Electronic Supplementary Material (ESI) for ChemComm.


# *Ready Display of Antigenic Peptides in a Protein 'Mimogen'*

## Supporting Information

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**General**

**Chemicals:** All reagents, starting materials and solvents were purchased from commercial suppliers and used without further purification if not further mentioned.

**Methodes:** Proteins were purified using ÄKTA FPLC system UPC-900 (GE Healthcare) at 6 °C and elution was monitored by UV absorption. All buffers used were made with MQ $H_2O$ and sterile filtered (0.22 µm). LB-agar plates and LB media (Fisher Scientific) was used for protein expression experiments. Stocks with transformed cells were kept in glycerol at -80 °C. Kanamycin (Fisher) and IPTG (Sigma-Aldrich) were prepared in $H_2O$-stocks according to the manufacturers and stored at -20 °C. For biological procedures media and plastic equipment were sterilised by autoclaving (121 °C, 20 minutes) in a LTE Scientific Falcon tabletop autoclave. Protein liquid chromatography-mass spectrometry (LC-MS) was performed on a Waters LCT Classic (ESI-TOF-MS) coupled to a Shimadzu 20 Series HPLC using a Merck Cromolith C18 column (50 x 2 mm). Water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid, were used as the mobile phase at a flow rate of 0.4 ml/min. The gradient was programmed to: 95% A (2 min isocratic) to 95% B after 8 min then isocratic for 3 min. The electrospray source parameters were: capillary voltage 3 kV and cone voltage 250 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 l/hr. Spectra were calibrated automatically. Total mass spectra were reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx software (v. 4.1 from Waters) according to manufacturer's instructions.

**Transformation**

The pET-28c vector containing the gen for Np276 G2F M61C was transformed into *Escherichia. Coli* (*E. coli*) BL21 (DE3) cells (Novagen) using heat shock. Therefore, 10 µL BL21 (DE3) cells were mixed with 2 µL plasmid at 0 °C. After 25 minutes the cells were heated to 42 °C for 30 seconds and immediately cooled to 0 °C again. After four minutes 80 µL SOC media was added, the cells were plated on previously prepared agar/kanamycin (50 µg/mL) petri dishes and incubated overnight at 37 °C.

**Pre-culture and Inoculation**

The *E. coli* BL21 (DE3) cells containing the desired plasmid were grown in 10 mL of freshly prepared and autoclaved Luria-Bertani (LB) medium (BD 244620, Difco) containing a final concentration of 50 µg/mL of kanamycin. The cells were incubated overnight at 37 °C. The cells were next transferred into 800 mL of fresh LB broth (containing a final concentration of 50 µg/mL of kanamycin) and were incubated at 37 °C with vigorous shaking until reaching an OD600 of 0.6 - 0.8 (typically 3 - 4 h).

**Induction and Cell Harvesting**

To the cells was added 800 µL of a previously prepared 1M IPTG (isopropyl-β-Dthiogalactoside) stock solution. The cells were stirred at 16 °C for 13 h or at 30 °C for 6 h after which they were harvested *via* centrifugation (8000 rpm, 10 min, 4 °C), resuspended in LB media (10 mL) and harvested again

*via* centrifugation (8000 rpm, 10 min). The cell pellet was stored at -20 °C awaiting lysis and purification.

**Mini-Preparation**

Mini-preparation was performed from a 10 mL pre-culture using a QIAGEN mini-prep Kit following the manufacturer's specifications and stored at -80 °C.

**Cell Lysis**

The *E. coli* cell pellets containing overexpressed Np276 G2F M61C protein were suspended in 25 mL loading buffer (40 mM imidazole, 0.5 M NaCl, 20 mM Tris, 2 mM DTT, 5% glycerol, pH = 7.8), disrupted upon addition of DNase I (25 mg), egg white lysozyme (0.25 mg) and protease inhibitor cocktail tablet (half a tablet, Complete EDTA free, Roche) under gently shaking for 1 h at 0 °C followed by pulse sonication. Sonication was carried out at 0 °C for a total of 5 cycles (60%, 30 sec. on, 60 sec. off, 0.5 sec. pulses). The lysate was transferred to a cooled centrifuge tube and the cell debris was removed via centrifugation (20000 rpm, 45 min, 4 °C).

**Protein Purification**

The supernatant from cell-lysis containing Np276 G2F M61C was first purified by Ni-NTA affinity column chromatography using an ÄKTA system equipped with a freshly loaded HisTrap fast flow column (5 mL, GE Healthcare Life Sciences) with a flow of 3 mL/min and the following step gradient: 0% B 20 CV, 5% B 2 CV, 7% B 2 CV, 10% B 2 CV, 20% B 2 CV, 100% B 10 CV. Loading buffer A: 40 mM imidazole, 0.5 M NaCl, 20 mM Tris, 2 mM DTT, 5% glycerol, pH = 7.8. Buffer B: 0.5 M imidazole, 0.5 M NaCl, 20 mM Tris, 2 mM DTT, 5% glycerol, pH = 7.8. The protein was eluted when switching to 100% B. The UV-active fractions were further analysed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis.

The fractions containing the desired protein were combined and concentrated into size 6 mL exclusion buffer (0.5 M NaCl, 20 mM Tris, 2 mM DTT, 5% glycerol, pH = 7.8) using a Vivaspin centrifugal filter (10k MWCO, Santorius AG). Size exclusion chromatography was performed using an ÄKTA system equipped with a Superdex 75 16/60 column and a flow rate of 1 mL/min. The UV-active fractions were further analysed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. The desired protein was concentrated to approximately 2.2 mg/mL using a Vivaspin centrifugal filter (3k MWCO, Santorius AG), aliquoted, flash frozen and stored at -80 °C until use.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

For analysis of the protein by SDS-PAGE, 10 µL of the desired sample was mixed with 3 µL 4x sample buffer (mixed with BME) and loaded onto the SDS-page (pre-casted Novex Bis-Tris gels (4-12 % or 12%, Invitrogen). As a size reference, 5 µL prestained protein ladder (Bioman cat # PREP1025) were added onto one lane. The elution was carried out with 170 V of continuous power for approximately 50 min. For staining, the plate was removed and the gel was incubated at room temperature for

15 minutes in a developing tray containing InstantBlue (Expedeon Ltd.) stain. Finally, the staining solution was decanted and the gel was washed with water prior to being scanned.

