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Investigating the candidacy of LPS-based glycoconjugates to prevent invasive meningococcal disease: chemical strategies to prepare glycoconjugates with good carbohydrate loading

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Abstract In previous studies protective antibodies that could facilitate bactericidal killing of *Neisseria meningitidis* (*Nm*) serogroup B strains were derived from immunisation with glycoconjugates prepared from *O*-deacylated lipopolysaccharide (LPS-OH) *via* direct reductive amination between the reducing end of the oligosaccharide molecule, created by treatment with alkaline phosphatase, and amino functionalities on the CRM_{197} carrier protein. These glycoconjugates proved difficult to prepare because the presence of amide linked fatty-acyl groups results in glycolipids that are relatively insoluble and aggregate. Therefore, we have examined several strategies to prepare glycoconjugates in order to identify a robust, consistently reproducible strategy that produces glycoconjugates with a high loading of LPS derived oligosaccharides. Initially we used completely deacylated LPS molecules, but lacking phosphoethanolamine (PEtn) from the core OS as the strong basic conditions required to completely deacylate the LPS would modify the PEtn residue. We utilised a squarate linker and conjugated *via* the reducing end of the carbohydrate antigen following removal of the glycosidic phosphate to amino groups on CRM_{197}, however carbohydrate loading on the carrier protein was low. Glycoconjugates were then produced utilising amidases produced by *Dictyostelium discoideum* (*Dd*), which partially remove N-linked fatty acids from the lipid A region of the *Nm* LPS molecule, which enabled the retention of the PEtn residue. LPS-OH was treated with *Dd* amidase, the reducing glycosidic phosphate removed, and using a cystamine linker strategy, conjugated to the carrier protein. Carbohydrate loading was somewhat improved but still not high. Finally, we have developed a novel conjugation strategy that targets the amino functionality created by the amidase activity as the attachment point. The amino functionality on the PEtn residue of the inner core was protected *via* a novel blocking and unblocking strategy with t-butyl oxycarbonyl. A maleimide-thiol linker strategy, targeting lysine residues on the carrier protein did not result in high loading of the carbohydrate molecules, however when we targeted the carboxyl residues we have consistently obtained a high loading of carbohydrate antigens per CRM_{197}, which can be controlled by variation in the amount of activated carbohydrate utilised in the conjugation reaction.

Keywords *Neisseria meningitidis* · Conjugate vaccine · LPS

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Introduction

There is currently no effective vaccine, appropriate for routine immunisation programmes, to protect infants against *Neisseria meningitidis* serogroup B (*Nm*B) disease. Due to the similarity of *Nm*B capsule to human antigens a glycoconjugate of *Nm*B capsular polysaccharide is unlikely to be used due to potential problems with autoimmunity and safety and moreover has been found to be poorly

immunogenic. Current vaccine strategies are based on targeting outer membrane components including: modified capsular polysaccharide [1, 2], outer membrane vesicles [3, 4], attenuated vaccines [5], common antigens identified in *Neisseria lactamica* [6] and outer membrane proteins identified from genomic and signature tagged mutagenesis approaches [7]. Some of these candidates are in clinical trials (*N. lactamica* OMV [8], PorA [9], Novartis genome derived vaccine [10]).

Strategies are being explored to use inner core lipopolysaccharide (LPS) that has been shown to be conserved in the majority of *Nm*B strains, accessible to antibodies and able to elicit functional antibodies (Abs) against *Nm*B strains [11–14]. The proof of principle that such a strategy could work has been demonstrated from functional studies with monoclonal antibody (mAb) L3B5 [12] and promising results with conjugates made from *O*-deacylated LPS (LPS-OH) derived from an immunotype L3 *galE* mutant [13]. These studies have demonstrated that it is possible to elicit functional, protective antibodies in mice against specific *Nm*B wild-type strains.

To date, our group and others have identified a limited number of inner core glycoforms elaborated by *Nm* LPS to represent 98% of *Nm*B disease-causing strains [15]. Our approach is to show proof *in* principle with one of these glycoforms, although the final vaccine would be expected to consist of a multivalent cocktail of a few LPS glycoforms conjugated to a carrier protein.

The original chemical methodology developed with LPS-OH had a number of problems including aggregation and solubility, which reflect the amphiphilic nature of the LPS-OH molecule and precluded the reproducible preparation of glycoconjugates with high carbohydrate loading. We have therefore examined several alternative conjugation strategies in order to develop a robust reproducible strategy that consistently results in glycoconjugates with a high loading of the carbohydrate antigen.

Materials and methods

Growth of bacteria and preparation of purified LPS

Media and growth conditions

Nm immunotype L3 MC58 strains *icsB / lpt3* (NRCC# 6172), *galE / lpt3* (NRCC# 6145) and *lgtB / lpt3* (NRCC# 6272), *icsB* (NRCC #), *galE* (NRCC # 4720), *lgtA* (NRCC # 6269) and *lgtB* (NRCC # 6270) where the gene indicated has been disrupted by the insertion of a kanamycin resistance cassette or in the case of *lpt3* mutants with an erythromycin cassette, were constructed in the laboratory of Prof. E.R. Moxon, Oxford University [16, 17]. The strains were all initially grown overnight on 50% Todd-Hewitt 50% Columbia (THC) agar plates (Difco) at 37°C and growths were used to inoculate 1 L of THC broth. Starter cultures were used to inoculate the 28 L fermenter (New Brunswick Scientific Microferm) with the same media, and grown as for starter cultures. The cultures were then grown at 37°C, with 24 $l min^{-1}$ aeration and stirring at 200 rpm for 18 h with 20% O_2 saturation. Cells were killed (2% phenol w/v, for 4 h) and harvested by using a Sharples continuous centrifuge.

Isolation and purification of lipopolysaccharide

Biomass following growth was frozen and lyophilised, and then washed with organic solvents (2 × acetone, 2 × ethanol, 2 × petroleum ether). The lipopolysaccharide (LPS) was isolated from 30 g of the washed cells by the hot water/phenol method [18]. The aqueous phase was dialysed against water and lyophilised. The dried sample was dissolved in water to give a 1–2% solution (w/v) and treated with deoxyribonuclease I (DNase) (0.01 mg/ml) and ribonuclease (RNase) (0.01 mg/ml) for 3 h at 37°C, then treated with proteinase K (0.01 mg/ml) for 3 h. The dialysed, dried sample was dissolved in water to make a 1% solution and ultra-centrifuged at 45 K following a low speed spin at 8 K to remove any insoluble material. The LPS pellet from the 45 K spin and the 8 K-pellet material were redissolved separately in water and lyophilised.

Purification of CRM_{197}

Initially, CRM_{197} (Novartis Vaccines) was purified on a Sepharose 6B column with PBS (50 mM sodium phosphate, 100 mM NaCl, pH 7.5) as eluent and was concentrated by a Centriplus or ultra-15 spin column 10 KDa cut-off (Amicon), to remove the bulking agent sucrose used for storage, and quantified by the bicinchoninic acid (BCA) assay [19]. Subsequently, it was found that the spin column alone could be used without prior column fractionation.

Preparation of completely deacylated conjugates

De-acylation

LPS from the *galE / lpt3* mutant strain was de-acylated by dissolving in 4N KOH (~10 mg/ml.) and stirring at 125°C for 30 h. The solution was cooled on ice and neutralised with acetic anhydride, which served to re-*N*-acetylate the amino groups created by this procedure. Precipitated salt was removed by centrifugation (9 k, 15 min.) and the supernatant was applied to a Sephadex G-25 column

(Amersham 17-0033-02, medium) and eluted with water as eluent. Carbohydrate-positive fractions were pooled and freeze-dried. The resulting material was de-*O*-acetylated (to remove non-specific *O*-acetylation produced during the re-*N*-acetylation step) by treatment (10 mg/ml) in 0.1M NaOH at 22°C for 2 h and purified on a Sephadex G-25 column in water, and lyophilised, to afford deacylated, *N*-acetyl LPS (KOH'd LPS; 110 mg) in ~15% yield. This was confirmed by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy and capillary-electrophoresis-electrospray-mass spectrometry (CE-ES-MS) analyses.

De-phosphorylation

The resulting KOH'd LPS material was de-phosphorylated by dissolving (~10 mg/ml.) in 0.1M NH_4HCO_3 buffer at pH 8.0 with shrimp alkaline phosphatase (Amersham Pharmacia) at 37°C for 24 h. The resulting material was de-salted on a Sephadex G-25 column in water, and lyophilised, to give KOH'd alk. P'd LPS in ~80% yield as indicated by ^{1}H-NMR and CE-ES-MS analysis.

KOH'd LPS material from *lgtB / lpt3* and *icsB / lpt3* LPS were treated as above with alkaline phosphatase (Sigma) (~200 units alk. P / mg KOH'd LPS) and stirred at 54°C for ~16 h. The solution was heated to 100°C for 5 min., cooled and centrifuged at 14 K for 10 min. The supernatant was freeze-dried.

Amination

The aldehydo functional group of the KOH'd alk. P'd LPS was aminated by dissolving the dried carbohydrate (8 mg/ml) in 10% DMSO, 2M NH_4OAc in MeOH and $NaCNBH_3$ was added to a concentration of 10 mg/ml. at 50°C for 72 h. The MeOH was evaporated under N_2 and the product lyophilised and purified on a Sephadex G-25 column in water. Following lyophilisation, the KOH'd alk. P'd aminated LPS was obtained in ~80% yield.

