Supporting Information

Enhanced Immunogenicity of Multivalent MUC1 Glycopeptide Antitumor Vaccines Based on Hyperbranched Polymers

M. Glaffig,[a] B. Palitzsch,[a] N. Stergiou,[b] C. Schüll,[a] D. Straßburger,[a] E. Schmitt,[b] H. Frey,[a] and H. Kunz[a]

1. Abbrevations

BSA bovine serum albumine
Fmoc Fluorenyl-9-methoxycarbonyl
DIPEA Diisopropylethylamin
HATU *O*-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluroniumhexafluoro phosphate
HBTU *O*-Benzotriazol-1-yl-*N,N,N',N'*-tetremethyluronium hexafluorophpsphate
HOAt *N*-Hydroxy-7-azabenzotriazol
HOBt 1-Hydroxy-benotriazole
NMM *N*-Methylmorpholine
NMP *N*-Methyl-2-pyrrolidone
Sp Spacer
TFA Trifluoroacetic acid
TIS Triisopropylsilane
In NMR: the 1-letter code for amino acids is used

2. Materials

Unless otherwise indicated, all commercially available materials (Aldrich, Acros, Roth) were used without further purification. All solvents were reagent grade or HPLC grade. For peptide synthesis, all preloaded resins were obtained from Rapp Polymere GmbH (Tübingen, Germany). The protected Fmoc-amino acids were obtained from Orpegen Pharma (Heidelberg, Germany) and peptide grade solvents were purchased form Iris Biotech (Marktredwitz, Germany). Phosphate buffered saline (PBS) was obtained from Fisher BioReagents containing 137 mM NaCl, 11.9 mM phosphates and 2.7 mM KCl.

3. Instrumentation

^{1}H NMR (600 MHz) and ^{13}C NMR (151 MHz) spectra were registered on Bruker 600 MHz FT NMR spectrometer in D_2O as solvent. Chemical shifts (δ) are given in ppm relative to Trimethylsilane. Solid-phase peptide synthesis (SPPS) was performed using ABI 433A peptide synthesizer (Applied Biosystems, Carlsbad, USA) with a 200 UV/Vis detector (Perkin Elmer) to control the synthesis process.
Separation and purification of the peptide was performed by a semi-preparative reversed-phase HPLC system (Knauer, Berlin, Germany) consisting of two pumps (Smartline 1000), a two-channel degasser, a dynamic mixer and a semi-preparative column (Phenomenex, Torrance, USA) with the dimension 250 mm x 30 mm filled with Luna RP-C18(2), 100 Å, Axia, 10 µm. Signals were detected with a UV/Vis-detector (Smartline 2500) at 214 nm and a RI-detector (Smartline 2400). All runs were

performed at flow rates of 10.00 mL/min. As eluent, a mixture of acetonitrile HPLC grade (VWR International, West Chester, USA) and Milli-Q-water both acidified with 0.1% TFA was used.

The purification of vaccine **1** and the corresponding BSA-conjugate was performed by ultrafiltration through a polyethersulfone-membrane (P30) (Millipore Corporation, Billerica, USA) with a cut-off of 30 kDa.

For analytical SEC measurements, hexafluoro-2-propanol (HFIP) containing 3.0 g/L of potassium trifluoroacetate was used as solvent using a PU 2080+ pump, an auto sampler AS1555 and an RI-detector RI2080+ from JASCO. Columns packed with modified silica were obtained from MZ-Analysentechnik: PFG columns, particle size 7 µm, porosity 100 Å.

ESI-MS analyses were carried out by a Navigator Instrument from Thermoelectronics with sample concentrations of 0.1 mg/mL, 0.75 mL/min flow rate, cone voltage 70 V, 45 V or 35 V and nitrogen flow rate 300 L/min.

Specific rotation were measured on Polarimeter 241 (Perkin-Elmer, Massachusetts, USA) at wavelength of λ = 546 nm und λ = 578 nm in a 10 cm polarimeter cell and extrapolated to the sodium-D-line (λ = 589.5 nm). Solvent, temperature and the concentration (g/100 mL) are given for the individual compounds.

4. Syntheses

4.1 Solid-phase (glyco)peptide synthesis of MUC1-P2 peptide **3**

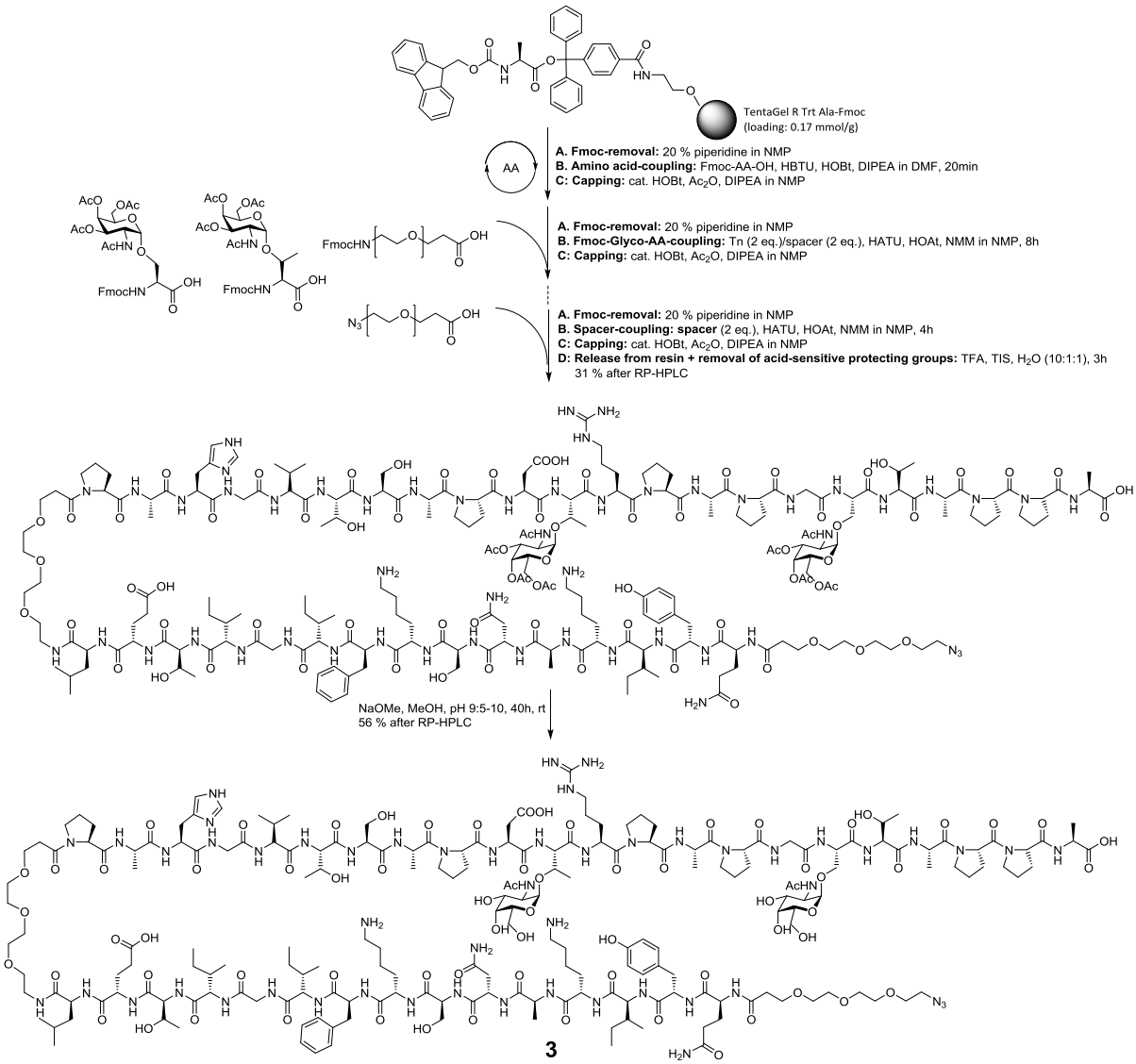
Peptide **3** was synthesized in a peptide synthesizer by standard solid-phase synthesis of Fmoc chemistry starting from Fmoc-Ala-Trt-Tenta Gel R resin (Rapp Polymere, Tübingen, 588.2 mg, 0.1 mmol, loading: 0.17 mmol/g). All Fmoc-protected amino acids with their corresponding side chain-protecting groups (acid-labile) were applied, each with an excess of 10 eq. Fmoc removal was performed using a solution of 20% piperidine in NMP. Couplings of the amino acid were carried out following the standard protocol by using HBTU (1.0 mmol), HOBt (1.0 mmol) and DIPEA (2.0 mmol) in DMF for 20 min at room temperature. The unreacted free amino groups were capped by acetylation using cat. HOBt in Ac_2O/DIPEA/NMP. The coupling of saccharide-amino acid building block (Tn antigens) and the two spacers were performed manually in the vessel of the peptide synthesizer. To this end, Tn-Ser antigen (131.3 mg, 0.2 mmol, 2.0 eq.) Tn-Thr antigen (134.1 mg, 0.2 mmol, 2.0 eq.), Fmoc-spacer (88.7 mg, 0.2 mmol, 2.0 eq.) and azide-spacer (49.44 mg, 0.2 mmol, 2.0 eq.) were added to the resin in a solution of HATU (91.2 mg, 0.24 mmol, 2.4 eq.) and NMM (55 µl, 0.5 mmol, 5.0 eq.) in NMP (2 mL). The reaction mixture was strongly shaken for 8 h in the case of the glycosyl amino acids and for 4 h in the case of each spacers (30 s vortex, 30 s stop), followed by filtration and washing with NMP and dichloromethane. The coupling of the amino acids (Fmoc-Gly-OH and Fmoc-Asp-OH) following the saccharide amino acids were performed twice by standard protocol (double coupling) without intermediate capping. After completion of the peptide assembly in the synthesizer, the resin was transferred to a Merrifield-solid phase-vessel and treated with a solution of TFA/TIS/water (10:1:1, 12 mL), shaken for 3,5 h to remove all acid-labile protecting groups und to remove the peptide from resin. After filtration and two washing steps with TFA (5 mL) for 5 min, toluene (20 mL) was added and removed afterwards under reduced pressure. After that, the residue was co-distilled twice with toluene (20 mL) and lyophilised. The acetyl-protected glycopeptide was purified by semi-preparative RP-HPLC to yield 151 mg (0.031 mmol, 31 %).

