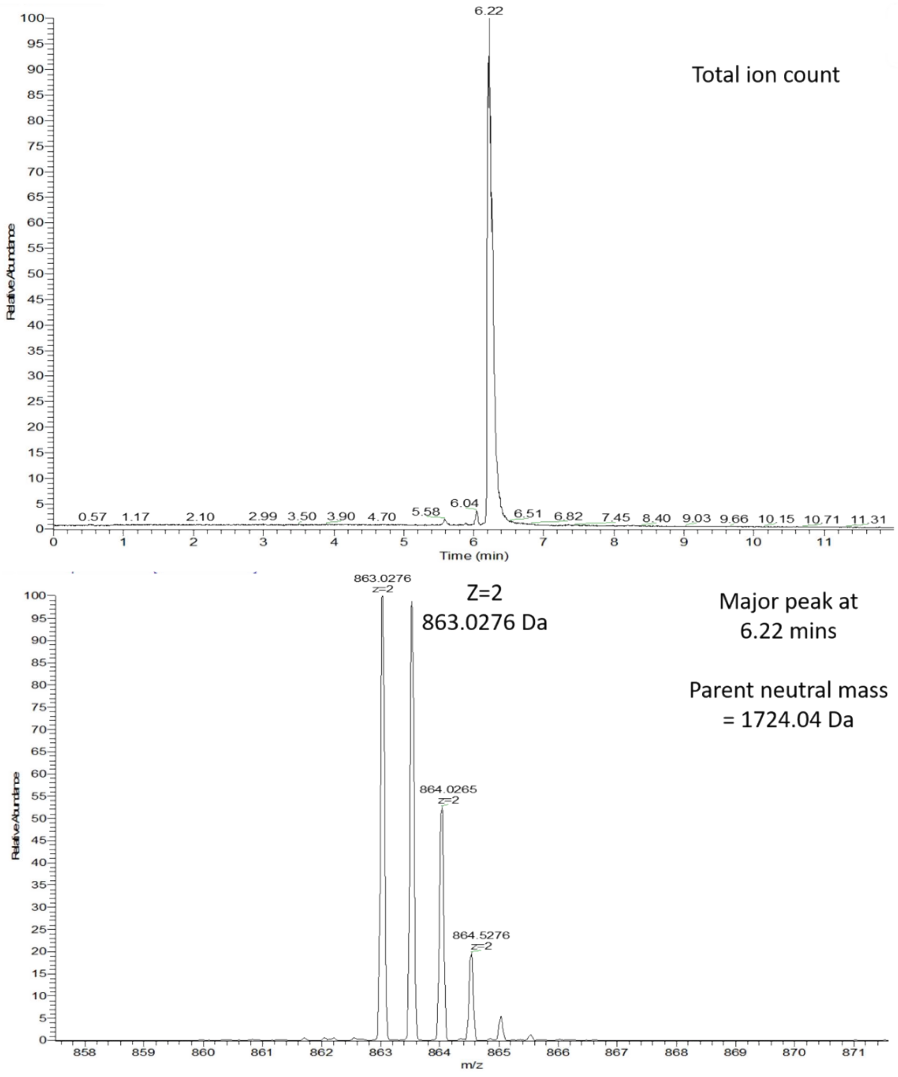


Fig. S5 Full structure and characterization of the PADRE peptide by LCMS.

(iv) Azido CLEC9A (CLEC9A): $C_{117}H_{181}N_{29}O_{34}$, expected mass =2536.33 Da. Found, (LC-MS) = 508.4751 Da $[M+5H]^{5+}$, 635.3426 Da $[M+4H]^{4+}$ and 846.7876 Da $[M+3H]^{3+}$, (MALDI-TOF) = 2537.300 Da $[M+H]^{+}$ and 2559.295 Da $[M+5H]^{5+}$. Purification gradient, 20% to 100% solvent B over 60 minutes.