Attachment of linker molecule

3,4-Diethoxy-3-cyclobutene-1,2-dione (squarate) (Aldrich) was attached to the resulting amino group by dissolving the dried carbohydrate in 75% MeOH in water (4 mg/ml) and adding 10 ul/ml of squarate at pH >8 (adjust with triethylamine (~1–2 ul)) for 2 h at 22°C, monitoring the pH every15 min. The MeOH was evaporated under N_2 and the product lyophilised and purified on a Sephadex G-25 column in water, and lyophilised, to give KOH'd alk. P'd aminated squarate activated oligosaccharide in ~90% yield. CE-ES-MS analyses confirmed the attachment of the squarate linker.

Conjugation to protein carrier

CRM_{197} (~15–20 mg) was reacted with a 25 × molar excess of the squarate linked carbohydrate in 1 ml of 0.02 M sodium borate buffer by stirring at 22°C for 72 h at pH 9.2. Squarate activated carbohydrate was added at initiation of the reaction and for the *galE / lpt3* molecule half of the carbohydrate was added after 24 h. After 24, 48 and 72 h an aliquot was removed and examined by MALDI-MS and SDS-PAGE and Western blot with a carbohydrate specific monoclonal antibody (mAb LPT3-1 [15]). The final reaction mixture was purified on a Sepharose 6B column with PBS (50 mM sodium phosphate, 100 mM NaCl, pH 7.5) to achieve separation of free oligosaccharide from conjugated carbohydrate. The fractions corresponding to the product peak were concentrated by an ultra-15 spin column 10 K cut-off (Amicon). The final volume of conjugate was quantified for protein by the BCA assay [19] and for carbohydrate by the PhOH / H_2SO_4 method [20].

Preparation of conjugates utilising Dd amidase and alkaline phosphatase

De-O-*acylation*

Purified LPS from the *galE* mutant strain was treated with anhydrous hydrazine as described previously to prepare O-deacylated LPS (LPS-OH) [11]. The composition of the LPS-OH was confirmed by ES-MS analysis.

De-N-*acylation*

LPS-OH was the substrate for amidase enzymes produced by *Dictyostelium discoideum* (*Dd*) in order to prepare de-*N*-acylated, de-*O*-acylated LPS (LPS-ONH). Briefly, *Dd* was grown to logarithmic phase (3×10^{6} cells / ml) in liquid TM media (10 g trypticase, 5 g yeast extract, 10 g glucose in 1 L of 2×10^{-3} M potassium phosphate buffer, pH 6.5) at 22°C. The cells were harvested and washed twice with PBS and immediately suspended in PBS with phenol-killed and PBS washed cells of *Klebsiella aerogenes* (*Ka*) to continue their growth for 24 h at 22°C now utilising *Ka* as a food source with LPS-OH from *Nm galE* supplied as an additional substrate. The ratio of the number of bacterial cells and *Dd* cells that optimised *N*-deacylation of the purified LPS-OH substrate was standardised to 1×10^{11} *vs.* 5×10^{7} cells / ml respectively. The suspension was pelleted by centrifugation at 10,000 g and the supernatant was purified on a spin column (Amicon) with a 10 kDa cut off membrane. Two water washes were also collected. The flow through material, which contains the partially *N*-

deacylated LPS-OH (LPS-ONH) was then lyophilised, re-dissolved in water and applied to a Sephadex G-25 column and eluted with water. The resulting carbohydrate fractions were pooled and lyophilised. The resulting material was examined by CE-ES-MS and NMR in order to monitor the extent of de-*N*-acylation.

De-phosphorylation

LPS-ONH (10 mg/ml) was treated with recombinant alkaline phosphatase (Roche) for 30 h at 37°C at pH 8.0 in 0.1 N NH_4CO_3 buffer in order to remove the mono-phosphate esters. The mixture was boiled to denature the phosphatase and centrifuged (10,000 g, 15 min.) and the supernatant was applied to a Sephadex G-25 column and eluted with water. The resulting carbohydrate fractions were pooled and lyophilised. The resulting material LPS-ONPH was examined by CE-ES-MS and NMR.

Attachment of linker molecule

Nm galE LPS-ONPH (~ 25 mg) was reacted with a 30 × molar excess of cystamine at 37°C for 72 h in a solution of 0.1 M $NaHCO_3$, pH 8.4 in the presence of 25 mg of $NaCNBH_3$. The reaction mixture was then applied to a Sephadex G-25 column and eluted with water. The resulting carbohydrate fractions were pooled and lyophilised. The resulting material was reduced with 200 mM dithiothreitol (DTT) in 0.1 M $NaHPO_4$ at pH 8.1 for 1 h at 22°C. The reaction mixture was then applied to a Sephadex G-25 column and eluted with water. The resulting carbohydrate fractions were pooled and lyophilised and examined by CE-ES-MS.

Activation of protein carrier and conjugation

In order to conjugate the protein carrier molecule CRM_{197} to the cystamine-tagged carbohydrate it was necessary to modify the amino groups on the CRM_{197} protein by treatment with *N*-succinimidyl-bromo-acetate. This was achieved by mixing CRM_{197} (5 mg/ml) in 0.1 M $NaHPO_4$, 1 mM EDTA, 0.02% sodium azide, pH 7.1 cooled to 4°C, with *N*-succinimidyl-bromo-acetate (15 molar equivalents in DMF to 10% of the final volume) at 22°C for 2 h. The reaction mixture was then applied to a Sephadex G-25 column and eluted with 0.1 M $NaHPO_4$, 5 mM EDTA, 0.02% sodium azide, pH 6.0 and stored at 4°C until used. The degree of activation of the protein carrier was monitored by MALDI. The activated solution of protein carrier (~5 mg/ml) was adjusted to pH 7.5 with NaOH and a 50 X molar excess of lyophilised carbohydrate was added and left at 22°C for 17 h. The reaction was cooled to 4°C and then a 50 X molar excess of cysteine was added in order to cap any free bromo-acetate groups remaining following the conjugation reaction. The reaction mixture was purified down a Sepharose 6B column with PBS (50 mM sodium phosphate, 100 mM NaCl, pH 7.5) / citrate (10 mM) to achieve separation of free oligosaccharide from conjugated carbohydrate. The fractions corresponding to the product peak were concentrated by an ultra-15 spin column 10 K cut-off (Amicon). The final volume of conjugate was quantified for protein by the BCA assay [19] and for carbohydrate by the PhOH / H_2SO_4 method [20]. The conjugate was examined by SDS-PAGE (see below) and Coomassie staining, and by Western blotting with a carbohydrate specific monoclonal antibody (mAb B5 [11]).

Preparation of conjugates utilising Dd amidase without reducing end ring opening

De-O-*acylation*

Purified LPS from the *galE* mutant strain was *O*-deacylated as described above.

De-N-*acylation*

LPS-OH was the substrate for amidase enzymes produced by *Dd* as described above, but with slight modification. Briefly, *Dd* cells of strain AX3 were grown axenically in liquid nutrient medium [21] at 22°C to a density of 2–3 × 10^6 cells /ml. The cells were pelleted, resuspended and washed twice in Sorensen's buffer (14.6 mM KH_2PO_4, 2 mM Na_2HPO4, pH 6.3). The cells were resuspended at 2×10^8 cells / 3 ml of Sorensen's buffer and transferred to a conical flask. 2 mg of LPS-OH were added for each 3 ml of 2×10^8 *Dd* cells and incubated at 24°C overnight with shaking (120 rpm). The cells were pelleted (3 K, 2 min.) and the resulting supernatant was pelleted (13 k, 30 min.). This supernatant was passed through a 10 KDa molecular mass cut-off spin column (Amicon ultra-15, Millipore), at 4,000×g at 4°C for 20 min. The spin column step was repeated twice, washing with 15 ml of distilled water (centrifuge as above). The flow through sample which contains the partially *N*-deacylated LPS-OH (LPS-ONH) was lyophilised. The lyophilised material was eluted from a Sephadex G-25 column and lyophilised. The resulting material was examined by CE-ES-MS and NMR in order to monitor the extent of de-*N*-acylation.

Protection of PEtn residue

The LPS-ONH (4 mg/ml) was dissolved with 8 molar equivalents of NaOH and 0.6 molar equivalents of di-tert-

butyl dicarbonate (Boc) (Aldrich) was added dissolved in the same volume of DMSO (BDH Chemicals) and stirred for 75 min. at 22°C. The mixture was extracted 3× with ethyl acetate (6 ml / 10 mg CHO). The aqueous layer was lyophilised. The sample was eluted from a Sephadex G-25 column, eluting with water and the product peak was lyophilised. The product was monitored by CE-ES-MS.