For the deprotection of the carbohydrate, the still acetyl-protected peptide was dissolved in methanol (20 mL) and a solution of sodium methoxide was added dropwise to set pH of 9.5-10. After stirring for two days, the solution was neutralised with acetic acid and lyophilised. The residue was purified by semi-preparative HPLC to yield 84.7 mg (0.0184 mmol, 65 %).

HPLC: Rt = 25.2 min (H2O + 0.01 % TFA, MeCN + 0.01 % TFA, 95:0 (0 min), 60:40 (30 min), 0:100 (40 min)).
ESI-MS (pos.), m/z (100%): 1151.31 ($[M+4H]^{4+}$, calc.: 1151.09), 1534.58 ($[M+3H]^{3+}$, calc.: 1534.45).
Specific optical rotation: $[\alpha]_D^{23}$ = +281.9 (c = 0.10, MeOH).
HR-ESI-MS, m/z: 1150.5955 ($[M+4]^{4+}$, calc. 1150.5926, 2.5 ppm).

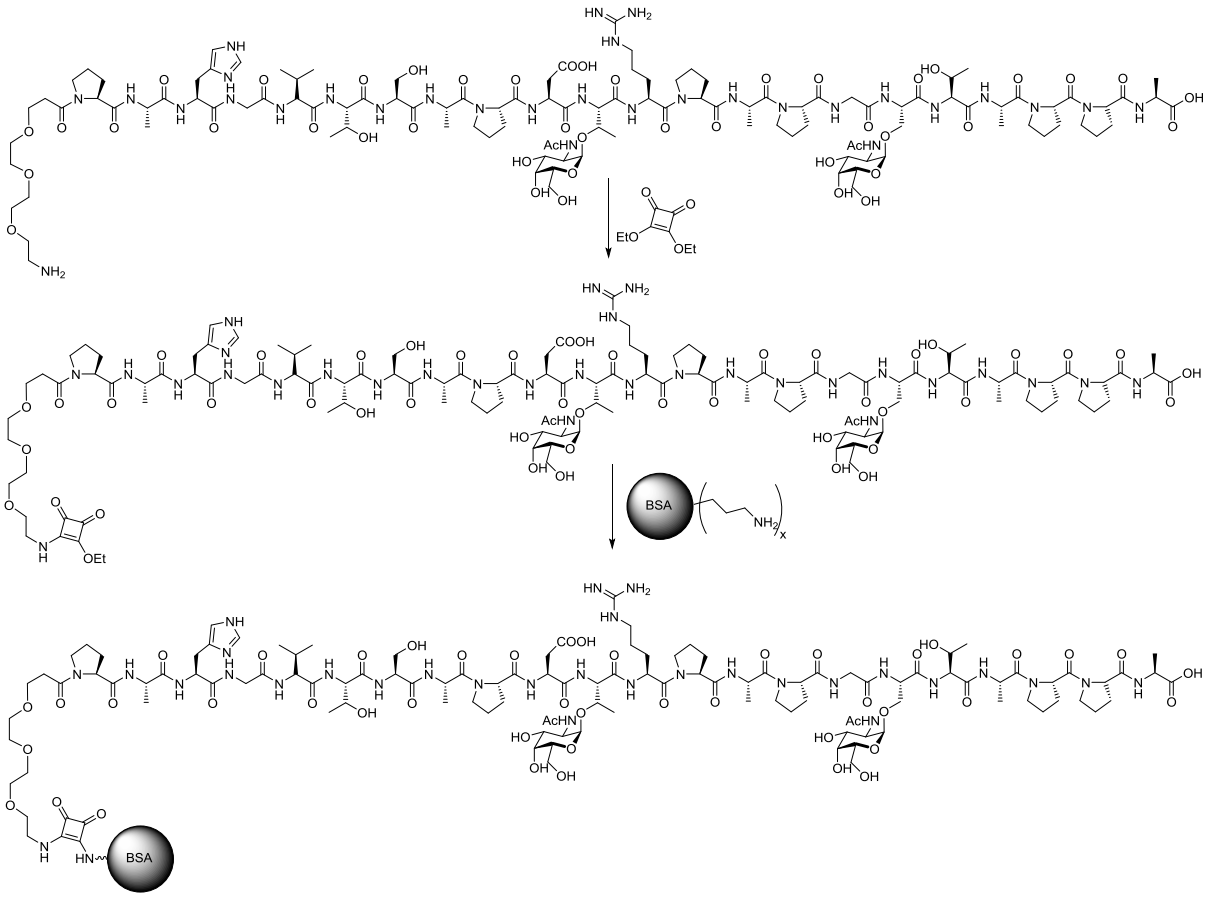
^{1}H NMR (600 MHz, D_2O, COSY, HSQC, HMBC), δ (ppm), pp= 8.58 (s, 1H, $H^{Im\text{-}H2}$), 7.28 - 7.18 (m, 6H, $H^{Im\text{-}H4}$ {7.28}, F^{ar}), 7.05 (d, 2H, *J* = 6.9 Hz, Y^{ar}), 6.77 (d, 2H, *J* = 6.76 Hz, Y^{ar}), 4.88 - 4.70 (under D_2O signal: 2 x Tn^{H1} {4.84, 4.80}), 4.73 - 4.69 (m, 1H, D^{α}), 4.68 - 4.45 (m, 7H, H^{α} {4.64}, Y^{α} {4.67}, N^{α} {4.65}, 2 x K^{α} {4.62}, R^{α}, F^{α}), 4.40 - 4.15 (m, 28H, 6 x P^{α}, 3 x T^{α}, Q^{α} {4.38}, E^{α} {4.37}, 3 x S^{α} {4.33}, Tn^{β} {4.32}, 6 x A^{α} {4.29, 4.22, 4.18}, 3 x T^{β} {4.17}, 3 x I^{α} {4.18}), 4.13 - 4.07 (m, 2H, V^{α}, L^{α}), 4.06 – 4.01 (m, 2H, 2 x Tn^{H2}), 3.99 - 3.85 (m, 9H, 6 x G^{α}, 2 x Tn^{H5} {3.96}, 2 x Tn^{H4} {3.91, 3.90}), 3.83 - 3.80 (m, 2H, 2 x Tn^{H3}), 3.79 - 3.66 (m, 22H, 3 x S^{β}, 6 x P^{δ}, 2 x Tn^{H6} {3.70}), 3.66 - 3.53 (m, 26H, $Sp^{CH2\text{-}O}$, H^{β} {3.61}), 3.44 - 3.37 (m, 4H, $Sp^{CH2\text{-}NH}$, $Sp^{CH2\text{-}N3}$), 3.19 - 3.12 (m, 4H, Y^{β}, R^{δ}), 3.10 - 2.67 (m, 10H, D^{β}, N^{β}, 2 x K^{ε}, F^{β} {2.96}), 2.40 (t, 2H, $J_{E\gamma,E\beta}$ = 7.2 Hz, E^{γ}), 2.35 - 2.14 (m, 4H, $Sp^{CH2\text{-}CO}$), 2.10 - 1.72 (m, 38H, V^{β} {2.07}, Q^{γ} {2.05}, 2 x CH_3-AcNH {1.99, 1.98}, E^{β}, L^{γ} {1.94}, Q^{β} {1.90}, L^{β} {1.80}, 6 x P^{β}, 6 x P^{γ}), 1.71 - 1.48 (m, 15H, 3 x I^{β}, 2 x K^{β} {1.65}, 2 x K^{δ} {1.58, 1.57}, R^{β}, R^{γ} {1.72 - 1.64}), 1.44 - 1.12 (m, 35H, 3 x I^{γ} {1.40}, 1 x A^{β} {1.38, d, $J_{\beta,\alpha}$ = 7.4 Hz}, 4 x A^{β} {1.31}, T^{γ} {1.20, d, 3H, $J_{T\gamma,T\beta}$ = 5.0 Hz}, 2 x K^{γ} {1.17}, 1 x A^{β} {1.16}, T^{γ} {1.15}), 0.93 - 0.85 (m, 15H, 3 x $I^{\gamma'}$, L^{δ}), 0.84 - 0.75 (m, 15H, 3 x I^{δ}, V^{γ}).