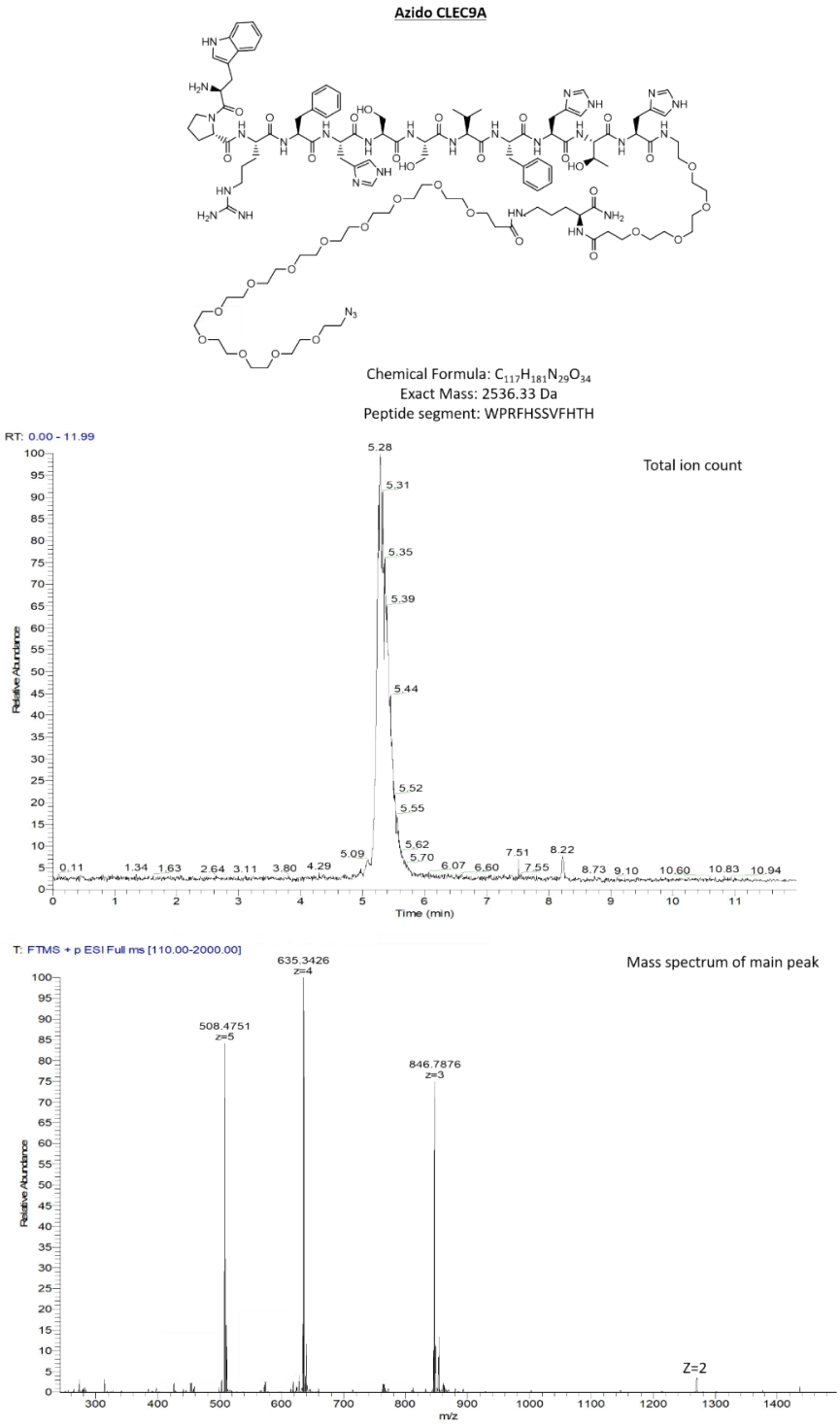


Fig. S6 Full structure and characterization of the CLEC9A peptide by LCMS.

1.3.8 Conjugation of N_3-PEG_{12}-Mannose to BH40-DBCO512 via the SPAAC reaction (Polymer amphiphile)

To BH40-DBCO512 (1 eq of alkyne units) in DMF was added the azido-PEG_{12}-mannose polymer (3 eq) dissolved in DMF. The resulting solution was left on a shaker at room temperature and an aliquot (50 μL) was withdrawn after 4 days and analyzed by FTIR (azide peak observed at 2100 cm^{-1}). The reaction was assumed to have reached the maximum conversion and the crude product was purified by dialysis against water for 3 days (MWCO 2 kDa). The solution obtained was lyophilized to obtain a very viscous oil which was analyzed by FTIR and ^{1}H NMR.

1.3.9 Preparation of polymer-peptide conjugates via conjugation of peptides and N_3-PEG_{12}-Mannose to BH40-DBCO512 via the SPAAC reaction

(i) Peptide conjugation: To BH40-DBCO512 (1eq of alkyne units) in DMF was added the CLEC9A peptide (0.075 eq) dissolved in DMF and the reaction was left to take place over 24 hours. The remaining peptides were added one at a time (0.1 eq) dissolved in DMF with a reaction time of 24 hours being allowed between additions. An aliquot (50 μL) was withdrawn and analyzed by to determine the extent of the reaction. Conversion was judged to be quantitative based on the disappearance of the peak due to the pure peptide in the HPLC chromatogram.

(ii) Polymer conjugation: To the polymer-peptide conjugation reaction solution (above) was added azido-PEG polymer (3 eq. based on starting alkyne units (excess)) dissolved in DMF. The resulting solution was left on a shaker for 3 days at room temperature. An aliquot (50 μL) was withdrawn and analyzed by FTIR (azide peak observed at 2100 cm^{-1}). The reaction was considered to have reached the maximum conversion and the crude product was purified by dialysis against water for 3 days (MWCO 3.5 kDa). The solution obtained was lyophilized to obtain a white solid which was analyzed by FTIR, ^{1}H NMR, SEC and AAA (ESI, Table S1).

Table S1. Amino acid analysis results of purified polymer-peptide hyperstars.

Amino Acid	nmoles	nms/50 ul	mole %	N (data)	N (exp)
Asx	1.030	1.001	11.57	3.2	3
Thr	0.292	0.284	3.28	0.9	1
Ser	0.475	0.462	5.33	1.5	1
Glx	0.324	0.315	3.64	1.0	1
Pro	0.387	0.376	4.35	1.2	1
Gly	0.000	0.000	0.00	0.0	0
Ala	1.517	1.475	17.04	4.8	6
Val	1.360	1.322	15.27	4.3	4
Ile	0.000	0.000	0.00	0.0	0
Leu	0.957	0.930	10.75	3.0	3
Tyr	0.390	0.379	4.38	1.2	1
Phe	0.901	0.876	10.12	2.8	3
His	0.000	0.000	0.00	0.0	0
Lys	0.828	0.805	9.30	2.6	3
Arg	0.443	0.431	4.98	1.4	1
Cysteic	0.000	0.000	0.00	0.0	0
MetSO2	0.000	0.000	0.00	0.0	0
Trp	0.000	0.000	0.00	0.0	0
Totals:					28

NB; The count for Phe (F) includes cyclohexylalanine(X).