Attachment of linker molecule

To introduce a maleimide functionality, Boc-protected carbohydrate (4 mg/ml) was dissolved in 100 mM sodium phosphate at pH 7.4 and a 1× molar equivalent of sulfo-GMBS (*N*-[g-maleimidobutyryloxy] sulfosuccinimide ester, Pierce) was added every 20 min. until 5 molar equivalents were added. The pH was adjusted to 7.4 and monitored every 20 min. adjusting as required with 0.1 M NaOH and the reaction was allowed to proceed for 2 h. To introduce a thiol moiety the Boc-protected carbohydrate (4 mg/ml) was dissolved in 100 mM sodium phosphate at pH 8.5 and a 1× molar equivalent of SATP (*N*-Succinimidyl-S-acetylthiopropionate, Pierce), dissolved in 50 ul of DMSO (BDH Chemicals) was added every 20 min. until 5 molar equivalents were added. The pH was adjusted to 8.5 and monitored every 20 min. adjusting as required with 0.1 M NaOH and the reaction was allowed to proceed for 2 h. The activated carbohydrates were eluted from a Sephadex G-25 column, eluting with water and the product peak was lyophilised. The product was monitored by CE-ES-MS.

De-protection of PEtn residue The Boc-protected carbohydrate molecule with the incorporated linker was dissolved in 20% trifluoroacetic acid (TFA, Fisher Scientific) at 6 mg/ml and left at 22°C for 2 h. The sample was eluted from a Sephadex G-25 column, eluting with water and the product peak was lyophilised. The product was monitored by CE-ES-MS.

Activation of protein carrier and conjugation

In order to conjugate amino groups on the protein carrier molecule CRM_{197} to the maleimide-tagged carbohydrate it was necessary to modify the amino groups on the CRM_{197} protein by treatment with 3, 3′-Dithiodipropionic acid di *N*-hydroxysuccinimide ester (DTSP, Pierce) in order to introduce a thiol moiety. This was achieved by mixing a 5 mg/ml solution of CRM_{197} in 0.1 M Na_2PO_4 buffer containing 1 mM EDTA and 0.02% sodium azide at pH 7.0 and pre-chilled to 4°C with fifteen molar equivalents of a solution of DTSP in DMF (10% of the aqueous volume). The reaction proceeded at 22°C for 2 h. DTT was added to a final concentration of 25 mM and the reaction proceeded for a further hour at 22°C and purified down a Sephadex G-25 column, eluting with 0.1 M Na_2PO_4 buffer containing 5 mM EDTA and 0.01% sodium azide at pH 7.0. Protein positive fractions were pooled and concentrated on an Ultra-15 10 kDa cut-off spin column (Millipore) and stored at 4°C.

In order to conjugate carboxyl groups on the protein carrier molecule CRM_{197} to the maleimide-tagged carbohydrate it was necessary to modify the carboxyl groups on the CRM_{197} protein (5 mg in 2 ml of 100 mM 2-(*N*-morpholino) ethanesulphonic acid (MES, Aldrich) at pH 5.2) by reaction with an 1,800 × molar excess of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Pierce) and a 1,500 × molar excess of adipic dihydrazide hydrazide, (ADH, Pierce) in 2 ml of 100 mM MES, pH 5.2 overnight at 4°C. The product was purified on an Ultra-15 10 kDa cut off spin column, washing with 100 mM sodium phosphate pH 6.8, and concentrated to approximately 0.5 ml and stored at 4°C. The activated protein was characterised by MALDI-MS as described below. The ADH activated CRM_{197} was diluted to 2 ml in 100 mM sodium phosphate, adjusting the pH to 6.0, and reacted with a 100× molar excess of *N*-succinimidyl-S-acetylthiopropionate (SATP, Pierce) dissolved in 0.1 ml of DMSO, and was allowed to react for 45 min. at 22°C in the dark. The sample was purified on a G-25 column, eluting with 100 mM sodium phosphate pH 6.8 The product peak was concentrated to approximately 0.5 ml using an Ultra-15 10 kDa cut off spin column and stored at 4°C. The activated protein was characterised by MALDI-MS as described below.

The acetate group protecting the thiol moiety was removed in 100 mM sodium phosphate pH 6.8 containing 250 mM hydroxylamine hydrochloride (JT Baker) at 22°C for 1.5 h. The sample was purified on a G-25 column, eluting with 100 mM sodium phosphate pH 6.8. Protein positive fractions were pooled and concentrated on an Ultra-15 10 kDa cut-off spin column (Millipore) and stored at 4°C.

For each conjugation reaction the appropriately activated carbohydrate and protein were left to react at 22°C for 5 h at pH 6.8 in 100 mM sodium phosphate with a second addition of carbohydrate provided so that a 50 × molar excess of carbohydrate was used in total. The reaction was allowed to continue overnight at 4°C and concentrated to ~1 ml. as described above. The concentrate was washed and concentrated a further four times using Dulbecco's PBS (Gibco) containing 10 mM sodium citrate (Sigma). The final concentrate was stored at 4°C. The glycoconjugates were characterised by MALDI-MS, SDS-PAGE and Western blotting as described below.

Analytical methods

Sugars were determined as their alditol acetate derivatives by GLC-MS as described previously [22].

Mass spectrometry and Nuclear Magnetic Resonance Spectroscopy

Capillary electrophoresis electrospray mass spectrometry (CE-ES-MS), and NMR spectroscopy were performed as described previously [23]. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra were obtained using a Voyager DE-STR mass spectrometer (Applied BioSystems, Foster City, CA, U.S.A.). The instrument was operated in positive, linear ion mode under delayed extraction conditions (200 ns) using an accelerating voltage of 25 000 V. Each spectrum is the average of approximately 150 laser shots. The matrix used was 3,5-dimethoxy-4-hydroxy cinnamic acid (sinapinic acid), prepared at a concentration of 10 μg/μl in 30% acetonitrile and 0.1% formic acid (v/v). Analyte concentration varied from approximately 1–35 pmol/μl and these solutions were either spotted directly on the MALDI target in a 1:1 ratio with matrix or diluted up to 1: 10 with matrix prior to sample spotting.

SDS-PAGE and Western blotting

The conjugates were separated on 10% Tris-HCl pre-cast gels under reducing conditions with the buffer system of Laemmli [24] and trans-blotted to polyvinylidene fluoride (PVDF) membranes. Membranes were immuno-stained with carbohydrate specific mAbs LPT3-1 [15] or B5 [11], followed by alkaline phosphatase conjugated anti-mouse IgG and developed with Alkaline Phosphatase Substrate Kit (Bio-Rad). SDS-PAGE was stained with Bio-Safe Coomassie.

Results

LPS purification

LPS was isolated from the mutant strains by standard methods. Sugar analysis by gas-liquid chromatography mass spectrometry (GLC-MS) examination of the derived alditol acetates revealed the expected ratios and confirmed the purity of the LPS as inferred from the absence of RNA derived ribitol.

Production and characterisation of conjugates

Glycoconjugates with completely deacylated LPS-derived oligosaccharides

The LPS was activated for conjugation according to a strategy that included complete de-acylation of the lipid A region, removal of the glycosidic phosphate, amination and attachment of the squarate linker. Each step was monitored by MS and the final activated carbohydrate is shown in Fig. 1 where the peak at *m/z* 869.0 is indicative of the squarate activated oligosaccharide. Conjugation to the protein moiety *via* squarate linker chemistry was carried out as detailed above, and the purified conjugate was examined by MALDI-MS (Fig. 2a and Table 1) and SDS-

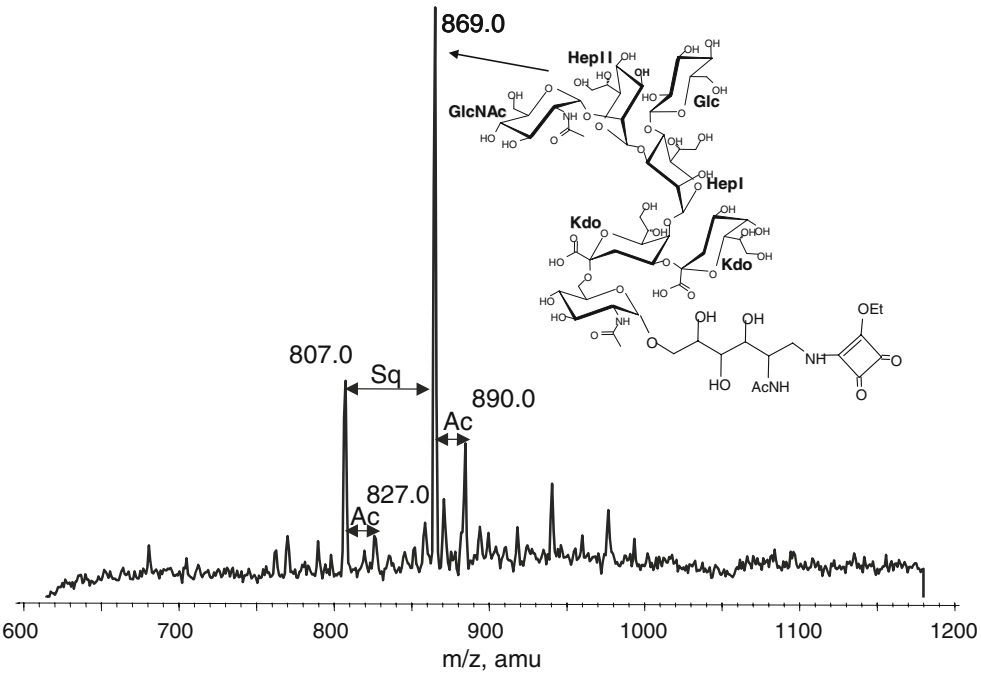


Fig. 1 CE-ES-negative ion MS analysis of deacylated / dephosphorylated / aminated LPS molecule from *Nm* mutant strain *galE / lpt3* following linkage to squarate linker molecule. Inset is a schematic of the squarate substituted molecule

PAGE with Western blotting with a carbohydrate specific monoclonal antibody LPT3-1 (data not shown) [15]. MALDI-MS analysis of the *galE* / *lpt3*-CRM_{197} conjugate gave a peak with a maximum at 60,736 Da consistent with the incorporation of 1–2 carbohydrate molecules (Fig. 2a).