^{13}C-NMR (151 MHz, D_2O, HSQC, HMBC), δ (ppm) = 177.64 - 162.46 (45C, C=0), 156.71 ($R^{C=NH}$), 154.46 ($Y^{C\text{-}OH}$), 136.17 ($H^{Im\text{-}C5}$), 133.48 ($H^{Im\text{-}C2}$), 130.51, 130.50 (2 x Y^{ar}), 129.15, 128.75, 128.71, 128.38, 128.36, 127.16 (6 x F^{ar}), 119.30 (Y^{ar}), 117.35 ($H^{Im\text{-}C4}$), 115.41 (2 x Y^{ar}), 98.58, 97.91 (2 x C-1), 71.48, 71.45 (2C, $S_{Tn}{}^{\beta}$, $T_{Tn}{}^{\beta}$), 71.37, 71.31 (2 x C-5), 69.66 - 68.74 (13C, 10 x $Sp^{CH2\text{-}O}$, 3 x S^{β}), 68.54, 68.42 (2 x C-4), 68.14 (2C, 2 x C-3), 67.05 (2C, 2 x $Sp^{3\text{-}CH2}$), 66.93, 66.54, 66.17 (3C, 3 x T^{β}), 61.35 (S^{α}), 61.17, 61.11 (2 x C-6), 60.98 (2C, 2 x S^{α}), 60.50, 60.11, 60.09, 59.92, 59.53, 59.33, 58.90, 58.88, 58.45, 58.28 (14C, 6 x P^{α}, E^{α}, 2 x K^{α}, L^{α}, 3 x I^{α}, V^{α}), 57.11, 55.95, 55.19, 54.90 (6C, 2 x Tn^{α}, 3 x T^{α}, F^{α}), 53.65, 53.33, 53.16, 52.62, 51.08, 50.42, 50.13, 49.88, 49.85, 48.65, 48.63, 48.11, 47.92, 47.74, 47.72 (20C, R^{α}, H^{α}, 6 x P^{δ}, 6 x A^{α}, Y^{α}, D^{α}, Q^{α}, C-2 {49.69, 49.68}, N^{α}), 47.66 (2C, $Sp^{CH2\text{-}N3}$, $Sp^{CH2\text{-}NH}$), 42.42, 42.32, 42.27 (3 x G^{α}), 40.47, 39.93, 39.15, 39.13, 38.94 (5C, R^{δ}, Y^{β}, 2 x K^{ε}, D^{β}), 36.80, 36.27, 36.03, 36.01, 35.63, 35.20, 34.11, 34.07, 30.92, 30.16, 29.76, 29.69, 29.42, 29.34, 29.20 (15C, N^{β}, F^{β}, Q^{β}, 3 x I^{β}, 2 x $Sp^{CH2\text{-}CO}$, R^{β}, R^{γ}, V^{β}, E^{γ}, L^{β}, 2 x K^{β}), 28.04, 27.43, 26.77, 26.35, 26.29, 26.16, 26.06, 24.73, 24.66, 24.62, 24.56, 24.39, 24.28, 24.16, 22.31 (20C, 6 x P^{β}, 6 x P^{γ}, H^{β}, E^{β}, L^{γ}, Q^{γ}, 2 x K^{γ}, 2 x K^{δ}), 22.12, 22.06 (2 x CH_3 (AcNH)), 21.95, 20.79, 18.88, 18.83, 18.72, 18.48, 17.66 (8C, 3 x T^{γ}, $V^{\gamma a}$, $T_{TN}{}^{\gamma}$, 3 x I^{γ}), 16.41, 16.30, 16.05, 15.46, 15.41, 15.37 (6C, A^{β}), 14.85, 14.66, 14.62 (3C, $I^{\gamma'}$), 10.29, 10.15, 9.92 (6C, 3 x I^{δ}, $V^{\gamma b}$, L^{δ}).