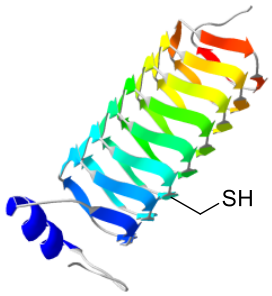
**DNA sequencing**

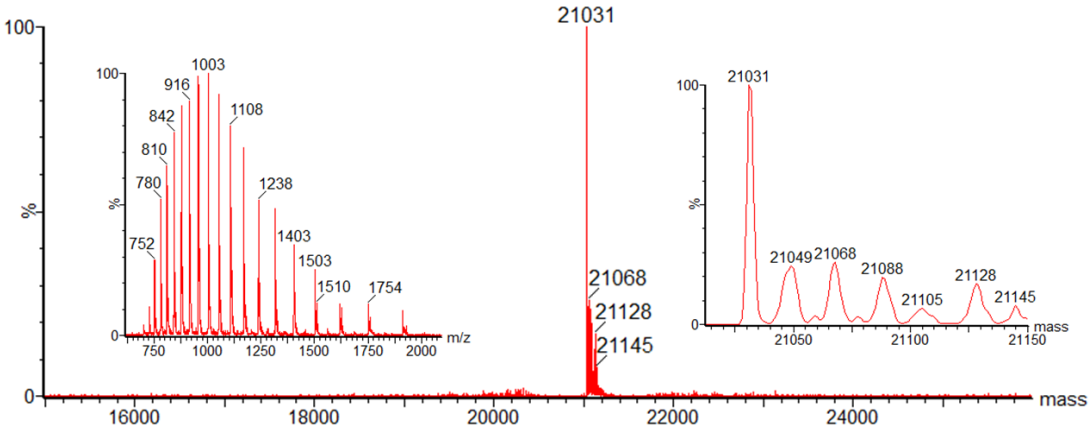
The DNA sequencing was performed by *Source BioScience LifeScience*. Therefore, 15 µL of Np276 G2F M61C pET-28 c (+) plasmid from Mini-Preparation were send to Source BioScience.

**Characterisation of Np276 G2F M61C (3)**

Expression level: 24.3 mg/L (16 °C 13 hours); 37.1 mg/L (30 °C 6 hours)

Calculated Molecular Weight (inclusive of N-terminal Met): 21031




Amino Acid sequence:

```
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        70         80         90        100        110        120
CLDEANLQQA NLSRADLSGA TLNGADLRGA NLSKADLSDA ILDNAILEGA ILDEAVLNQA

       130        140        150        160        170        180
NLKAANLEQA ILSHANIREA DLSEANLEAA DLSGADLAIA DLHQANLHQA ALERANLTGA

       190                                                    200
NLEDANLEGT ILEGGNNNLA T
```

T7 Forward Sequence:

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T7 Reverse Sequence:

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T7 Reverse and compliment sequence:

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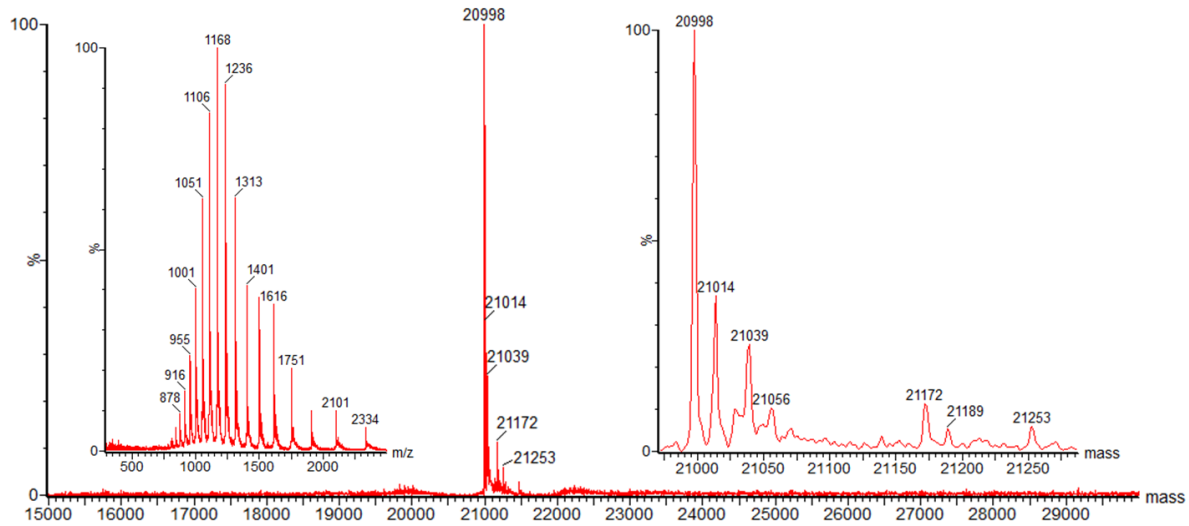
**Formation of Np276 G2F Dha61 (4)**

First the Np276 G2F M61C (**3**) was transferred to phosphate buffer (50 mM, pH = 8.0) using PD G25 columns with a final protein concentration of about 1.5 mg/mL. Then DTT (50 equiv., 0.5 M in water) was added and the solution was shaken for 20 minutes at room temperature. The reduced protein was purified by size exclusion chromatography using PD G25 columns. The protein concentration was checked by NanoDrop before 2,6-dibromoheptanediamide (500 equiv., 0.5 M in DMF) was added. The reaction was shaken for 2 hours at 37 °C and purified by size exclusion chromatography using PD G25 columns. The concentration of Np276 G2F Dha61 was determined by NanoDrop and increased using *Vivaspin* sample concentrators if required. The purified Np276 G2F Dha61 (**4**) was shock frozen and stored at – 80 °C until further modification.