Glycoconjugates prepared with Dd amidase and alkaline phosphatase treatment

The extent and specificity of de-*N*-acylation achieved during treatment with Dd amidases was effectively monitored by MS (data not shown) and NMR (Fig. 3). MS analysis revealed the complete removal of one *N*-linked fatty acid and some partial removal of a second *N*-linked fatty acid. This was confirmed by a tandem mass spectrometry technique. The nature of LPS-OH and derived molecules is such that fragmentation is enhanced between the lipid A region and the core oligosaccharide molecule, with the size of the fragmented lipid A region being indicated in the resulting mass spectrum. The intact LPS-OH molecule from strain *galE* fragmented to give ions for the lipid A region of *m/z* 952^{-} and 475^{2-}, corresponding to two glucosamine sugars, two phosphate groups and two *N*-linked fatty acid moieties. However, MS/MS analysis of the

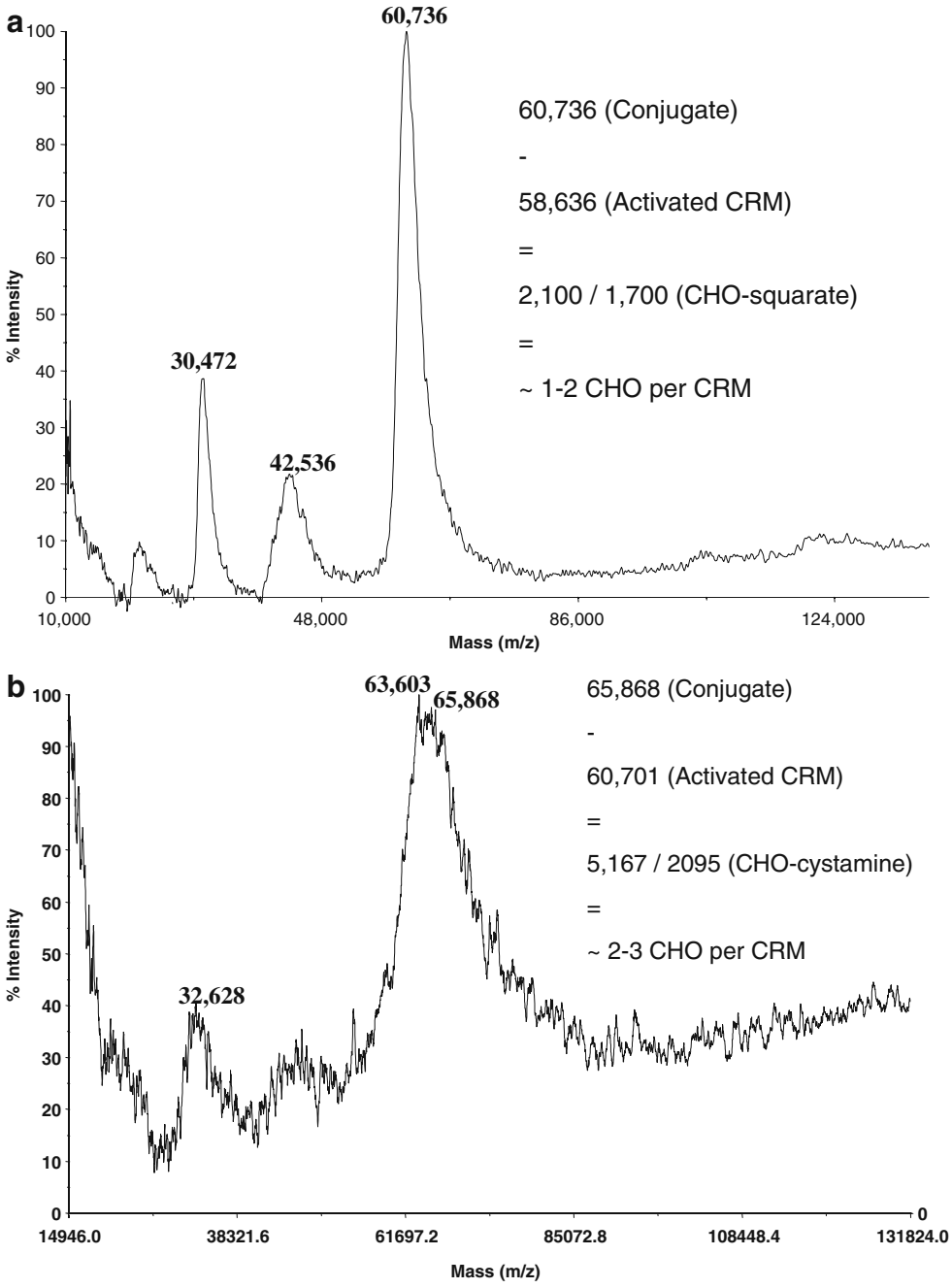
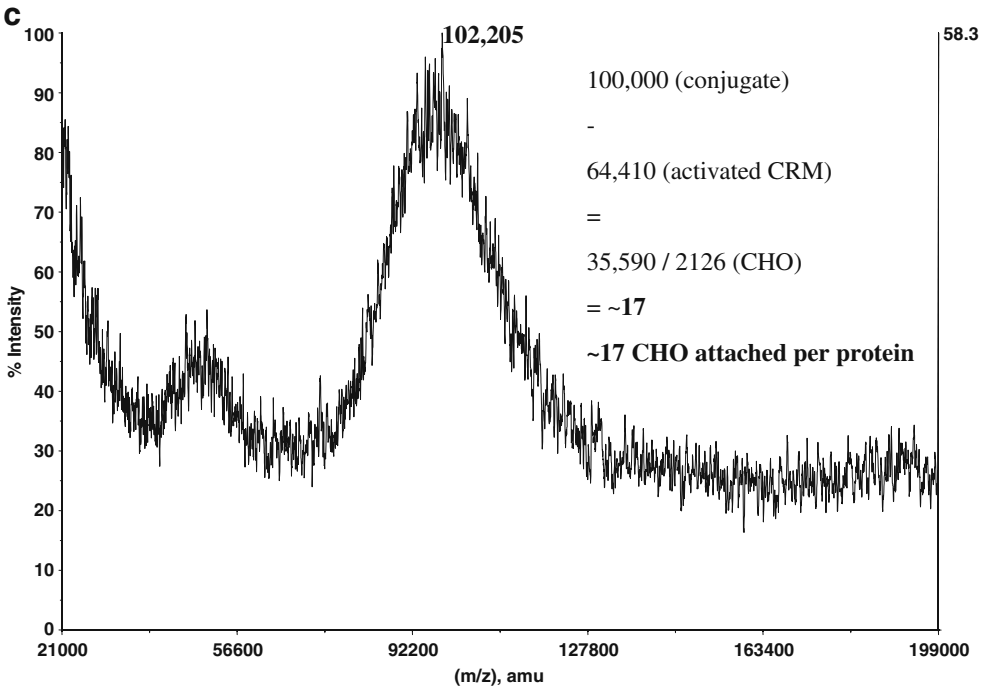


Fig. 2 MALDI-MS analysis of **a**) *galE* / *lpt3*-SQ-CRM conjugate, **b**) *galE*-CYS-CRM conjugate, **c**) *galE*-GMBS-ADH-SATP conjugate

Fig. 2 (continued)



doubly charged ion at *m/z* 1018.7^{2-} from the LPS-ONH molecule gave a singly charged ion for the lipid A region of *m/z* 725^{-}. This ion of *m/z* 725^{-} corresponds to two glucosamine sugars, two phosphate groups and one *N*-linked fatty acid moiety, thus illustrating that a *N*-linked fatty acid has been removed from the lipid A region of the LPS-OH following exposure to the *Dd* amidase. Proof that the *N*-linked fatty acid on the reducing glucosamine residue of lipid A had been removed was obtained by NMR experiments. Compared to the unresolved spectrum of *O*-deacylated LPS (Fig. 3a), a well-resolved ^{1}H-NMR spectrum was obtained (Fig. 3b) for the Dd amidase treated material, consistent with a water-soluble molecule being produced following removal of at least one *N*-linked fatty acid. A 2D COSY spectrum (data not shown) identified the H-2 resonances. The upfield shift of the H-2 resonance of the glucosamine bearing the glycosidic phosphate to 3.4 ppm, was consistent with the removal of the *N*-linked fatty acid. A ^{13}C-^{1}H HSQC experiment (data not shown) confirmed that this H-2 resonance was on a carbon residue attached to a nitrogen atom by virtue of its diagnostic ^{13}C shift of ~54 ppm. It can also be noted that signals indicative of the retention of the PEtn residue are still observed at intensities consistent with stoichiometric attachment. Therefore treatment with amidases from *Dd* has created a water soluble molecule, fully amenable to subsequent steps in the