4.2 Synthesis of B-cell epitope (MUC1)-BSA conjugate for ELISA microtitre plate coating[i]

For ELISA analyses, the microtitre plates were coated with bovine serum albumin (BSA)-conjugated glycopeptides which contains only the MUC1 B-cell epitope of peptide **3**. Therefore, the MUC1-glycopeptide with an amine-spacer at the N-terminus was synthesised on solid-phase as previously described. Squaric acid diethyl ester were added to the fully deprotected glycopeptide. The resulting squaric ester monoamide was subsequently conjugated to BSA. The synthetic process and analytic data is described in recent literature.[i]



4.2 Synthesis of the hyperbranched polyglycerol[ii]

The hyperbranched polyglycerol HP(G-GPE) **5** was synthesised by copolymerization of glycidol (G) with the alkyne-containing oxirane monomer glycidyl propargyl ether (GPE) in a ratio of 13:8 according to reference.[ii] NMR spectra (HSQC-TOCSY, HMBC) of hPG(G_{13}-GPE_8) **5** is given in ref. ii.

4.3 The two spacers[iii] and vaccine **2**[iv] were synthesised as described recently.

4.4 Coupling of P2-MUC1 glycopeptide **3** to hyperbranchend P(G-GPE) **5** to afford vaccine **1**

In a Schlenk tube, HbP(G_{13}-GPE_8) **5** (1.78 mg, 0.74 µmol) was dissolved in 500 µL Millipore water under argon atmosphere. To this solution, a mixture of MUC1-P2 peptide **3** (30.0 mg, 6.52 µmol) in 500 µL Millipore water was added dropwise. After three freeze-pump-thaw cycles for solvent degassing, copper sulfate (0.95 mg, 5.93 µmol) and sodium ascorbat (5.87 mg, 29.64 µmol) in 300 µL were added. Following one additional freeze-pump-thaw cycle, the reaction mixture was stirred at 40 °C for four days under argon atmosphere. Ion exchange resin (*Lewatit® Monoplus S100H*) was added and the reaction mixture was shaken for 24h. The solution was purified by ultrafiltration (polyethersulfone-membrane, cut-off 30kDa). Successful coupling of glycopeptide **3** to the polymer **5** was confirmed by SEC in HFIP as eluent. 17.2 mg of vaccine **1** was obtained.

5. Immunologic Experiments

5.1 Immunisation schedule

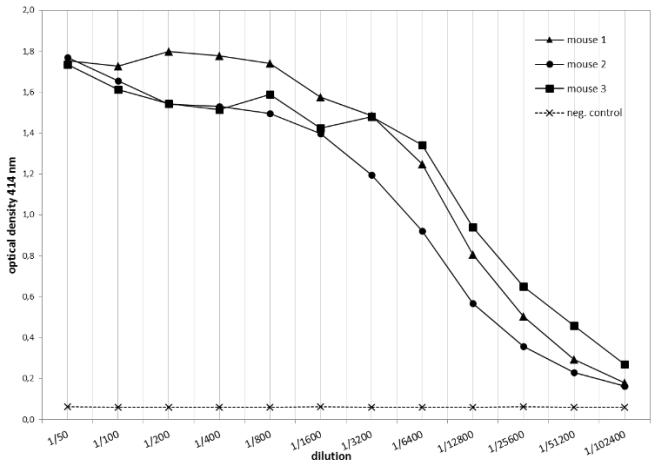
For the preparation of the vaccine adjuvant sample, 250 µL of the vaccine solution (62.5 µL of 12 mg/mL of the vaccine in 187.5 µL phosphate buffered saline (PBS)) were mixed with either 250 µL complete of Freund's adjuvant (CFA, Fa. DIFCO) or 250 µL of incomplete Freund's adjuvant (IFA, Fa. Sigma-Aldrich) by an ultrasound sonicator (Fa. Bandelin electronic UW 2070) for 30 s. The resulting emulsion was transferred to a syringe and stored on ice until administered.

Three female Balb/c mice of 6-10 weeks were immunised. They were kept under sterile conditions. At intervals of two weeks, the mice were immunised three times with 40 µL of the above-described emulsion: the first immunisation was administered subcutaneously with CFA, the following two boost immunisations were administered intraperitoneally with IFA. Five days after the two boost immunisations, blood was collected from tail vein and subsequently centrifuged twice at 10 000 rpm for ten minutes. After each centrifugation, the supernatant was collected in order to obtain the serum antibodies.

5.2 ELISA analysis

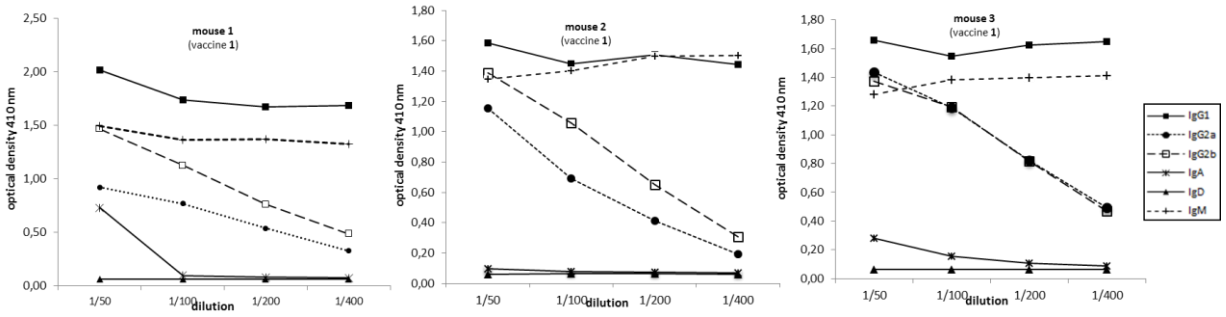
For analysing the titres of MUC1-specific induced antibodies, enzyme-linked immunosorbent assay (ELISA) was applied. To this end, 96-well plates (Fa. Nunc MaxiSorp® flat-bottom) were incubated with 50 µL per well of a solution of BSA-glycopeptide conjugate (see above, 2.5 µg/mL) in coating buffer (0.1 M Na_2HPO_4 in water, pH 9.3) at 37 °C. Washing steps (three times) were processed using 100 µL blocking buffer (1% BSA, 0.2% Tween-20 in PBS). In order to saturate free binding sites, the coated plates were subsequently incubated for 30 minutes with 50 µL blocking buffer at 37 °C. The antiserum was diluted in blocking buffer (1:50 for the first column of the 96-well plate and then serially diluted in a ratio of 1:1), then applied and incubated for one hour at 37 °C. After three additional washing steps, the samples were incubated with biotinylated sheep-α-mouse antibody (c = 0.48 µg/mL) for one hour at 37 °C in 50 µL blocking buffer. Again, the samples were washed three times and subsequently incubated for 30 minutes with streptavidin-horseradish peroxidase (c = 0.5 µg) in 50 µL blocking buffer. After washing three times, each well was treated with a solution of 1 mg/mL 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 0.01% hydrogen peroxide in citrate buffer (40 mM citric acid, 60 nM Na_2HPO_4 x H_2O, pH 4.5). After minutes of incubation at room temperature, the optical density of each well at 410 nm was measured with a spectrophotometer (Tecan Reader, Genios).