Calculated Molecular Weight: 20997



**Peptide Synthesis: 1 and 2**

Solid-phase peptide synthesis was performed either on a Liberty automated peptide synthesiser (CEM Corporation) or a Liberty Blue automated peptide synthesiser (CEM Corporation), using HBTU and DIPEA for the acid activation and 20% piperidine in DMF for the Fmoc deprotection, or were coupled manually using HATU and DIPEA as activator.

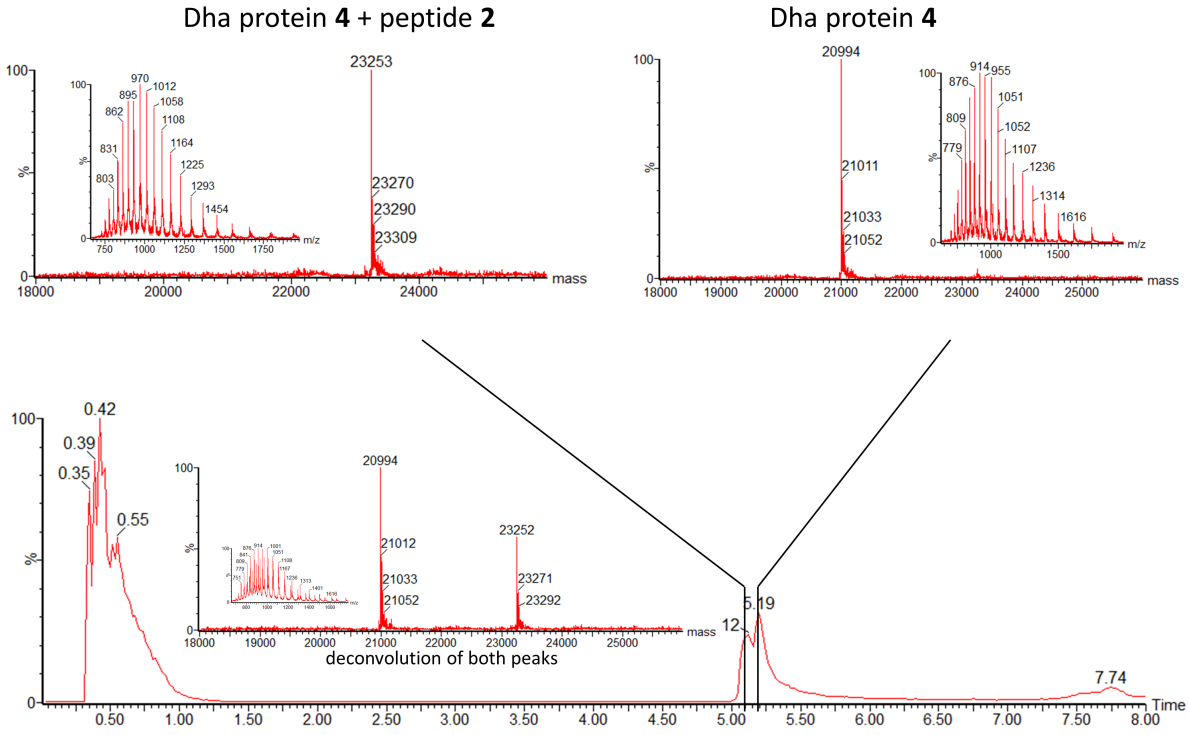
Mass spectrometry analysis

Peptide **1**: LRMS for $C_{93}H_{165}N_{31}O_{27}S^{2+}$ $[M+2H]^{2+}$ calcd.: 1090.1, found: .1190.4

Peptide **2**: LRMS for $C_{93}H_{166}N_{31}O_{30}PS^{2+}$ $[M+2H]^{2+}$ calcd.: 1130.1, found: .1130.4

**Conjugation of the antigenic peptides onto Np276 G2F 61Dha (4)**

Np276 G2F Dha61 (**4**) was first transferred to the desired reaction buffer using PD G25 columns. The protein concentration was determined by standard BCA protein assay and adjusted to about 1.1 - 1.3 mg/mL. The peptide (500 equiv., 0.2 M in water) was added to the protein solution and the reaction was shaken at 22 °C or 30 °C for 1.5 or 5 hours. The crude reaction mixture was purified by dialysis using *Slide-A-Lyzer MINI Dialysis Devices* (10K MWCO, Thermo Scientific) for 1.5 to 12 hours and analyzed by liquid chromatography mass spectrometry. Circular dichroism analysis confirmed the apparent stability of the platform under reaction conditions. Conversion could be monitored by LC-MS and where appropriate LC used to readily separate and recycle peptide and platform. Example reaction and separation for recycling:



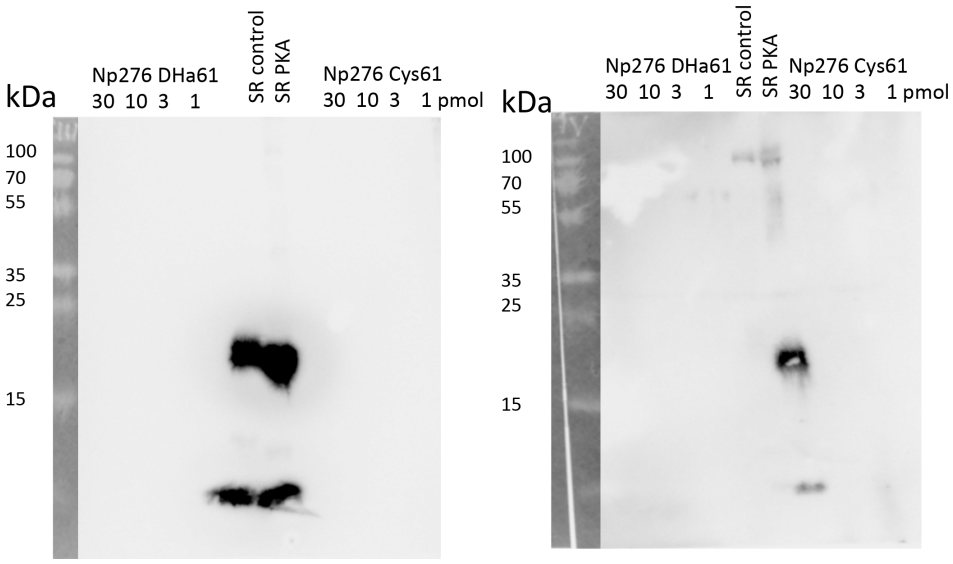
**Western Blot Analysis**

Prior to western blotting, samples were suspended in Laemmli sample buffer (0.125 M Tris pH 6.8, 10% glycerol, 0.04% bromophenol blue, 2% SDS, 5% β-mercaptoethanol). Samples and the molecular weight ladder (Thermo Scientific PageRuler™ Plus) were run down a 12% polyacrylamide gel (10.2 ml $ddH_2O$, 12 ml acrylamide 30%, 7.5ml 1.5 M Tris pH 6.8, 150 µl APS 10%, 150 µl SDS 20%, 20 µl TEMED, 30 ml total volume) with 4% stacking buffer (4.075 ml $ddH_2O$, 0.67 ml acrylamide 30%, 1.25 ml 0.5 M Tris pH 6.8, 25 µl APS 10%, 25 µl SDS 20%, 5 µl TEMED, 6 ml total volume) at 150V for 70 minutes in running buffer (25 mM Tris pH 6.8, 192 mM glycine, 0.1% SDS) at 4˚C. Samples were then transferred to a PVDF membrane using transfer buffer (25 mM Tris pH 6.8, 192 mM glycine, 10% methanol) for 2 hours at 4˚C, 150mA constant current. The membrane was then washed in blocking buffer (TBS pH 7.4, 0.1% Tween-20, 5% milk protein) for one hour, to block non-specific binding sites. Incubation with the primary antibody (A010-14, 1:5000 dilution factor) was for 16 hours at 4˚C in blocking buffer. Membranes were washed 5 times in wash buffer (TBS pH 7.5, 0.1% Tween 20) before being incubated with an HRP-conjugated secondary antibody (Goat anti-mouse, 1:5000 dilution factor) for 90 minutes at room temperature. Membranes were again washed 5 times in wash buffer, before being exposed to ECL reagent (Thermo Scientific Pierce™ ECL Western Blotting Substrate) and imaged on a CCD camera.

Membranes were stripped (for re-staining with antibody to Ser16-phosphorylated PLN, Badrilla Ltd, Leeds UK, catalogue No. A010-12) with pre-warmed stripping buffer (100mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCL pH 6.8) for 30 minutes at 60˚C, before being washed 5 times in wash buffer, and incubated with blocking buffer for 1 hour. Membranes were then stained with the A010-12 primary antibody in a 1:5000 dilution factor, followed by a goat anti-rabbit secondary antibody in a 1:5000 dilution factor.

**Sample contents:** Lane 0; molecular weight ladder (20 µl). Lane 1; $DBK_1$ 5 µl at 0.18 pmol/µl. Lane 2; Canine Sarcoplasmic Reticulum (CSR) 5 µl at 0.4 µg/ml. Lane 3: CSR + Protein Kinase A (PKA) 5 µl at 0.4 µg/ml. Lane 4: Peptide **5 (**unphosphorylated mimogen) 40µl at 0.07 pmol/µl. Lane 5: Peptide **6** (phosphorylated mimogen) 40 µl at 0.03 pmol/µl.

**Control blotting of scaffold:** Mimogen scaffold was not recognised by mAb to PLN (below left) or pAb to pPLN (Ser-16, below right). Np276-Dha61 and Np276-Cys61 (1-30pmol), and canine cardiac SR vesicle proteins (2 mg, containing ~6pmol PLN) were used; unphosphorylated (SR control) and PKA phosphorylated states (SR PKA) were separated by SDS-PAGE (13%) and transferred to PVDF membrane. PLN was detected by mAb A1 (1:5000 dilution); antibody failed to detect scaffold but detected both pentameric (~20kDa) and monomeric (~7kDa) forms of PLN (below left) Ser-16 phosphorylated PLN was detected by pAb PS-16 (1:5000 dilution); antibody failed to detect platform but detected both pentameric (~20kDa) and monomeric (~7kDa) forms of PLN (below right).


kDa
Np276 DHa61
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SR control
SR PKA
Np276 Cys61
30 10 3 1 pmol
100
70
55
35
25
15
kDa
Np276 DHa61
30 10 3 1
SR control
SR PKA
Np276 Cys61
30 10 3 1 pmol
100
70
55
35
25
15

**Mass spectra of conjugation of antigenic peptides 1 and 2 onto Np276 G2F 61Dha (4)**

MW (Np276 G2F Dha61): 20997

MW (protein conjugated with unphosphorylated peptide **1**): 23175

MW (protein conjugated with phosphorylated peptide **2**): 23255

*Minor impurities as well as oxidation of the N-terminal methionine present in all spectra derive from the protein expression (see characterisation of Np276 G2F M61C). There ration is consisted over all experiments and to not belong to any side reactions.