Table 1 MALDI-MS analyses of CRM_{197}, activated CRM_{197} and glycoconjugates

MALDI-MS data (kDa)/ Conjugate	CRM-BrAc *via* lysine	CRM-DTSP *via* lysine	CRM-ADH *via* carboxyl	CRM-ADH-SATP *via* carboxyl	CRM-SQ-*galE* / *lpt3* *via* lysine	CRM-cyst-*galE* *via* lysine	CRM-DTSP-*galE via* lysine	CRM-ADH-SATP-*galE via* carboxyl
CRM-SQ-*galE* / *lpt3*[a]	–	–	–	–	60.7 => 1–2 carbohydrate attached	–	–	–
CRM-cyst-*galE*	60.7 =>17 lysines activated	–	–	–	–	65.9 =>2–3 carbohydrates attached	–	–
CRM-DTSP-*galE*	–	60.4 =>24 lysines activated	–	–	–	–	60.6, 62.4 =>0–1 carbohydrate attached	–
CRM-ADH-SATP-*galE*[b]	–		62.2 =>25 carboxyls activated	64.8 =>20 ADH's activated	–	–	–	100.0 =>17 carbohydrates attached

[a] Other conjugates were also prepared using this methodology (loading in parentheses) *icsB/ lpt3* (1–2) and *lgtB/lpt3* (1–3)

[b] Other conjugates were also prepared using this methodology (loading in parentheses) *icsB* (9), *lgtA* (16) and *lgtB* (16)

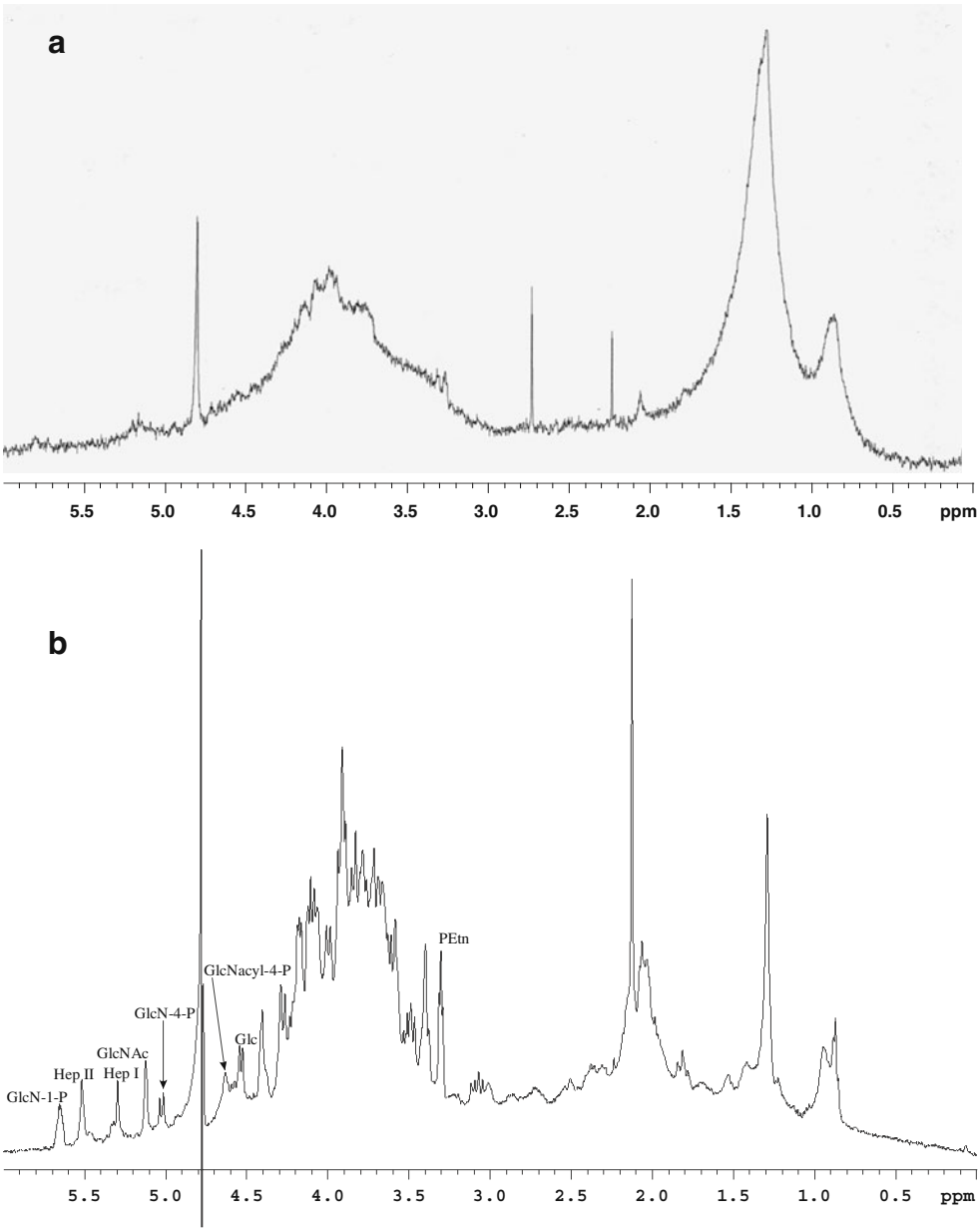


Fig. 3 1D-^{1}H-NMR spectroscopy of **a**) *Neisseria meningitidis* L3 *galE O*-deacylated LPS; **b**) *Neisseria meningitidis* L3 *galE O*-deacylated mono-*N*-deacylated LPS

conjugation strategy, and the immunologically important PEtn residue has been retained.

Following alkaline phosphatase treatment (data not shown) cystamine was added to the carbohydrate and following reduction the product was characterised by mass spectrometry (Fig. 4) revealing that the linker had been efficiently incorporated into the carbohydrate molecule. The protein carrier CRM_{197} was activated with approximately 17 bromo-acetyl groups being attached as deduced by MALDI-MS experiments (Table 1). Activated CRM_{197} was conjugated to the cystamine coupled carbohydrate and unconjugated bromo-acetyl groups were capped with cysteine as described.

Following conjugation the conjugate was purified as described in the Materials and Methods and examined by MALDI-MS (Fig. 2b and Table 1) and SDS-PAGE (data not shown). MALDI-MS analysis of the *galE* -CRM_{197} conjugate gave a peak with a maximum at 65,868 Da consistent with the incorporation of 2–3 carbohydrate molecules. The migration pattern of the unmodified CRM_{197}, activated CRM_{197} and conjugate are all consistent with an increase in size of the molecule. The conjugate reacted with the carbohydrate specific mAb B5 [11] in a Western blot (data not shown).

Preparation of conjugates utilising Dd amidase without reducing end ring opening

Amino and carboxyl targeted conjugates were prepared as detailed in the Materials and Methods where the scheme employed for the latter approach is illustrated in Fig. 5.

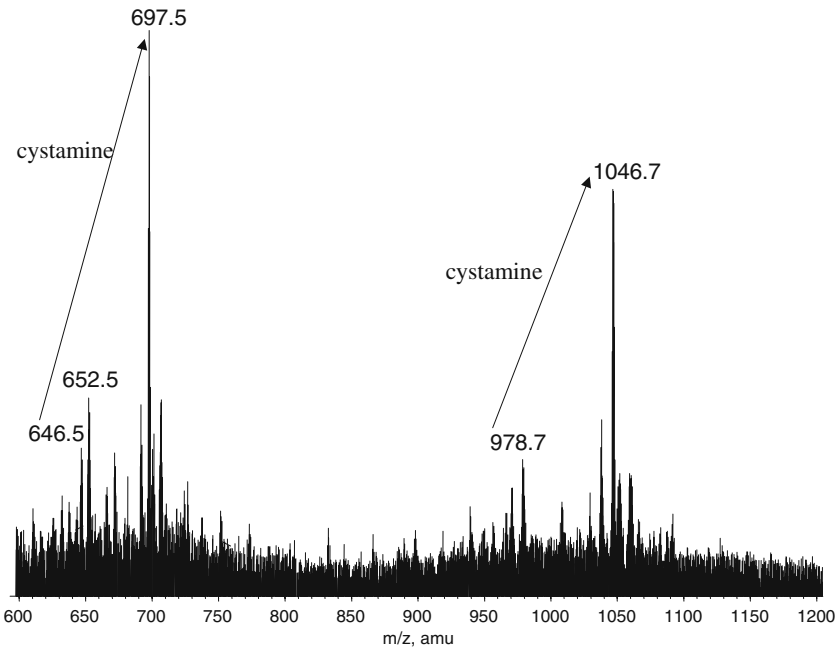


Fig. 4 CE-ES-MS-MS analysis of *Neisseria meningitidis* L3 *galE O*-deacylated mono-*N*-deacylated LPS following cystamine linker incorporation

Each step of the strategy was monitored by MS and or NMR as appropriate.

The extent and specificity of de-*N*-acylation achieved during treatment with *Dd* amidases was effectively monitored by MS and NMR as described above.