ELISA analysis of the antisera induced by vaccine **1**, five days after the last immunisation:



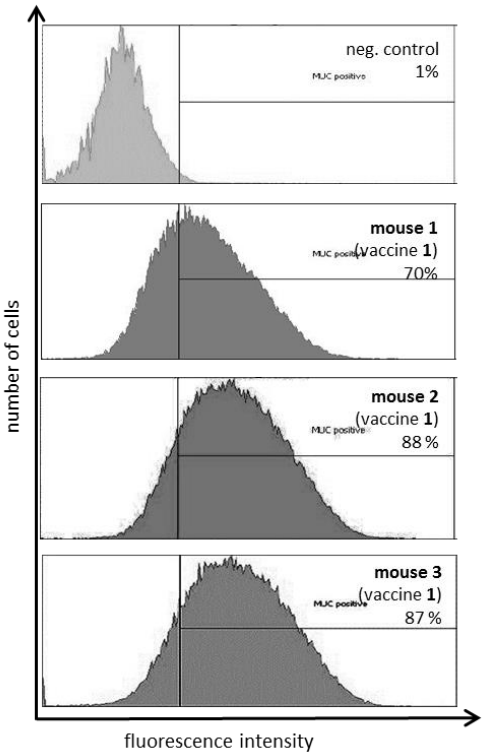
5.3 Antibody isotypes analysis

For the isotype determination of the induced MUC1-specific antibodies, ELISA was carried out using the protocol described above. The following secondary antibodies were used: biotinylated anti-mouse-IgM (Fa. eBioscience, clone eB121-15F9), biotinylated anti-mouse-IgD (Fa. eBioscience, clone 11-26), biotinylated anti-mouse-IgA (Fa. eBioscience, clone 11-44-2), biotinylated anti-mouse-IgG1 (Fa. BD Pharmingen, clone A85-1), biotinylated anti-mouse-IgG2a (Fa. BD Pharmingen, clone R19-15), biotinylated antimouse-IgG2b (Fa. BD Pharmingen, clone R12-3).



5.4 Analysis of the antibody binding to human breast cancer cells

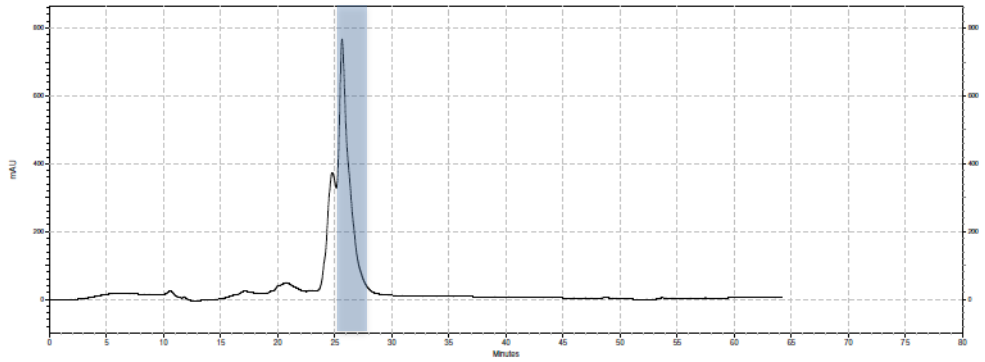
For analysing the binding ability of the antibodies to human breast cancer cells, T47D cells (1x10^5 cells per sample) were transferred to a 96-well plate. After washing the cells using 100 µL PBS, they were incubated with antiserum after the second boost immunisation (50 µL, diluted 1:400 in PBS) from immunised mice for 20 minutes at 4 °C. Then, the cells were washed two times with 100 µL PBS and afterwards incubated with a goat-α-mouse-IgG-Alexa Fluor 488 antibody (solution of 2 µg/mL in PBS) for 20 minutes at 4 °C. After two further washing steps with 100 µL PBS, the cells were taken up in 100 µL PBS and pipetted into a FACS tube. For each sample, 10^4 cells were analysed on a BD Biosciences FACSVerse machine.