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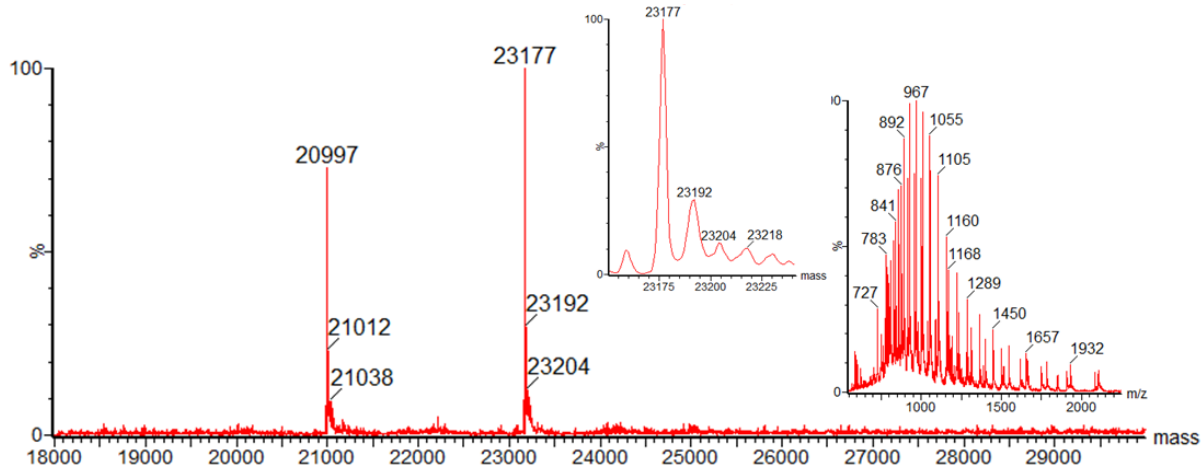


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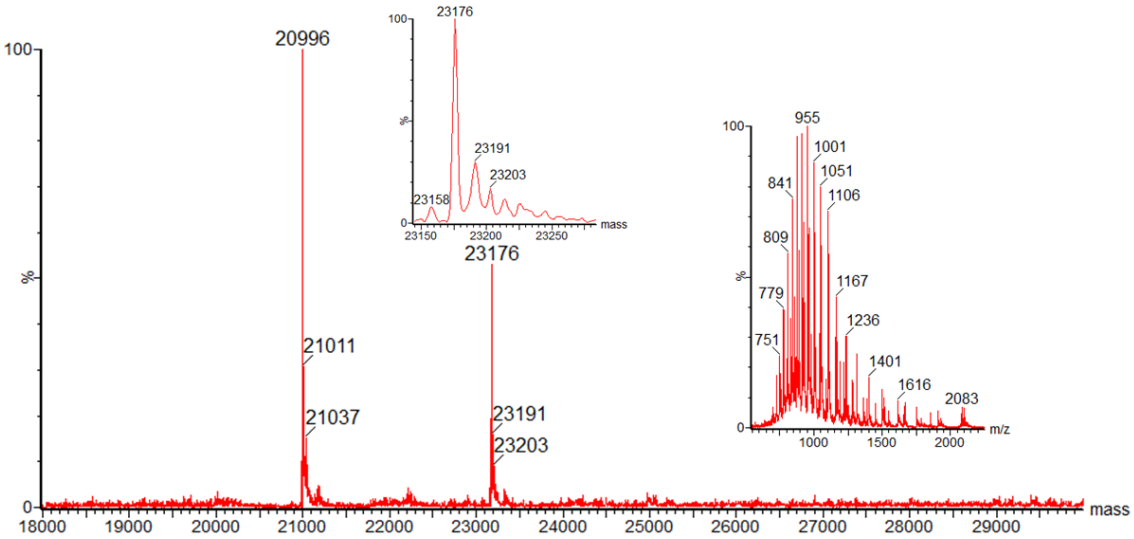

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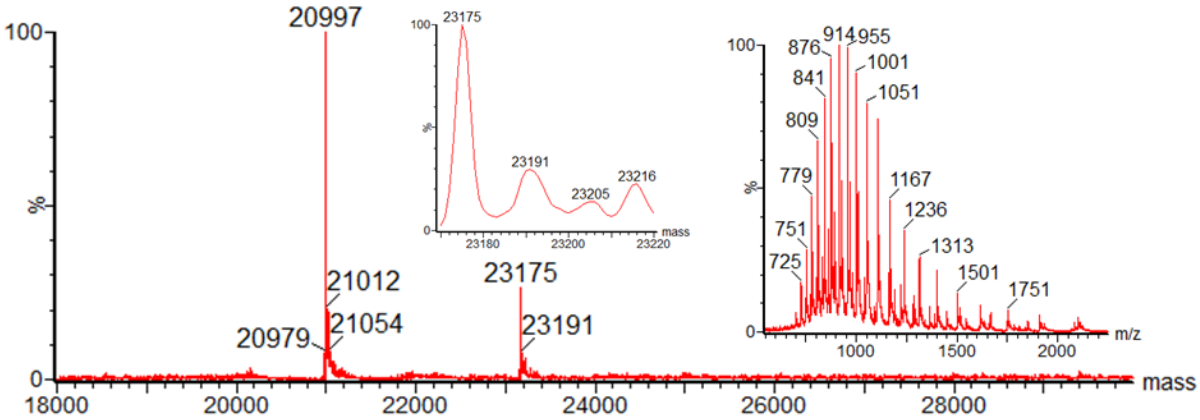