Following de-*N*-acylation, the PEtn residue on the *galE* derived molecule was selectively protected and the product was characterised by mass spectrometry (Fig. 6). MS analysis of the material before (Fig. 6a) and after (Fig. 6b) Boc protection illustrated that the molecule had increased in mass by 100 amu, consistent with the incorporation of one Boc moiety. Small amounts of glycoforms consistent with the incorporation of a second Boc group were observed (Fig. 6b). As evidenced by MS/MS analysis, the Boc protecting group was localised to the core oligosaccharide portion of the carbohydrate molecule as the mass of the de-*N*-acylated glucosamine disaccharide region (*m/z* 362.5^{2-}, 725.7^{-}) was consistent with no Boc incorporation and the mass of core OS (*m/z* 1192.0^{-}) was consistent with the incorporation of a Boc molecule (Fig. 6c). MS/MS analysis on the two Boc containing molecules (Fig. 6d) revealed that both the reducing glucosamine disaccharide (*m/z* 412.0^{2-}, 825.3^{-}) and the core OS (*m/z* 1192.0^{-}) had increased by 100 amu.

A maleimide containing linker, sulfo-GMBS, was attached to the *galE* molecule as detailed in the Materials and Methods and characterised by MS (Fig. 6e).

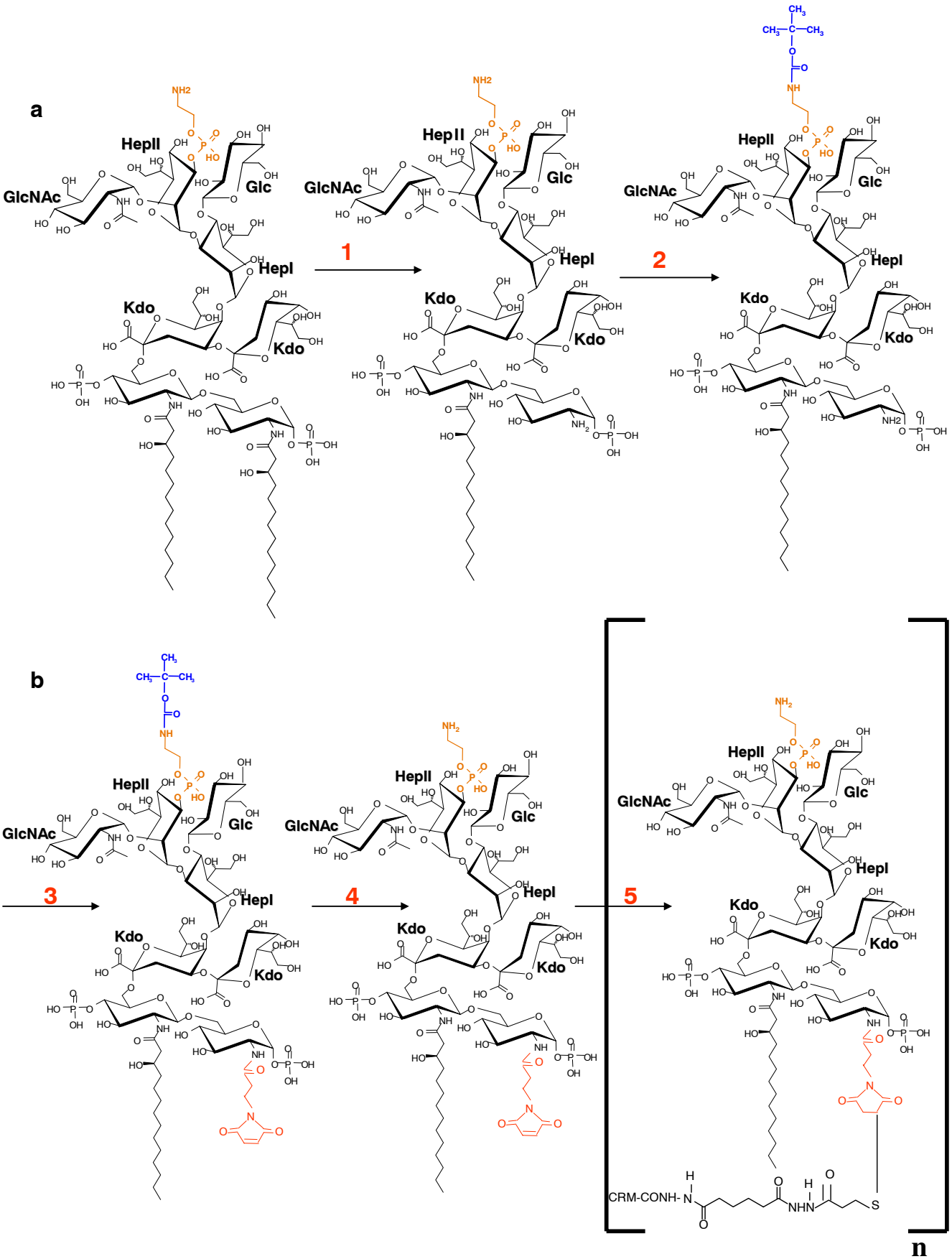
Following linker incorporation the t-Boc blocking group was efficiently removed from the PEtn residue of the *galE* derivative by treatment with 20% TFA with no effect on the remainder of the molecule as characterised by MS (Fig. 6f). A decrease in mass of 100 amu was observed, as evidenced by the identification of ions (*m/z* 550.3^{4-}, 733.8^{3-}, 1101.3^{2-}) consistent with the removal of the Boc protecting group, when compared to the Boc-protected molecule (Fig. 6e). Finally, the activated carbohydrate was examined by ^{1}H-NMR, which indicated that the maleimide linker was present on the carbohydrate molecule, by virtue of a sharp singlet at 6.8 ppm corresponding to the equivalent protons of the double bond of the maleimide ring (Fig. 7).

Lysine groups of CRM_{197} were activated with a thiol containing linker (DTSP) as described in the Materials and Methods and characterised by MALDI-MS which revealed that ~24 lysine residues had been activated as evidenced by a mass increase of ~2.1 kDa (Table 1).

CRM_{197} activated with a thiol linker (DTSP) was conjugated to the carbohydrate *via* the maleimide linker molecule sulfo-GMBS on the activated carbohydrate as described above. Conjugation products were purified as described and monitored by MALDI-MS (Table 1), SDS-PAGE and Western blotting (data not shown). It was apparent that only one carbohydrate molecule had been conjugated targeting lysine moieties, and for this reason carboxyl groups were then targeted.

Carboxyl groups of CRM_{197} were activated in order to elaborate a thiol moiety with the sequential addition of ADH and SATP as described in the Materials and Methods and characterised by MALDI-MS which revealed that ~30 carboxyl residues had been activated as evidenced by a mass increase of ~4 kDa (Table 1). CRM_{197} activated in

Fig. 5 Conjugation reaction scheme for carboxyl targeting, illustrating derivitisation of *Nm galE* LPS-OH; **a**): Step 1, *Dd* amidase de-*N*-acylation; Step 2, protection of PEtn residue; Step 3, linker incorporation; Step 4, de-protection of PEtn residue; Step 5 conjugation to activated protein carrier ▶