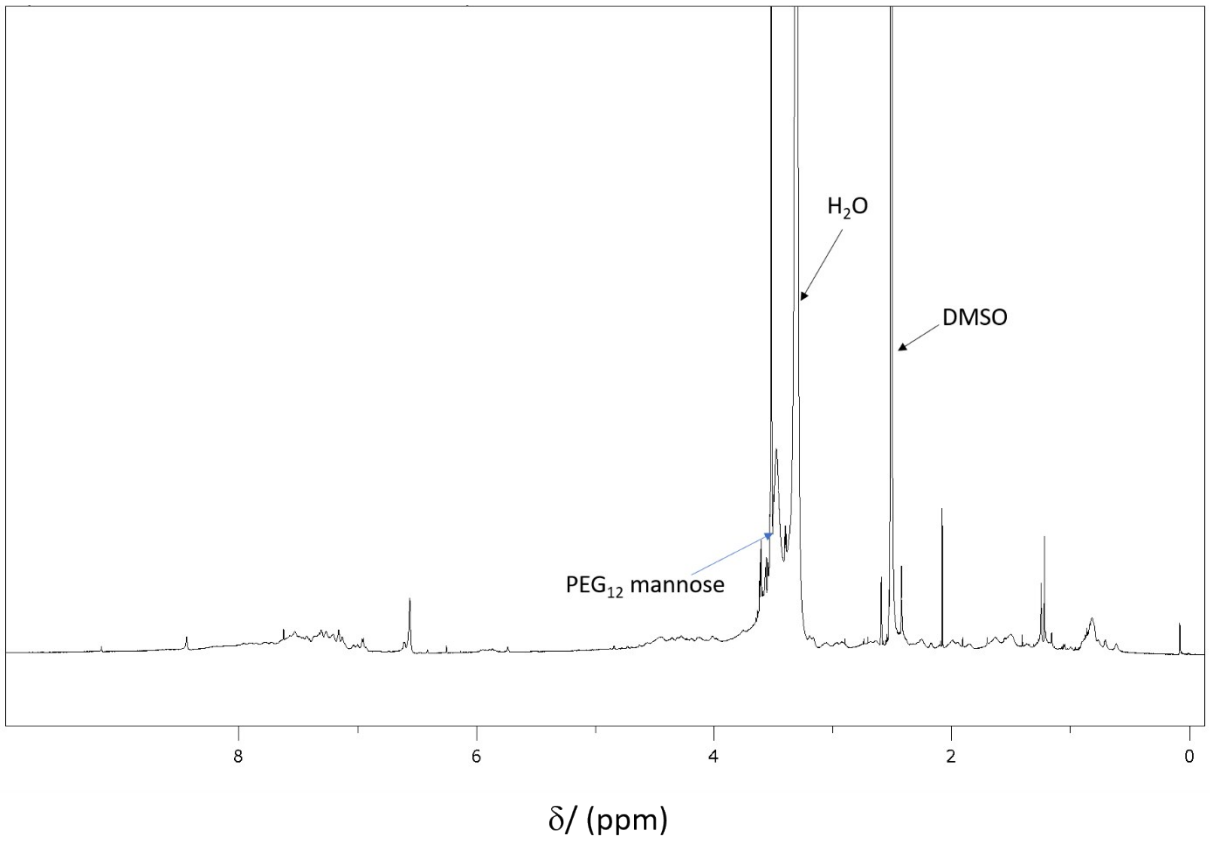


Fig. S7 ^{1}H NMR spectrum of purified polymer-peptide hyperstars showing the presence of PEG_{12} -mannose (remainder of the peaks are due to the peptides).

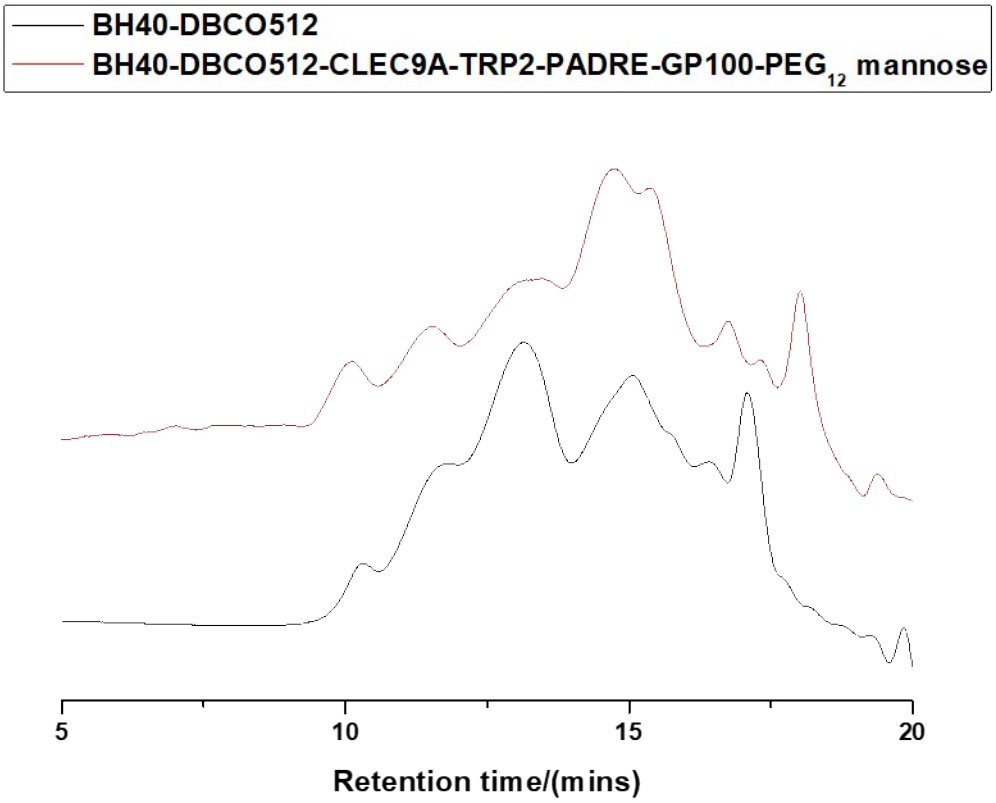


Fig. S8 SEC chromatograms of BH40-DBCO512 and the purified polymer-peptide hyperstar.

1.3.10 Aqueous solubilization of the polymer-peptide hyperstars

The polymer-peptide hyperstars (4 mg) was dissolved in previously degassed DMF (1 mL) and left to stir for 4 hours. Filtered (0.2 μm sterile filter), deoxygenated water (1 mL) was then added to the solution using a syringe pump (0.05 mL/min) while stirring at 500 rpm. The solution was placed in a dialysis bag and dialyzed against 4 liters of filtered deoxygenated deionized distilled water (Millipore Steritop, 0.45 μm, sterile filter) overnight (MWCO 3.5 kDa). The water was then replaced with filtered deoxygenated deionized distilled water and dialysis ensued for a further 6 hours after which the contents of the dialysis bag were collected and centrifuged at 1000 rpm for 2 minutes. Most of the solution was pipetted out with care being taken to avoid the bottom of the tubes in case traces of sedimented material were present. The volume of solution was adjusted to 4 mL (ca.1mg/mL material). An aliquot of the solution was obtained for analysis via TEM and another, mixed with 100 μL of PBS buffer (pH 7.4, 25 mmol NaCl), was analyzed via DLS (size and zeta potential).