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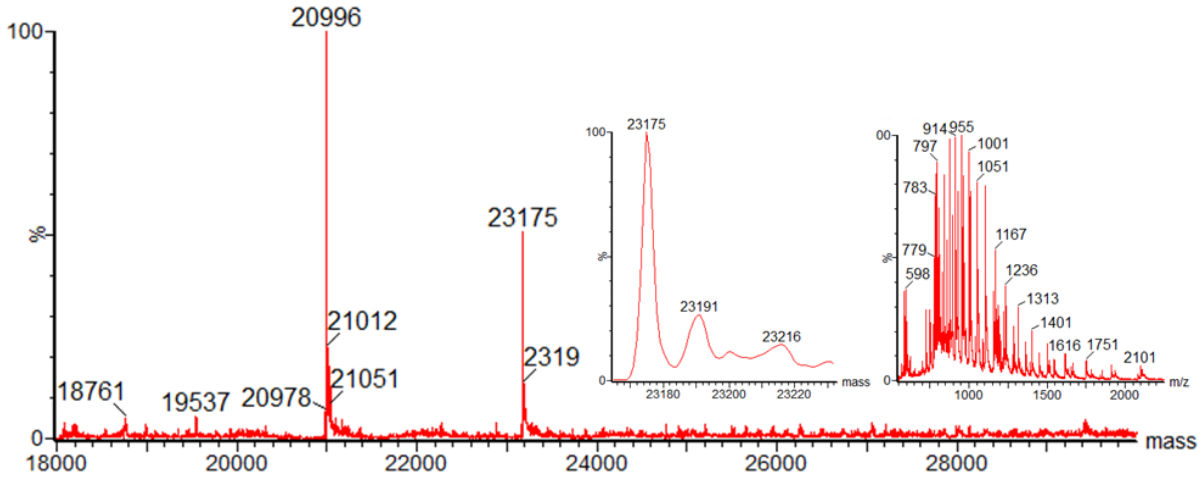


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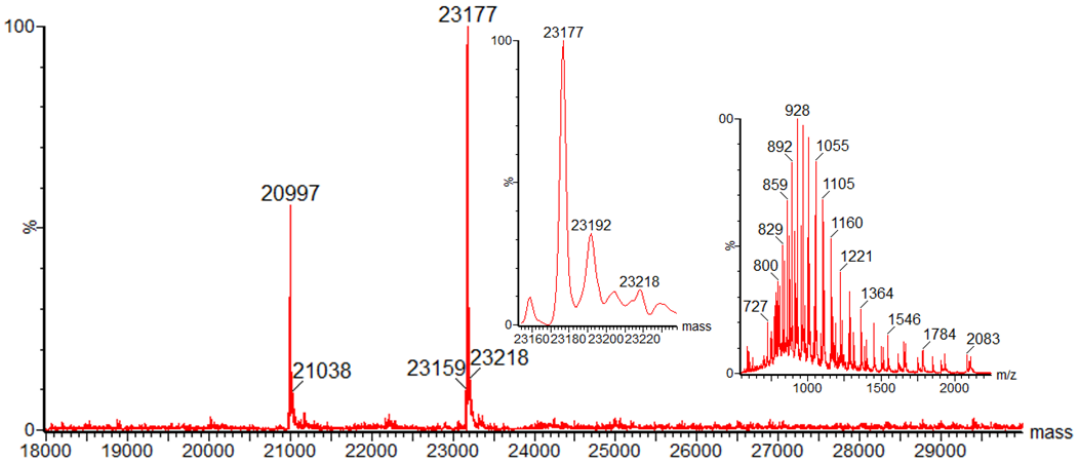

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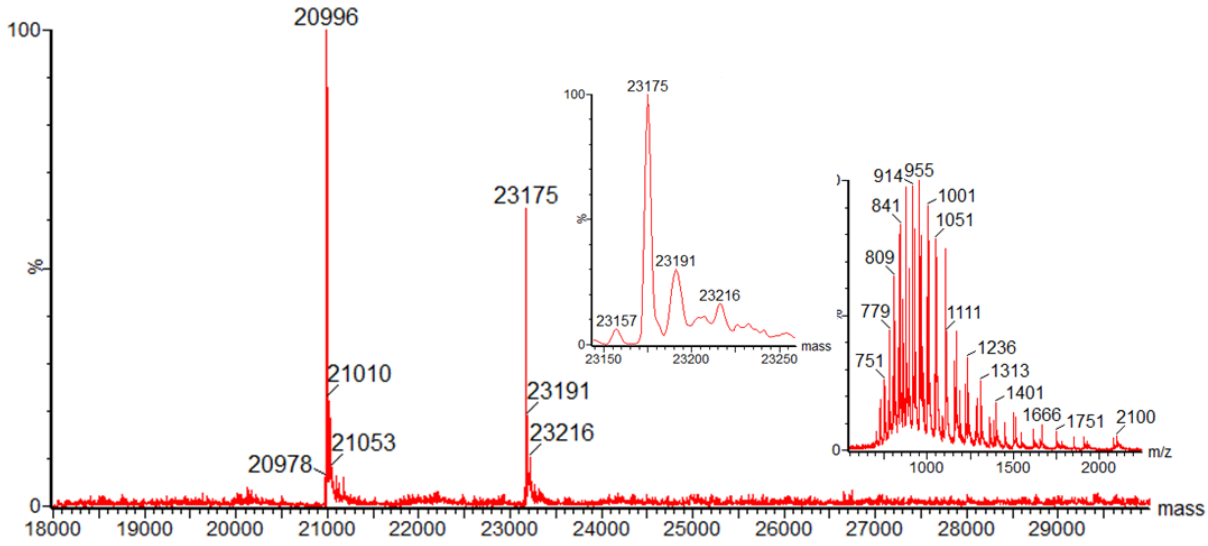