a
b
GlcNAc
HepII
Glc
HepI
Kdo
1
2
3
4
5
CRM-CONH-
n

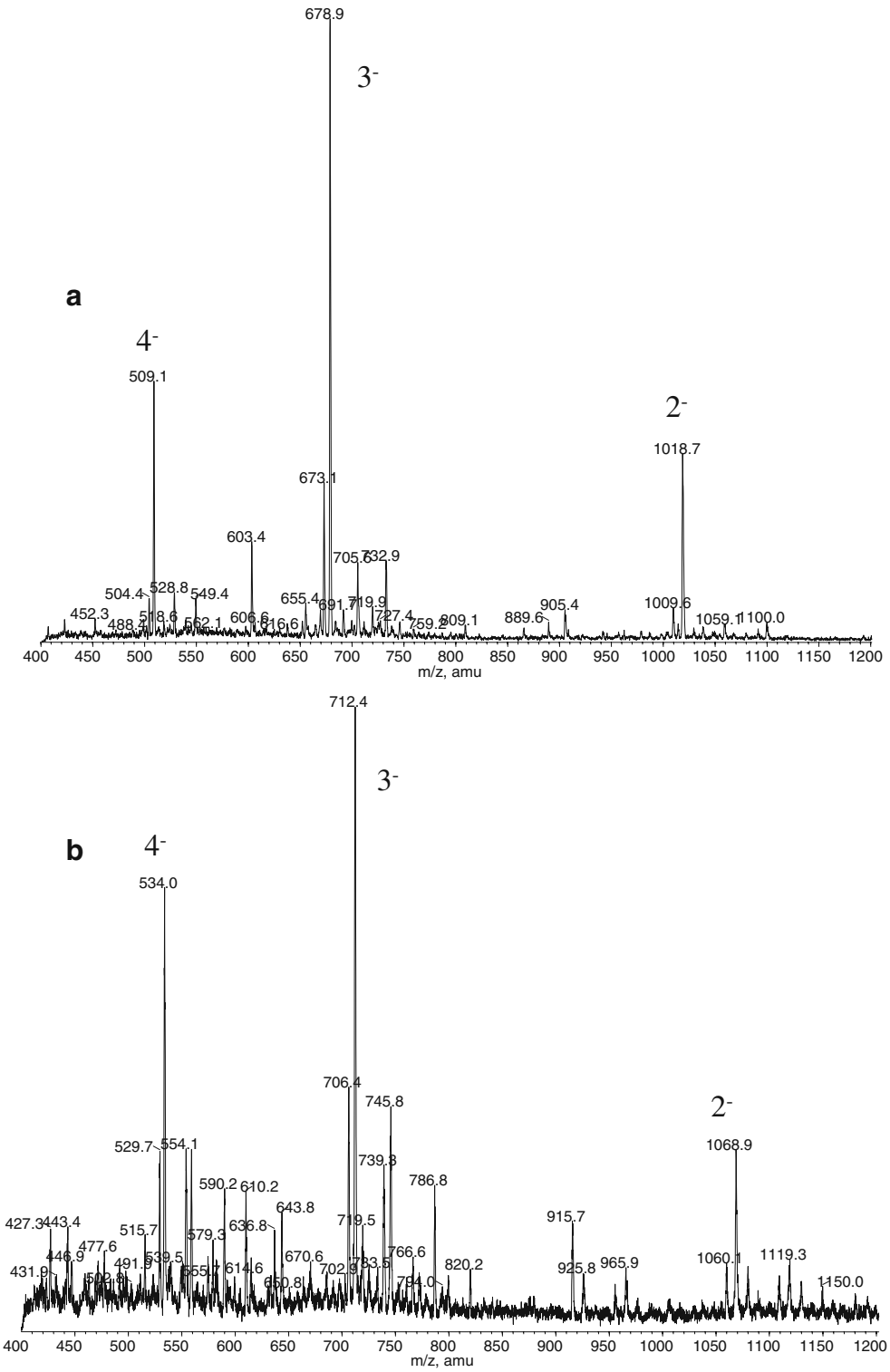


Fig. 6 CE-ES- negative ion MS analyses of *Neisseria meningitidis* L3 *galE O*-deacylated mono-N-deacylated LPS; **a**) before Boc protection, **b**) after Boc protection, **c**) MS /MS analysis of the one Boc containing molecule of *m/z* 712.4^{3-}, **d**) MS / MS analysis of the two Boc containing molecule of *m/z* 745.8^{3-} **e**) after introduction of a maleimide group, **f**) following removal of Boc protecting group from the maleimide activated molecule

this way was conjugated *via* two additions of the maleimide activated carbohydrate as described. Conjugation products were purified and monitored by MALDI-MS (Fig. 2c and Table 1), SDS-PAGE and Western blotting (Fig. 8). The SDS-PAGE migration pattern of the conjugate was consistent with a significant increase in the size of the molecule and Western blotting revealed strong recognition with mAb B5 (Fig. 8). This proved to be the case after both the first addition (Fig. 8, lane 5) and the second addition (Fig. 8, lane 6) of carbohydrate. MALDI-MS analysis revealed that approximately 11 oligosaccharides had been attached in the first carbohydrate addition (data not shown) and the final

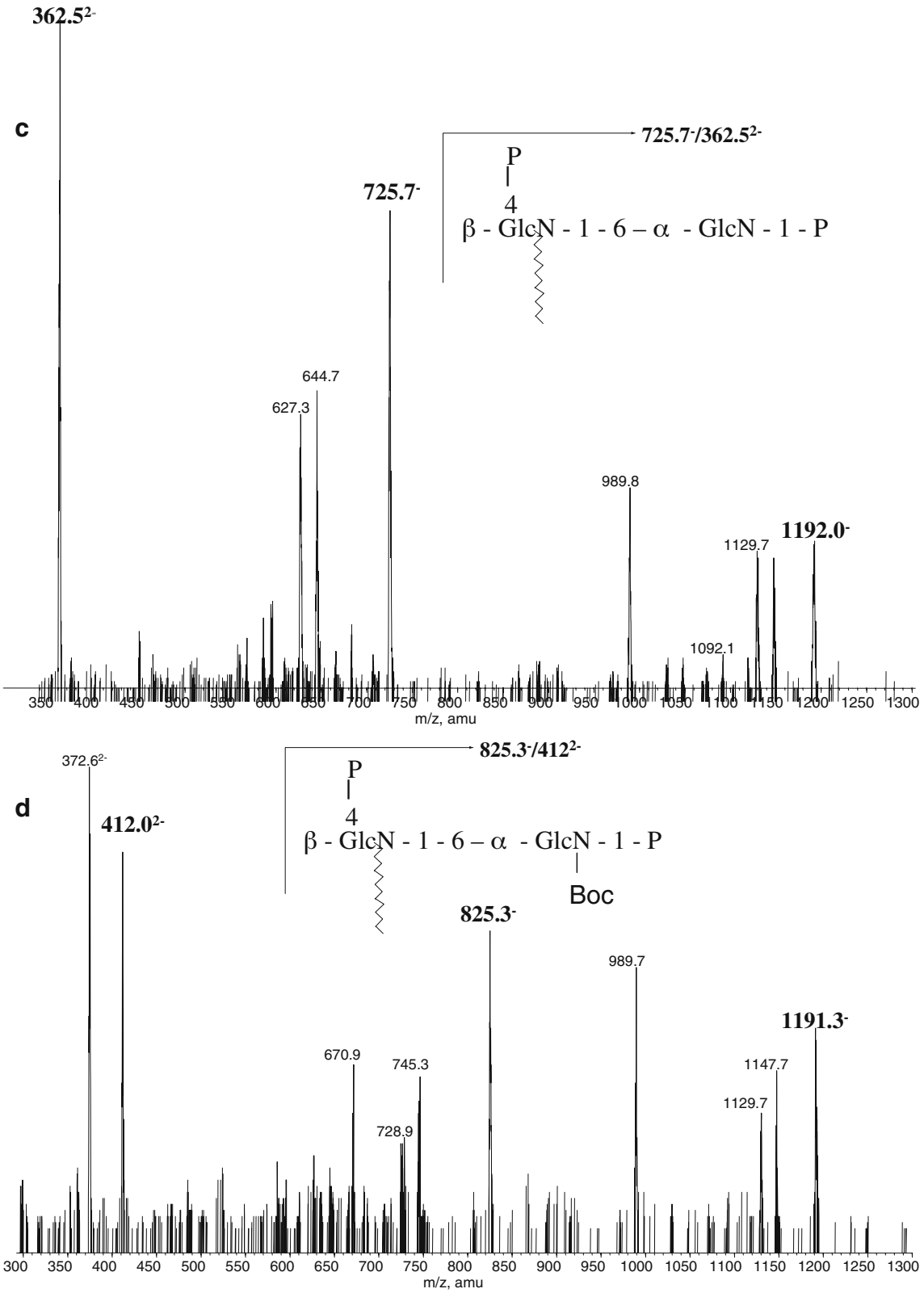


Fig. 6 (continued)

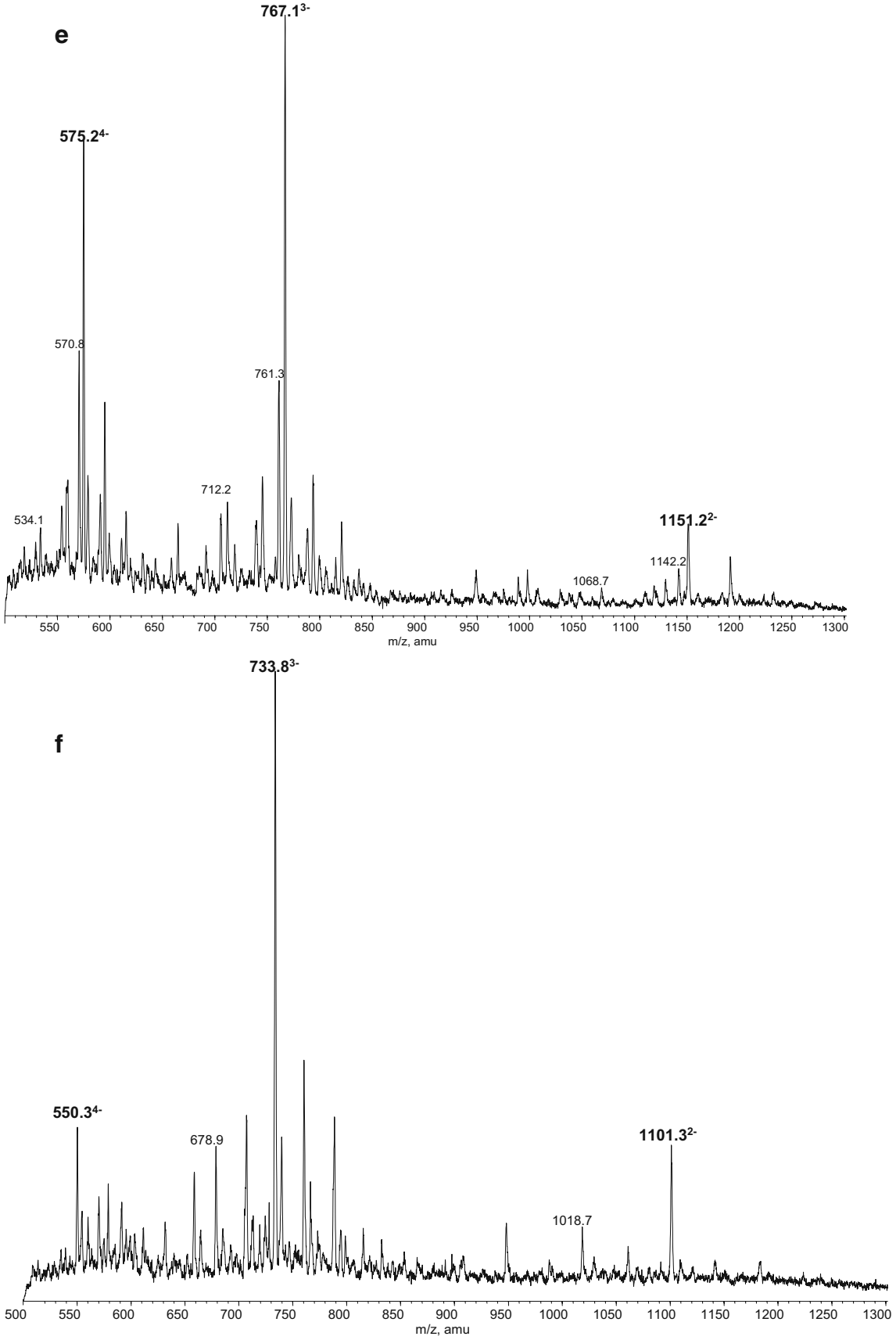


Fig. 6 (continued)