1.3.11 Cell Viability

(i) *Cancer cells:* B16-F10 murine melanoma cells (ATCC, Manassas, VA, #CRL-6475) were plated at 2000 cells in 100 μL media per well in 96-well tissue culture plates 24 hours prior to peptide/particle addition. Free peptide or particles were added to each well in 100 μL complete media. Cells were incubated continuously with either free peptide (0.5, 0.25, 0.1, 0.05 mg/mL) or particles (1, 0.5, 0.25, and 0.1 mg/mL) for either 24 or 48 hours at 37 °C in a 5% CO_2 incubator. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (Invitrogen, Carlsbad, CA) was added to media at a concentration of 0.5 mg/mL, and cells were incubated 2 hours at 37 °C in a 5% CO_2 incubator. Media was removed, and formazan crystals dissolved in DMSO (100 μL/well, Sigma Aldrich, St. Louis, MO). Absorbance was measured using a Tecan (San Jose, CA) Infinite® M1000 microplate reader.

(ii) *Antigen presenting cells:* RAW264.7 macrophages were purchased from American Type Culture Collection (ATCC, Manassas, VA, # TIB-71) and cultured in DMEM:F12 1:1 without HEPES (Invitrogen, Inc., Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (Corning Inc., Corning NY) and 1% HyClone Penicillin-Streptomycin (ThermoFisher Scientific). Cells were plated at 2500 cells per well in 96-well tissue culture plates 24 hours prior to peptide/particle addition. Free peptide or particles were added to each well in 100 μL complete media. Cells were incubated continuously with either free peptide (0.5, 0.25, 0.1, 0.05 mg/mL) or particles (1, 0.5, 0.25, and 0.1 mg/mL) for either 24 or 48 hours at 37 °C in a 5% CO_2 incubator. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (Invitrogen, Carlsbad, CA) was added to media at a concentration of 0.5 mg/mL, and cells were incubated 2 hours at 37 °C in a 5% CO_2 incubator. Media was removed, and formazan crystals dissolved in DMSO (100 μL/well, Sigma Aldrich, St. Louis, MO). Absorbance was measured using a Tecan (San Jose, CA) Infinite® M1000 microplate reader.

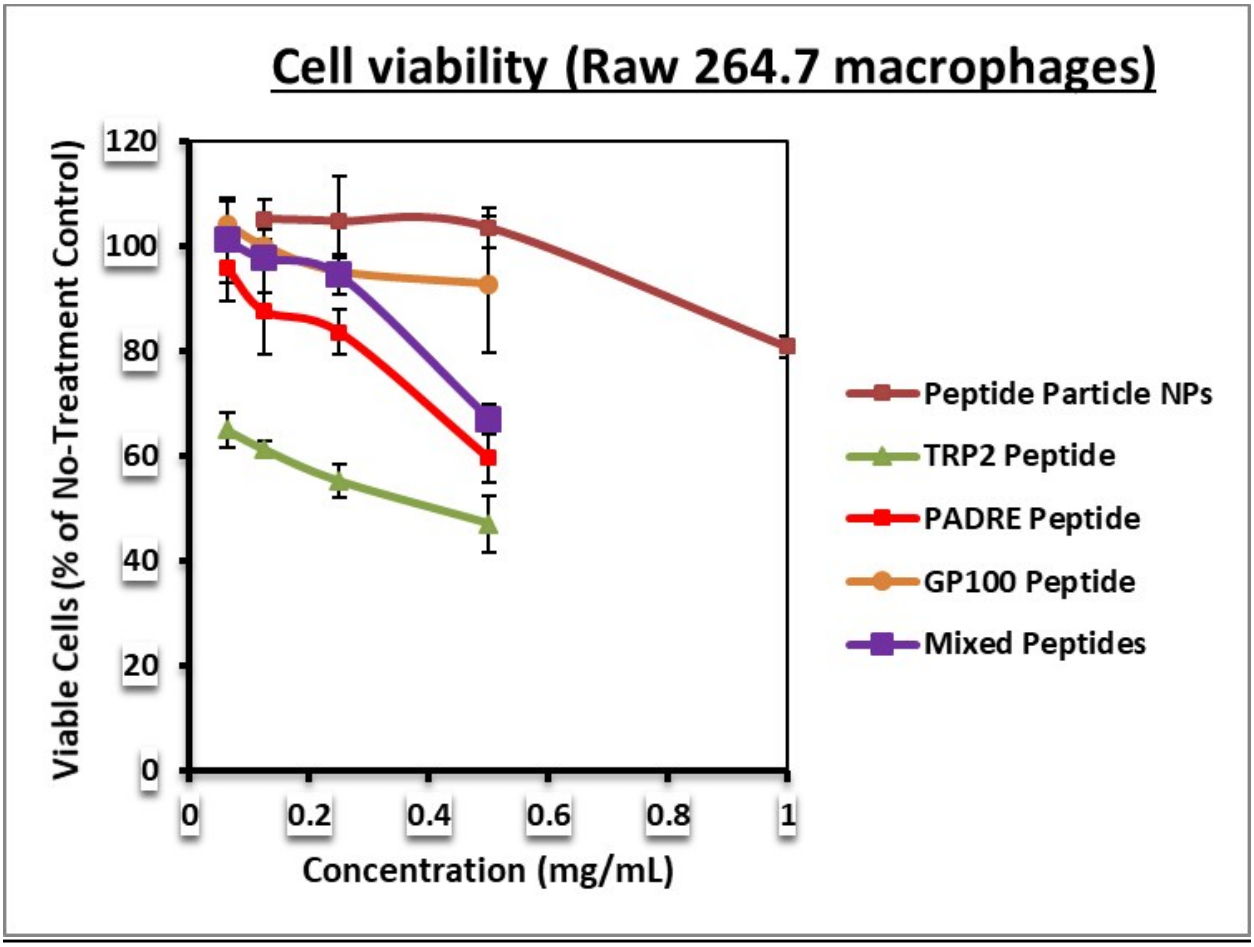
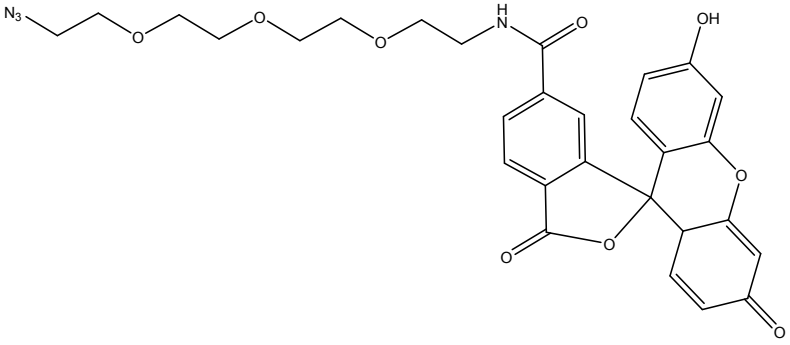