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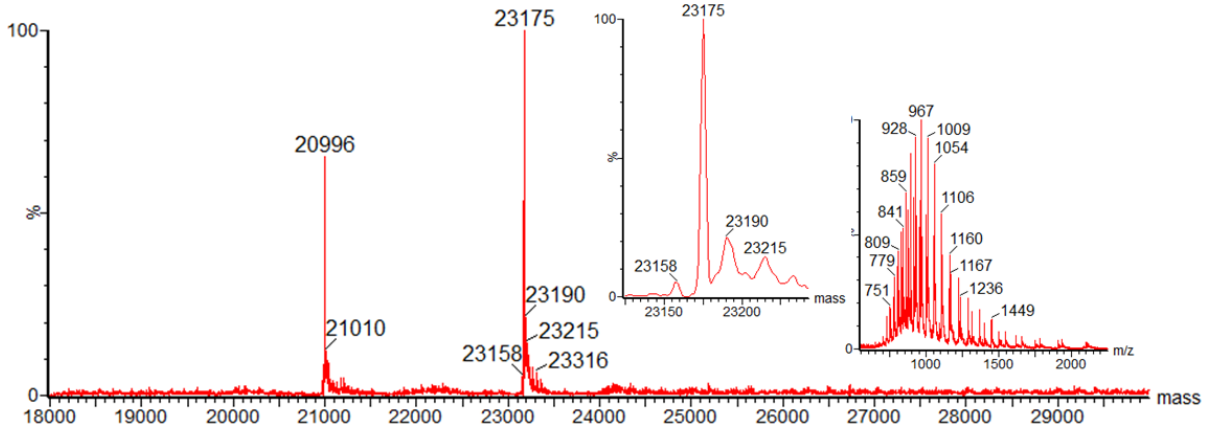


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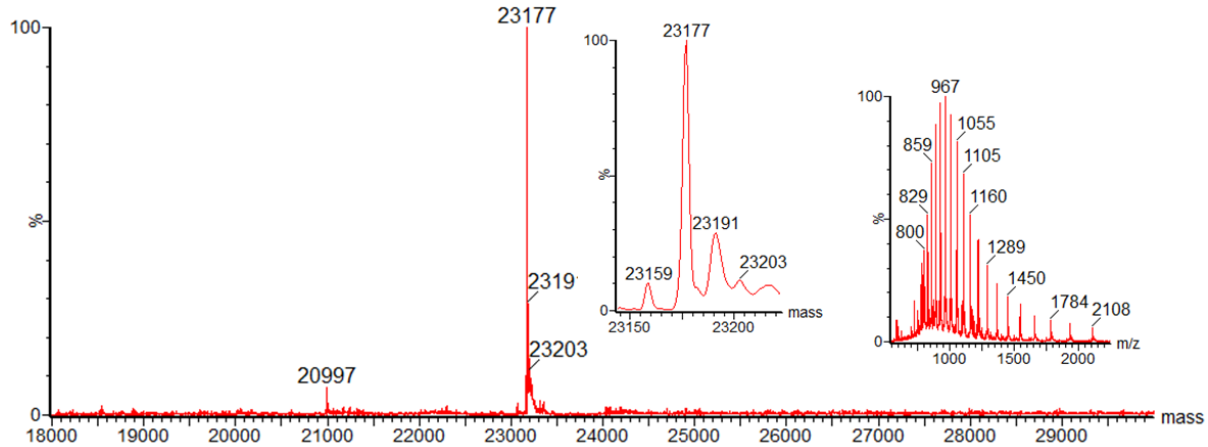

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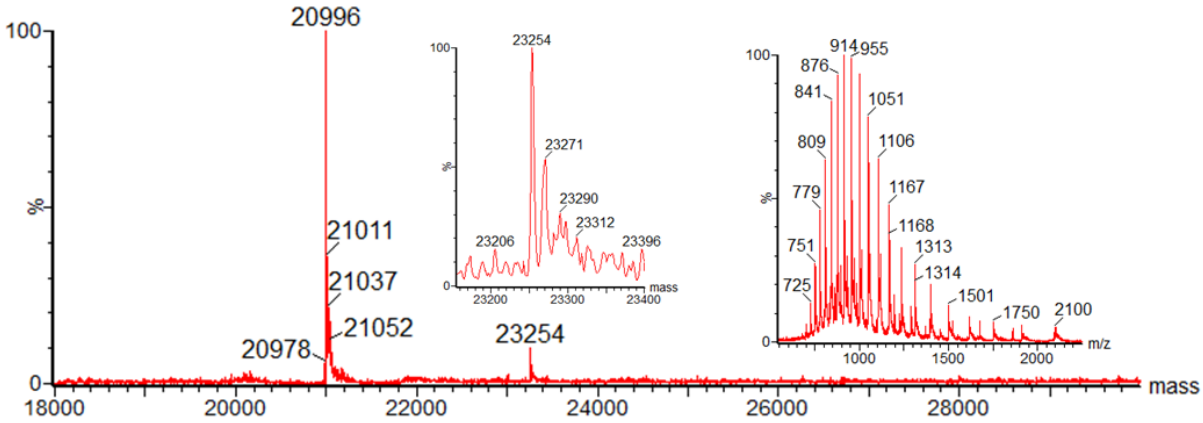