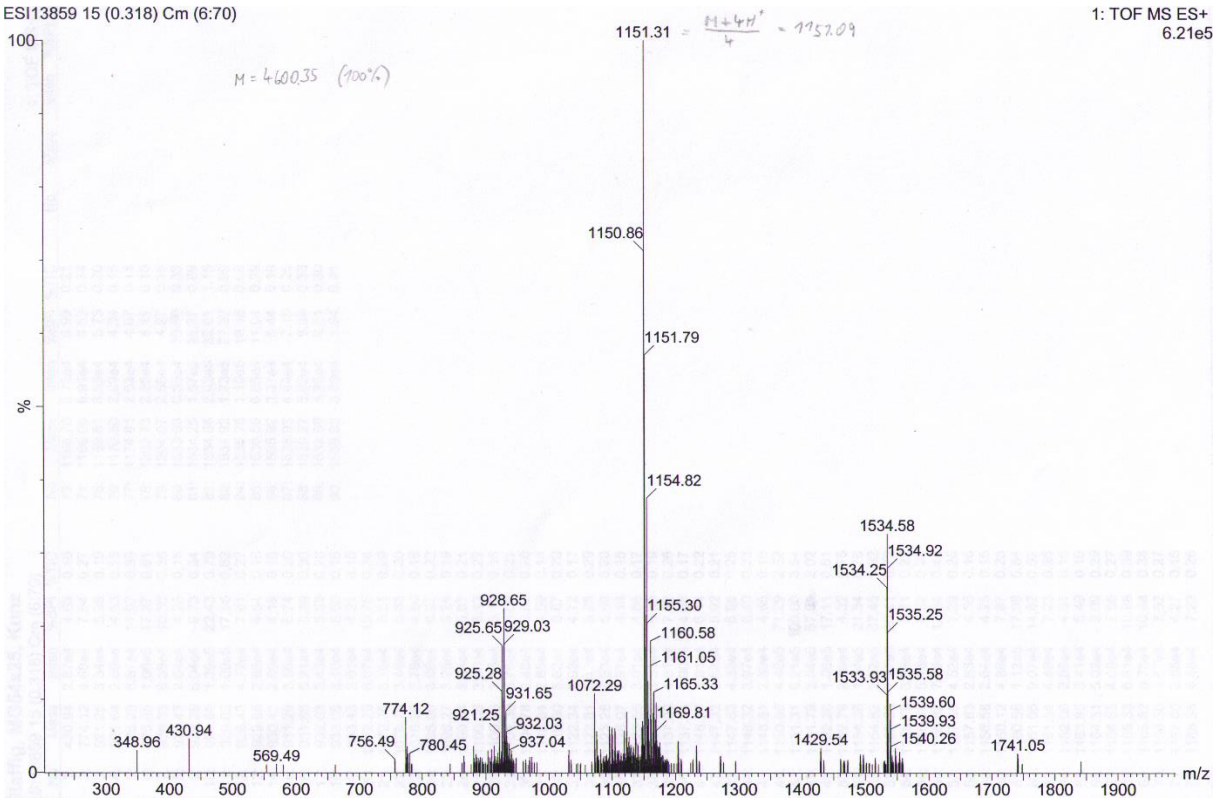
6. Characterisation Data

6.1 MUC1-P2 glycopeptide **3**

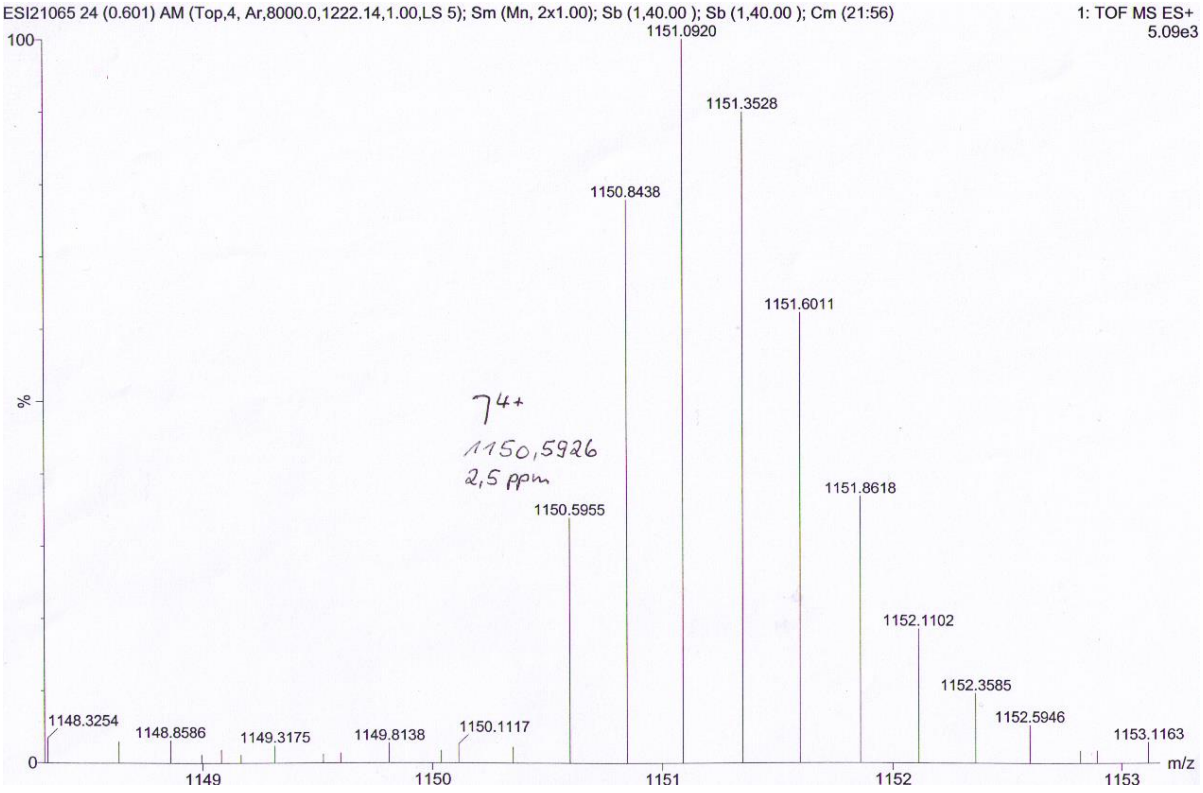
a) HPLC elugram: Rt = 25.2 min (H2O + 0.01 % TFA, MeCN + 0.01 % TFA, 95:0 (0 min), 60:40 (30 min), 0:100 (40 min)).



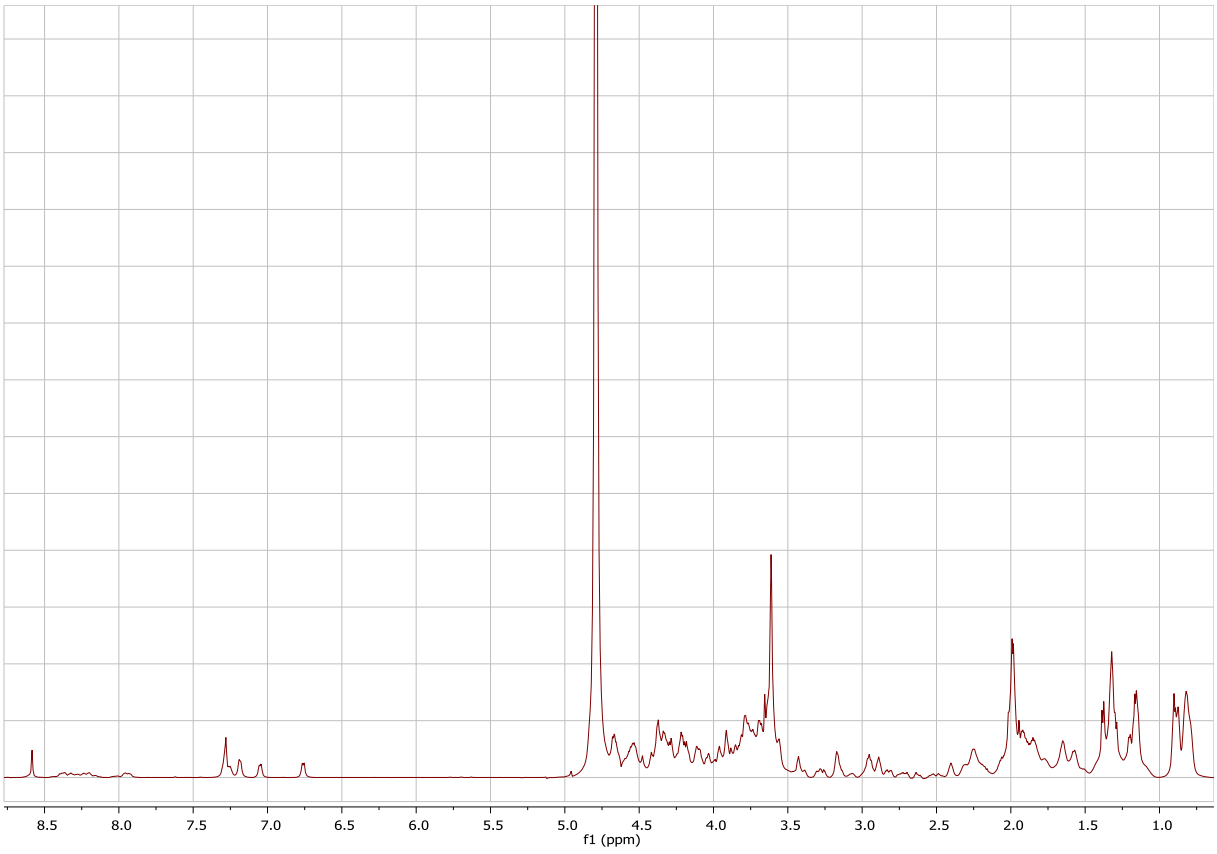
b) ESI-MS (pos.)