Fig. S9 Cell viability study results of the polymer nanoparticles and peptides after 48 hours of incubation with antigen presenting cells (Raw 264.7 macrophages). NB: The amount of the targeting peptide (CLEC9A) incorporated into the nanoparticles is nearly negligible thus its not included herein.

1.3.12 Synthesis of azido PEG_3-carboxyfluorescein



5/6-carboxyfluorescein succinimidyl ester (20.00 mg, 42.2 μmol) was dissolved in dry DMF (500 μL) followed by the addition of *O*-(2-Azidoethyl)-*O′*-methyl-triethylene glycol (10.1 mg,

1.1 eq) dissolved in dry DMF (500 μL) then triethylamine (50 μL). The resulting solution was left on a shaker at room temperature for 24 hours and an aliquot (50 μL) was withdrawn to determine the extent of reaction via HPLC. No peak was observed at the retention time expected for the pure carboxyfluorecsein dye (or its hydrolysed form) after 24 hours of reaction. The reaction was stopped and the product was purified via semi-preparative HPLC (5% to 90% solvent B over 60 mins, λ=495 nm) to afford the product as a bright yellow solid after freeze-drying of the collected fractions (azide peak observed at 2100 cm^{-1}). The compound mass was confirmed by mass spectrometry (m/z=577.19 Da $[M+H]^+$). Yield =13.2 mg, 95%.

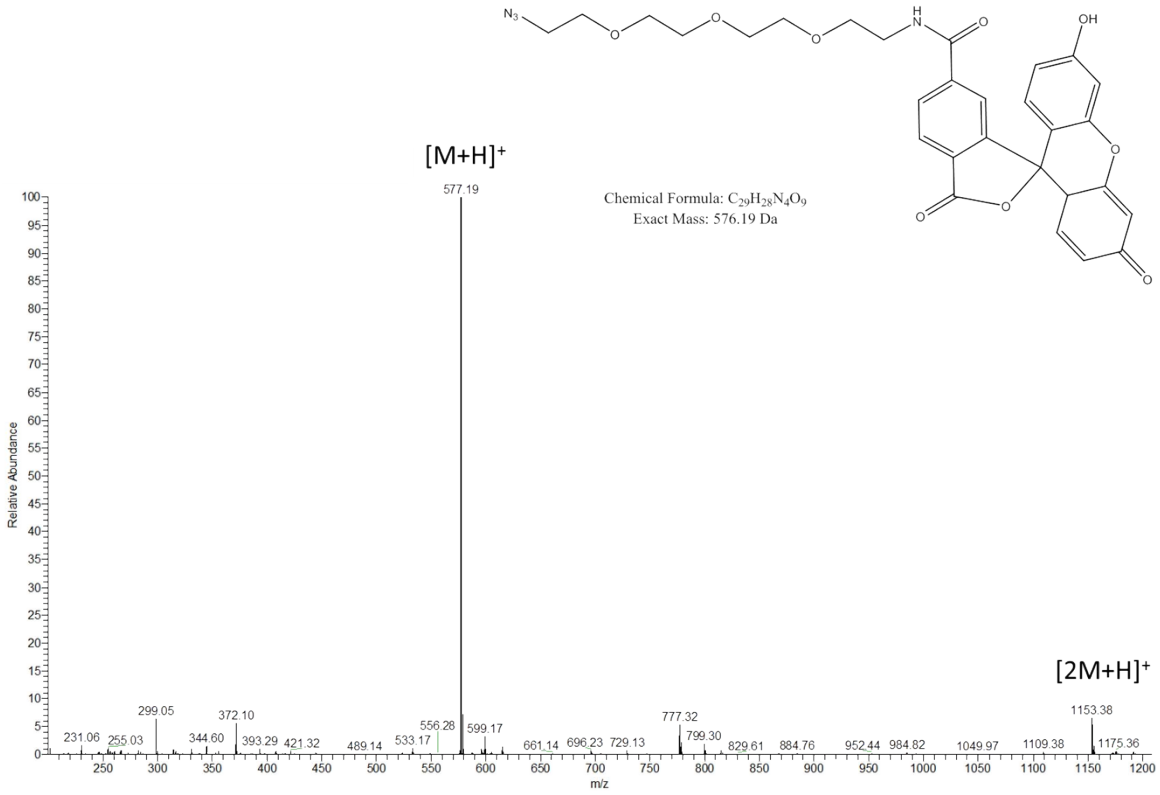


Fig. S10 Mass spectrum of azido PEG_3-carboxyfluorescein.

1.3.13 Preparation of fluorescently labelled hyperstars via conjugation of azido PEG_3-carboxyfluorescein, peptides and N_3-PEG_{12}-Mannose to BH40-DBC0512 via the SPAAC reaction

(a) Peptide nanoparticles sample

(i) Fluorescent dye conjugation: To BH40-DBCO512 (1 eq of alkyne units) in DMF was added the azido-PEG_{12}-carboxyfluorescein (0.2 eq) dissolved in DMF. The resulting solution was left on a shaker for 2 days at room temperature. An aliquot (50 μL) was withdrawn and analyzed by HPLC (Gradient 10% to 100% B over 45 min, λ=495 nm) to determine the extent of reaction. and GPC to determine the extent of the reaction. Maximum conversion was achieved based on the absence of the peak due to the pure dye in the HPLC chromatogram.