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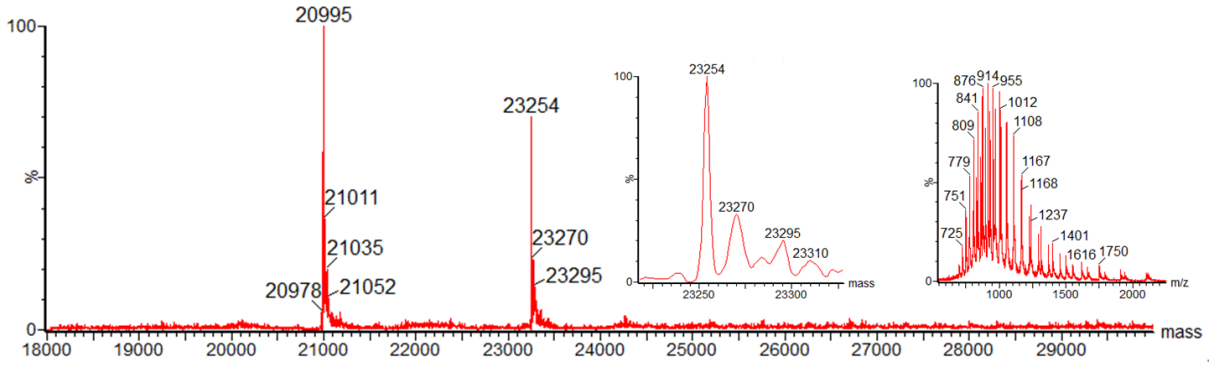
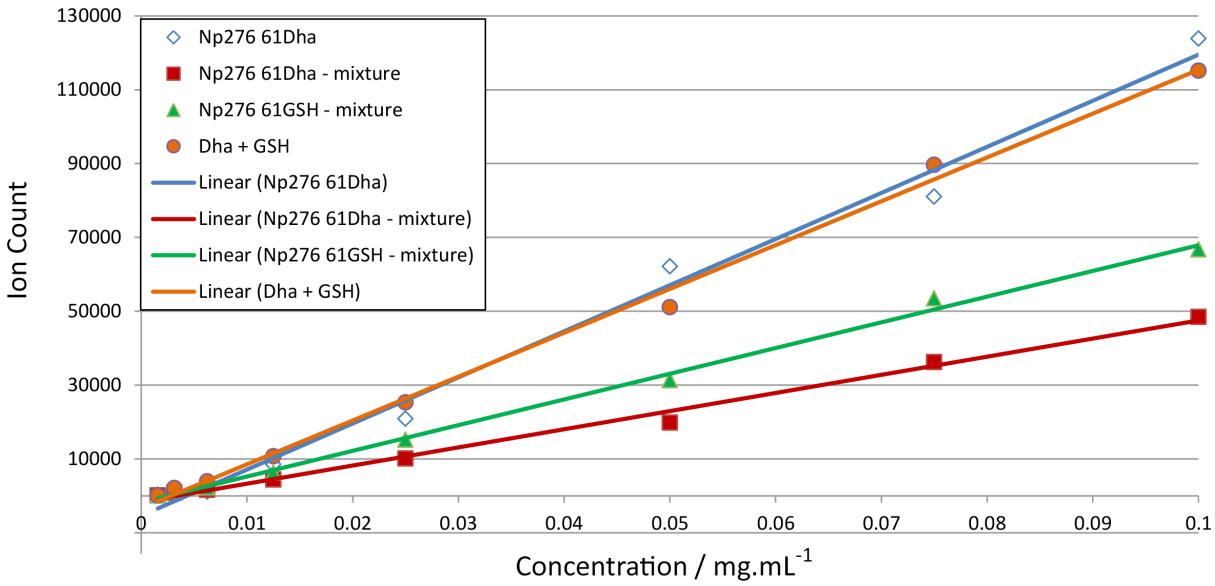



**Calibration of MS as a Measure of Conversion Yield**

To test the linear correlation of MS ion count with concentration as determined by ESI+ LC-MS we determined TIC at a range of concentrations for both platform Npβ-G2F-Dha61 (blue alone, red in presence of conjugate), conjugate displaying model peptide (here GSH at conjugation ratio of 41:59, green) at a range of different concentrations (0.1 mg/mL – 0.0015 mg/mL). All showed excellent linear correlation. In addition summation of ion counts gave exactly the same values for conjugate (orange overlay) as those determined for platform alone. In addition, the concentration of platform (red) and conjugate (green), calculated from this graph, are in perfect agreement with the MS data 41/59 for reaction conversion.




As well as this demonstration of good correlation between ion count and concentration in several systems (see for example quantitative on-protein chemistry kinetic determined by MS[1]), other detailed studies have shown that the ionization of proteins is dependent on the total surface composition, which is typically invariant even with large changes (even such as the attachment of many large glycans). Therefore, and in contrast to small molecule MS, relative intensities detected and assigned by MS for intact proteins (as used here) can therefore be taken as a generally good proxy for the relative abundances, in line with the well-known minor effects of post-translational modification on intact-protein analysis.[2]

**Unprocessed Western Blot Scans to Support Fig 3a**

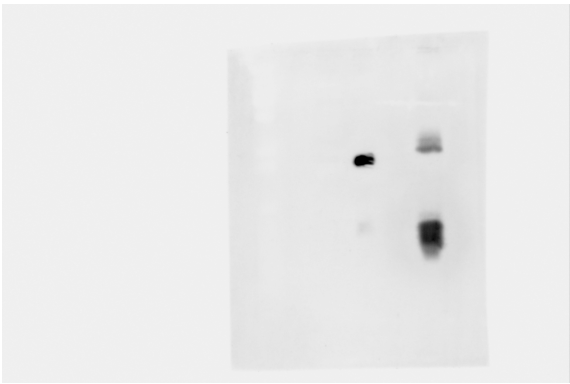
10 seconds exposure

20 seconds exposure

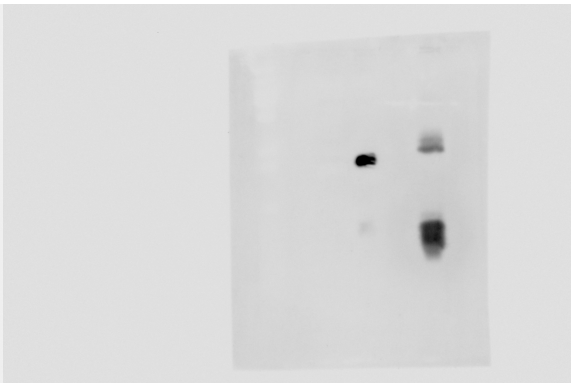
30 seconds exposure

60 seconds exposure

**Unprocessed Western Blot Scans to Support Fig 3b**



30 seconds exposure



60 seconds exposure

**References**

[1] Y.A. Lin, O. Boutureira, L. Lercher, B. Bhushan, R.S. Paton, B.G. Davis, *J. Am. Chem. Soc.* **2013**, *135*, 12156–12159.

[2] J.J. Pesavento, C.A. Mizzen, N.L. Kelleher, *Anal. Chem.* **2006**, *78*, 4271–4280.