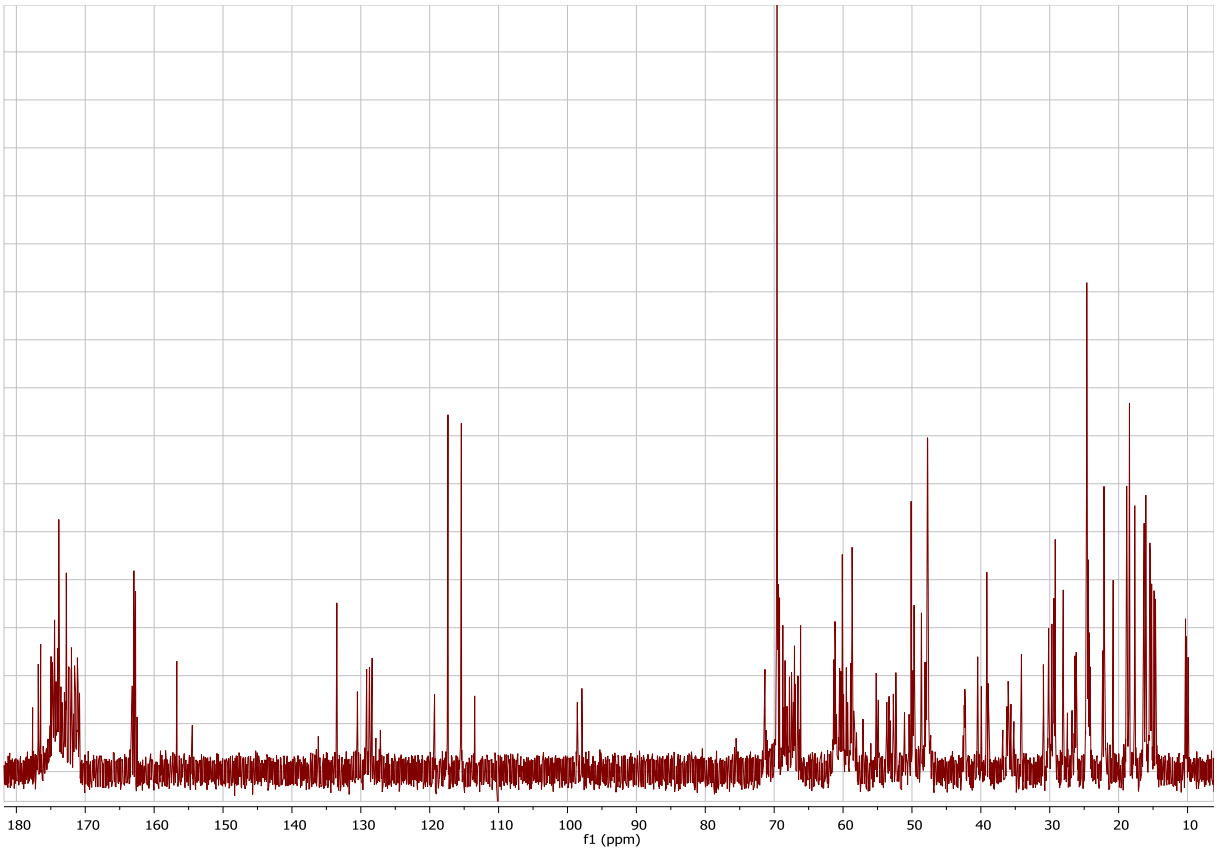
c) HR-ESI-MS



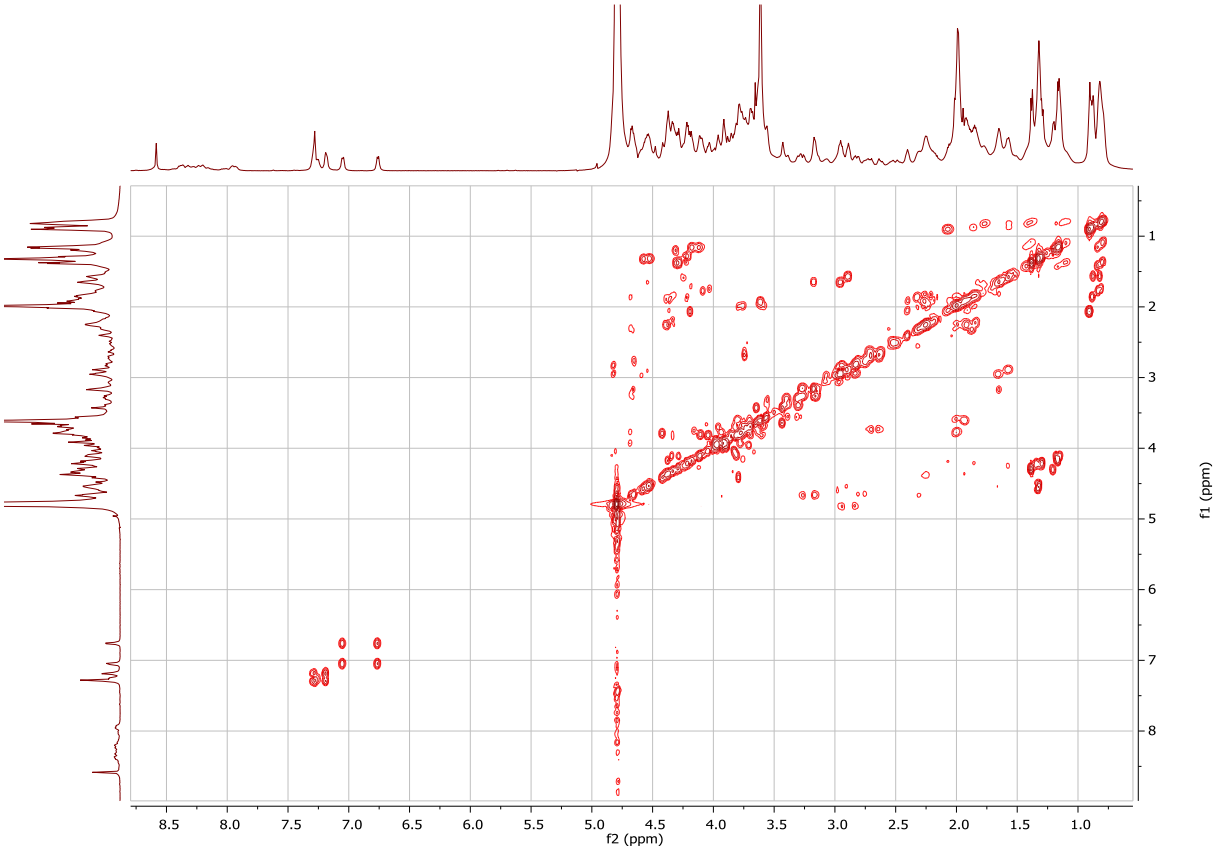
d) ^{1}H NMR (D_2O, 600 MHz)