(ii) Peptide conjugation: To dye the labelled BH40-DBCO512 (1 eq of alkyne units) in DMF was were added the peptides in a sequential manner starting with CLEC9A (0.075 eq) followed by the remaining peptides (0.1 eq of starting alkyne) as previously described herein. After addition of the last peptide, the resulting solution was left on the shaker for one day at room temperature. An aliquot (50 μL) was withdrawn and analyzed by HPLC determine the extent of the reaction. Maximum conversion was achieved based on the absence of the peak due to the peptides in the HPLC chromatogram.

(iii) Polymer conjugation: To the polymer-peptide conjugation reaction solution (above) was added azido-PEG_{12}-mannose polymer (3 eq based on starting alkyne units (excess)) dissolved in DMF. The resulting solution was left on a shaker for 3 days at room temperature. An aliquot (50 μL) was withdrawn and analyzed by FTIR (azide peak observed at 2100 cm^{-1}). The reaction was considered to have reached the maximum conversion and the crude product was purified by dialysis against degassed deionized water for 3 days (MWCO 3.5 kDa). The solution obtained was lyophilized to obtain a yellow solid which was analyzed by GPC and FTIR.

(b) Polymer nanoparticles sample

The same procedure as described above in (a) was followed while omitting step (ii).

1.3.14 Particle uptake and internalization

1.3.14.1 Nanoparticle formation

For *in vitro* experiments, nanoparticles (with and without fluorescent label) were prepared following the aqueous solubilization procedure described above. To obtain the nanoparticle solutions in PBS at the desired concentrations, 1 mg/mL, solvent exchange (water to PBS) was done by using Amicon centrifugal filters (MWCO 3 KDa) which were centrifuged at 4000 rpm. Nitrogen degassed filtered PBS (0.2 μm) buffer was added to replace the water after each concentration cycle.

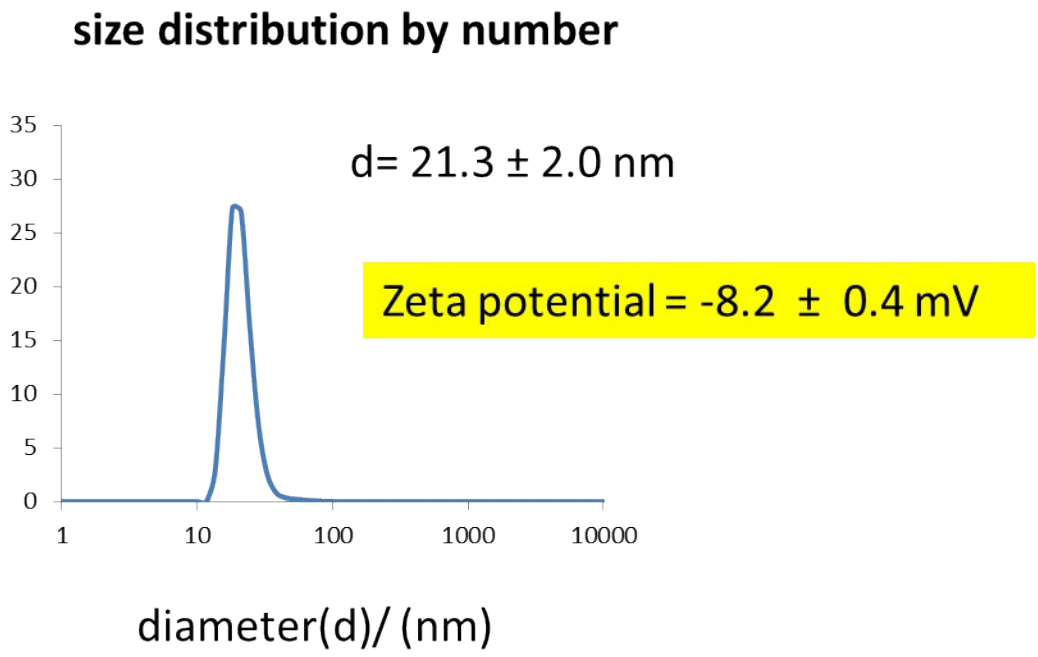


Fig. S11 Dynamic light scattering size distribution and zeta potential of the peptide nanovaccine. Results are an average of 5 measurements.

1.3.14.2 Cellular Internalization Studies

(a) Flow cytometry: Bone marrow derived DCs were plated at 2 x 10^5 cells/well in 24-well tissue culture plates for flow cytometry or at 4 x 10^5 cells/dish in 35-mm dishes for microscopy, 24 hours prior to experiments. Particles were incubated with cells continuously at 37 °C in complete media (100 µg/mL in 1 mL for imaging dishes and 300 µL for 24-well plates) for up to 24 h. For flow cytometry, cells were rinsed once with PBS and collected at 0, 4 and 24 hours in 170 µL TrypLE Express dissociation buffer (ThermoFisher Scientific, Waltham MA). Samples were then analyzed on a BD FACScan flow cytometer.

(b) Confocal Microscopy: Internalization of nanoparticles was confirmed using fluorescent confocal microscopy (Olympus FV3000). At day 6 of culture, cells were plated on a collagen-treated 35 mm glass bottom microwell dish (MatTek Corporation, Ashland, MA). Dendritic cells were stained with 0.1nM LysoTracker® Red DND-99 (Thermo Scientific) for 2 hours to stain lysosomal elements. Cells were subsequently washed twice with PBS. Following, cells were incubated with fluorescein-labeled peptide-conjugated nanoparticles or fluorescein-labeled bare nanoparticles at 100 µg/mL for 4 h in the presence of 0.1 nM LysoTracker® Red DND-99. Following incubation, cells were washed thoroughly with cold PBS and fixed with 4% methanol free paraformaldehyde (Thermo Fisher Scientific, Waltham, MA) for 15 minutes. Following fixation, cells were washed twice with PBS. To stain the cell nucleus, DCs were incubated in 10 ug of 4′,6-diamidino-2-phenylindole (DAPI) in 0.1% Tween-20 in PBS.