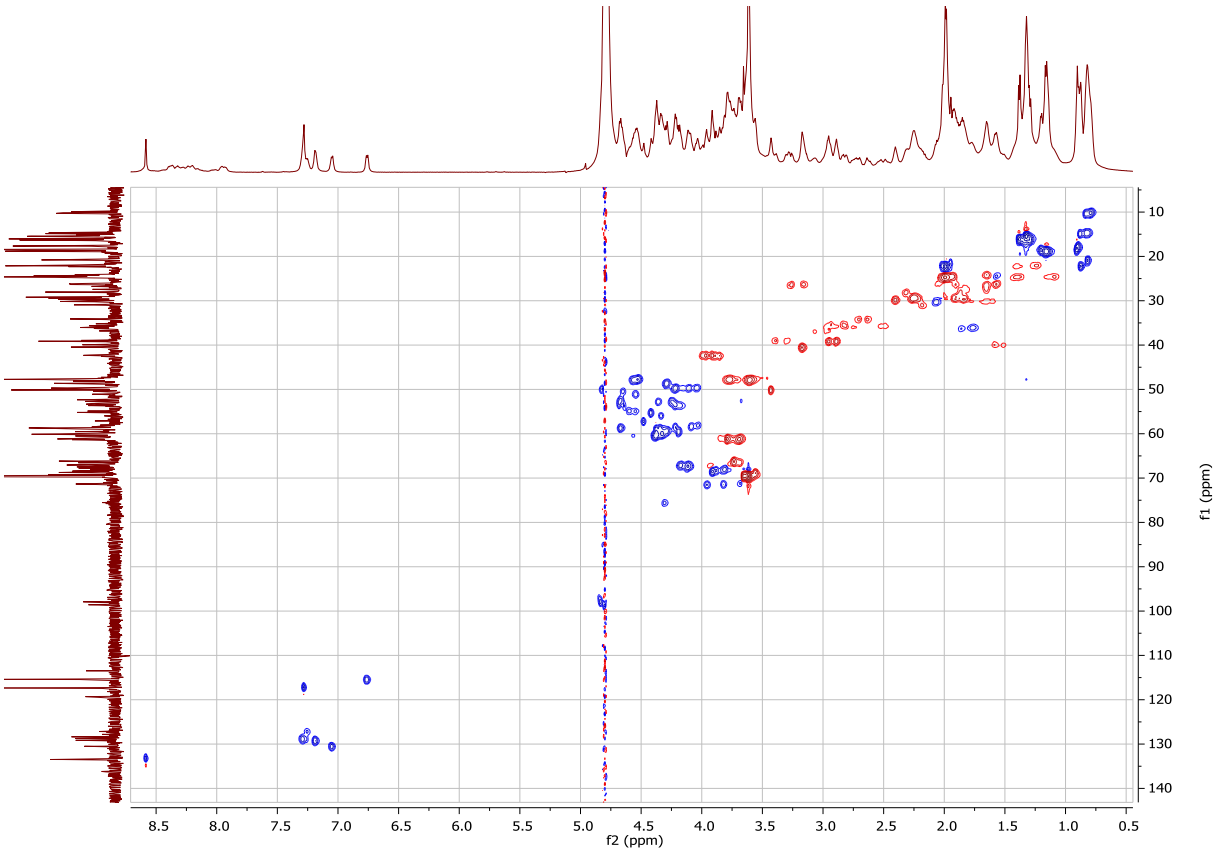
e) ^{13}C NMR (D_2O, 151 MHz)



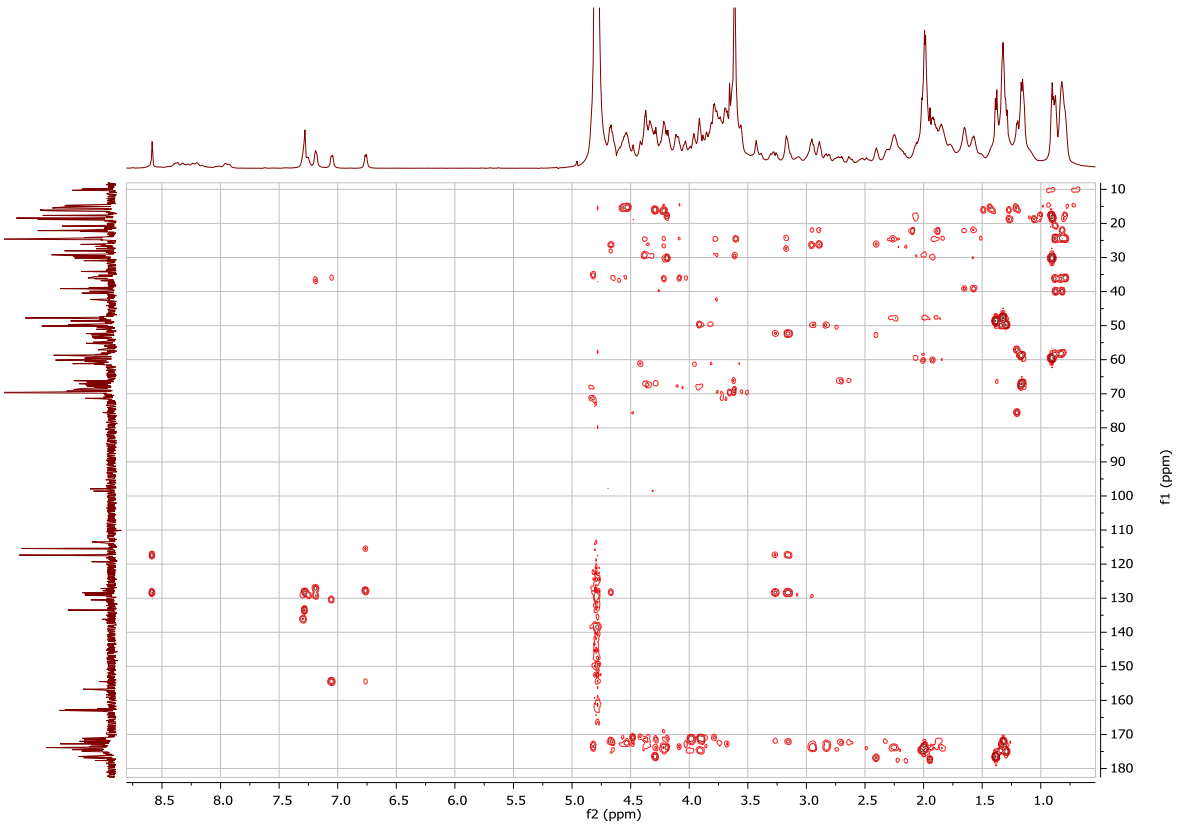
f) COSY (D_2O)



g) HSQC (D_2O)

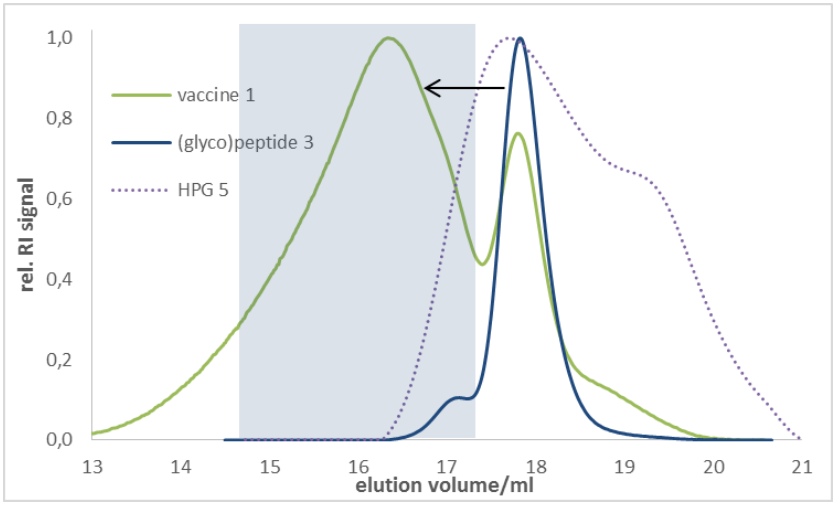


h) HMBC (D_2O)



4.2 MUC1-P2-hyperbranched polyglycerol vaccine **1**

SEC elugram, hexafluoroisopropanol as eluent



i B. Palitzsch, S. Hartmann, N. Stergiou, M. Glaffig, E. Schmitt and H. Kunz, *Angew. Chem. Int. Ed.*, 2014, **53**, 14245.

ii C. Schüll, T. Gieshoff and H. Frey, *Polym. Chem.*, 2013, **4**(17), 4730.

iii S. Keil, C. Claus,W. Dippold and H. Kunz, *Angew. Chem. Int. Ed.,* 2001, **40**, 366; L. S. Wong, S. J. Janusz, S. Sun, G. J. Leggett, J. Micklefield, *Chem. Eur. J.* 2010, **16**, 12234-12243.

iv M. Glaffig, B. Palitzsch, S. Hartmann, C. Schüll, L. Nuhn, H. Frey and H. Kunz, *Chem. Eur. J.*, 2014, **20**, 4232.