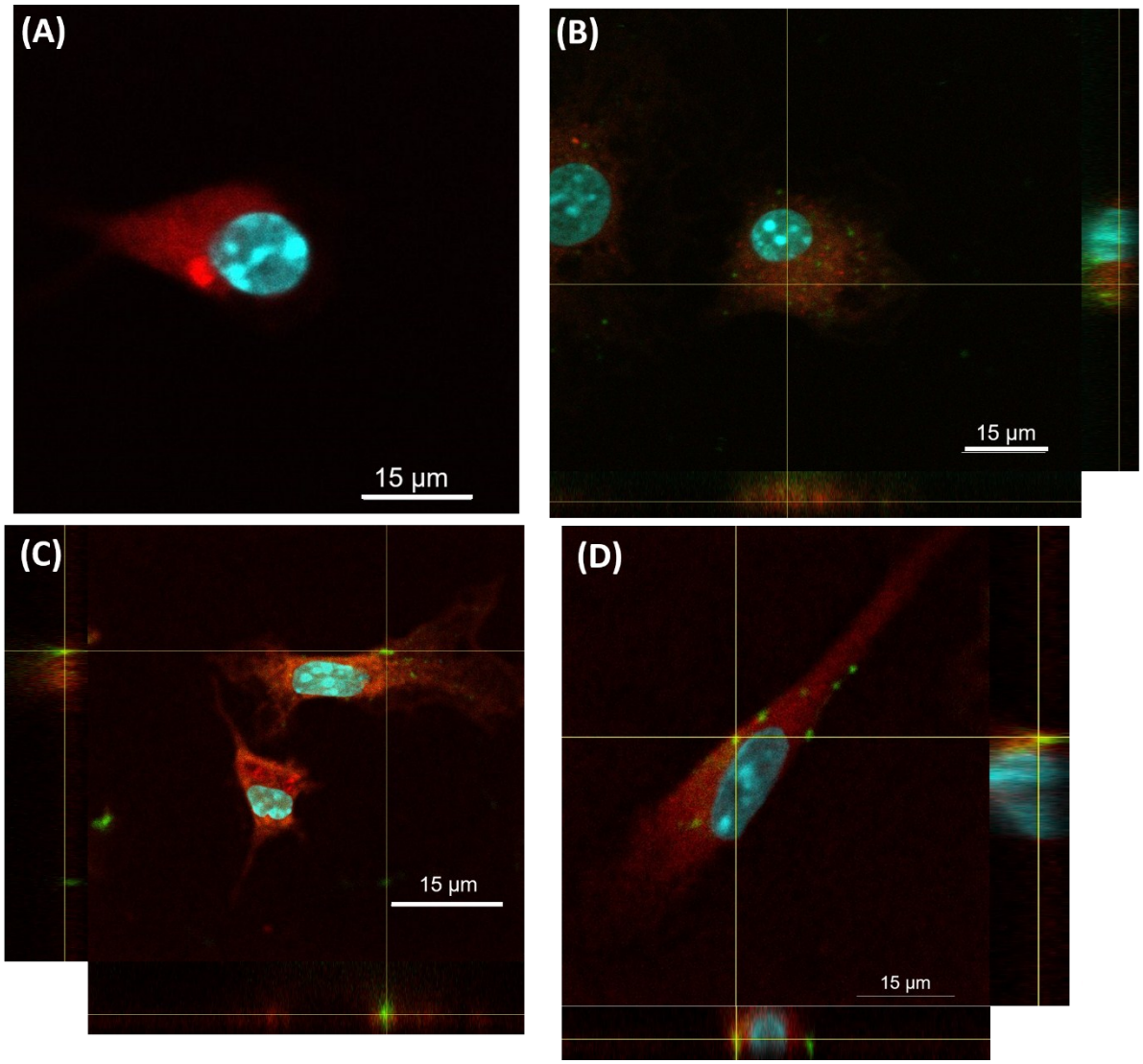


Fig. S12 Confocal microscopy images showing nanoparticle uptake of fluorescently polymer particles and peptide nanoparticles by DCs in vitro after 4 hours of incubation. Colocalization of the Lysotracker stain (red) and nanoparticles (green spots) can be clearly observed. The nucleus appearing as bright light blue was stained by DAPI. In (**A**) is the negative control (no particles), (**B**) polymer (no-peptide) nanoparticles and in (**C** and **D**), peptide-conjugatednanoparticles.

1.3.15 In vivo studies

(i) Nanoparticles preparation: Nanoparticles for *in vivo* experiments were prepared following the aqueous solubilization procedure described above. To obtain the nanoparticle solutions in PBS at the desired concentrations, solvent exchange (water to PBS) was effected by using Amicon centrifugal filters (MWCO 3 KDa) which were centrifuged at 4000 rpm. Nitrogen degassed filtered PBS buffer was added to replace the water after each concentration cycle.

Peptide presence and content was ascertained by AAA (1 μg/μL, amino acid count same as shown in AAA analysis table above).

(ii) Therapeutic treatment protocol: Vaccination was done on days 3 and 10 after B16OVA cells were injected. For the peptide nanoparticles treated group, 30 μL of a nanoparticle solution (1 μg/μL peptide) was injected via intradermal injection and the adjuvant, CpG (30 μL), was injected subcutaneously. The adjuvant only cohort was administered with CpG (30 μL). Non-treated mice were injected intradermally with saline only (30 μL). Once tumors were palpable, ultrasound was used to obtain the volume size of tumors twice per week Acuson Sequoia® 512 system (Siemens Medical Solution USA, Inc., Issaquah, WA). Mice were euthanized and tumors harvested before total tumor burden reached 2.0 cm.

(iv) Flow cytometry: Tumor-bearing mice were sacrificed 14 days after tumor cell implant and tissues processed by mechanical and enzymatic disruption to single-cell suspensions for immune cell profiling via flow cytometry. In order to exclude dead cells from analysis, live-dead cell staining with the LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Invitrogen, Carlsbad, CA) was done according to the manufacturer's instructions prior to all other antibody staining. Antibody panel combinations used to distinguish immune cell populations were $CD45^+$ (leukocytes) plus the following: $CD11b^+$, $F4/80^+$, $Gr\text{-}1^-$ (macrophages); $CD11c^+$, $MHCII^+$, $F4/80^-$ (dendritic cells); $CD3^+$ (T-cells); $CD3^+$, $CD4^+$ ($CD4^+$ T-cells); and $CD3^+$, $CD8^+$ ($CD8^+$ T-cells). Cell samples were fixed in Cytofix buffer (BD Biosciences), diluted to 1% paraformaldehyde (PFA) in PBS-/-, and run within 24 hours on either a FACScan or LSRII flow cytometer (BD Biosciences). All data were analyzed using FlowJo v10 software (TreeStar).

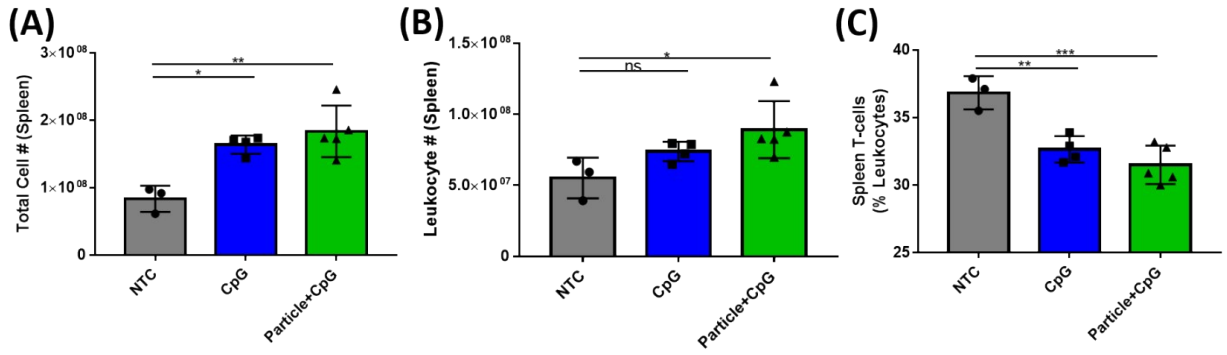


Fig. S13 Flow cytometry results of immune cell populations in the spleen. For statistical significance, $p \leq 0.05$ (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

(v) Data analysis: Kaplan-Meier survival plots were obtained using GraphPad Prism software and compared via the log rank (Mantel-Cox) test.

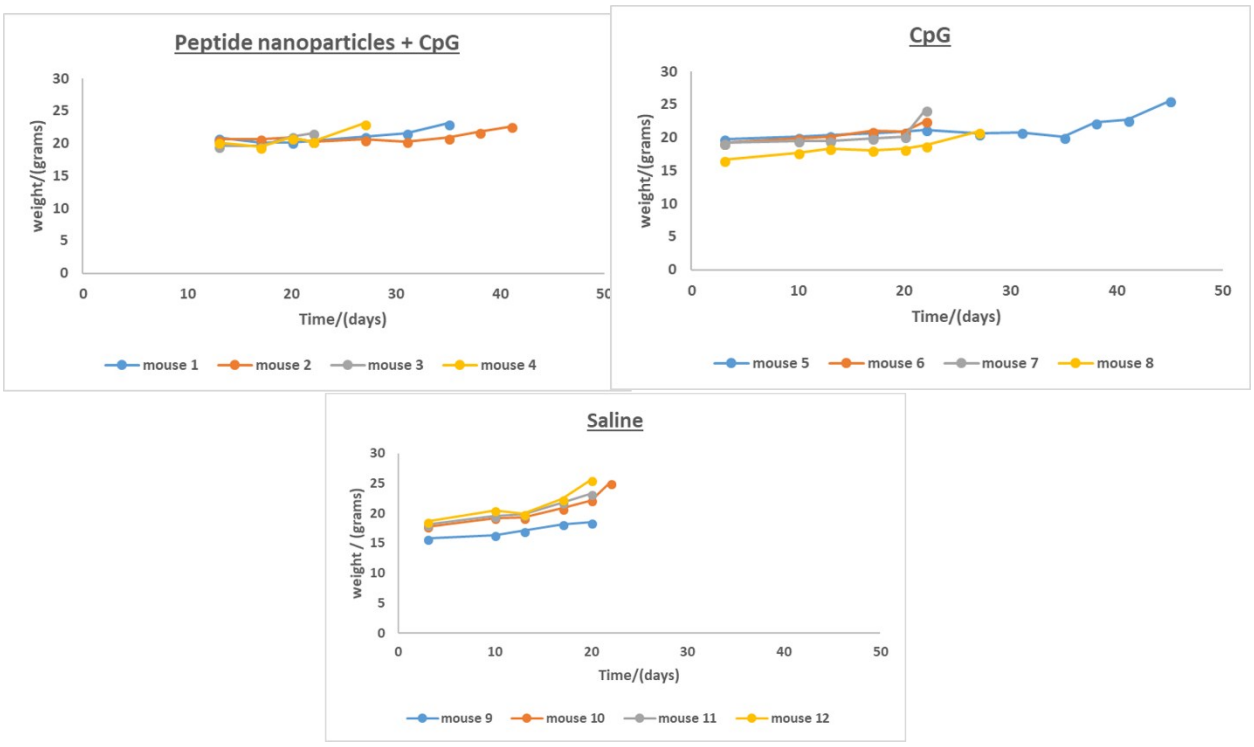


Fig. S14 Animal weights recorded from survival studies.

1.4 References

1. H. Kakwere, E. S. Ingham, R. Allen, L. M. Mahakian, S. M. Tam, H. Zhang, M. T. Silvestrini, J. S. Lewis and K. W. Ferrara, *Bioconjugate Chem.*, 2017, **28**, 2756-2771.