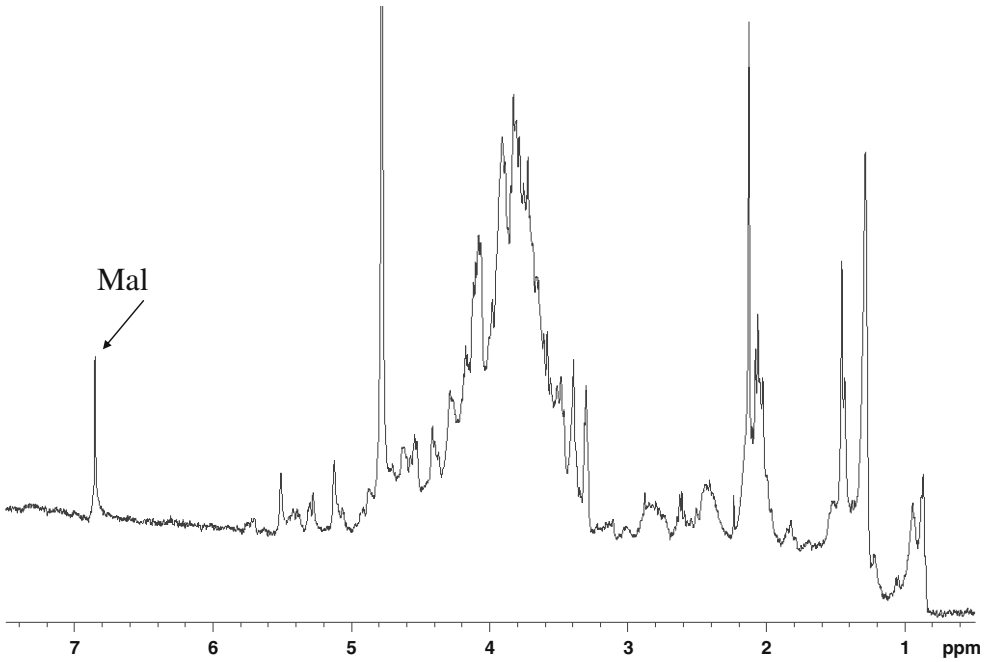


Fig. 7 1D-^{1}H-NMR spectroscopy of *Nm* L3 *galE* O-deacylated mono-N-deacylated LPS following maleimide linker incorporation

conjugate elaborated approximately 17 carbohydrates per protein carrier (Fig. 2c) as evidenced by the significant increase in mass of ~33 kDa when compared to the activated carrier protein (Table 1).

Discussion

This study describes the preparation of glycoconjugates by a variety of chemical methods. Initially, completely deacylated LPS of *N. meningitidis* mutant strain *galE / lpt3* was conjugated to CRM_{197}. The chemical methods employed to completely deacylate the LPS molecule would also cause the cleavage of the ethanolamine (Etn) portion of the PEtn residue, which would result in the concomitant retention or migration of the resulting phosphate residue, thereby introducing heterogeneity. Therefore, we used *lpt3* mutants to avoid this potential complication in the methodology. The aim was to produce completely deacylated molecules that were water-soluble and more amenable to steps involved in glycoconjugate production. Glycoconjugates were constructed *via* linkage to carrier protein CRM_{197} at the lipid A terminus, following removal of the glycosidic phosphate with alkaline phosphatase. This

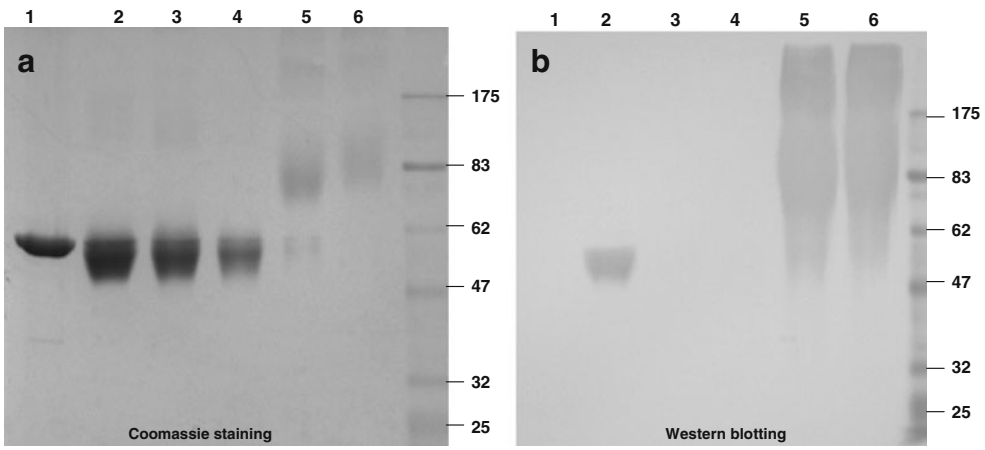


Fig. 8 Coomassie staining and Western blot analysis of *galE*-GMBS-ADH-SATP conjugate **a**) Coomassie staining of samples after equal protein loading. **b**) Western blot analysis of samples using B5 antibody at a dilution of 1:10^{6}. Lanes 1, 2, 3, 4, 5, 6 are CRM, CRM-ADH, CRM-ADH-SATP, CRM-ADH-SH, CRM ADH-GMBS-*Nm galE* conjugate after 1st CHO addition, CRM-ADH-GMBS-*NmgalE* final conjugate respectively

creates an aldehydo function on a terminal open chain sugar, which allows conjugation following amination with the bi-antennary linker molecule squarate.

This conjugation strategy has proved to be a robust and reproducible methodology for each of the carbohydrate structures utilised. Each step was successfully monitored and characterised by analytical techniques including mass spectrometry and NMR spectroscopy. However, the final loading was only 1–2 carbohydrates per CRM. Pozsgay *et al*. [25] examined the immunogenicity of glycoconjugates as a function of the length and loading of the carbohydrate antigen, and established that for an oligosaccharide of approximately 8 units a loading of 8 oligosaccharides per protein was required for optimal immunogenicity. Clearly different oligosaccharides are likely to have different immunogenicities, but nevertheless we theorised that only 1–2 oligosaccharides per CRM was too low.

Subsequently, we examined the utility of an isolated amidase from the slime mould *Dictyostelium discoideum* to enable the preparation of glycoconjugates with the immunologically important PEtn residues maintained. *Dd* produces a variety of degradative enzymes, including esterases and amidases, which specifically remove the fatty acids from the lipid A region without affecting the carbohydrate region of the molecule [26]. *Dd* acts on LPS in a specific sequential manner initially utilising esterases to remove the *O*-linked fatty acids and then removing the two *N*-linked fatty acids in turn, utilising amidases [26]. Amidase activity was used on isolated *O*-deacylated LPS from immunotype L3 *galE* mutant to create a water soluble molecule, in which one or both of the *N*-linked fatty acids were removed, in order to enable subsequent steps in strategies culminating with conjugation to CRM_{197}. We conjugated *via* a linker molecule cystamine, which involved sulphur chemistry to avoid potential involvement of the amino functionality on the PEtn molecule and in an effort to increase the efficiency of the conjugation reaction. These conjugation strategies were enhanced following the action of the *Dictyostelium* amidase as the resulting molecule is completely water-soluble and more amenable to these manipulations.

This conjugation strategy also proved to be a reproducible methodology with each step successfully monitored and characterised by analytical techniques including mass spectrometry and NMR spectroscopy.

In contrast to glycoconjugates prepared from *O*-deacylated LPS, the advantage of using water-soluble carbohydrate molecules was that it enabled us to remove any remaining non-conjugated carbohydrate species from the final conjugate molecules. As a result, this eliminated any interference of the free carbohydrate molecules in conjugate quantification. However once again, the final loading achieved of only 2–3 carbohydrates per CRM protein was thought to be too low.

The final strategy utilised a novel conjugation strategy that has facilitated the preparation of conjugates with a high loading of carbohydrate molecules (12–18) per carrier protein when carboxyl groups were targeted. We successfully utilised the amino functionality created at the reducing end of the carbohydrate following enzyme treatment with amidase from *Dictyostelium discoideum*. This amino group could then be directly targeted as the site of conjugation without having to use alkaline phosphatase to create an aldehydo functionality and open up the glucosamine residue at the reducing end of the modified LPS molecule. However, targeting the lipid A amino functionality then presented the challenge of selectively protecting the amino group of the immunologically important PEtn residue in the inner core OS. We achieved this by utilising a novel blocking strategy with di-tert-butyl dicarbonate, which preferentially attached to the inner core PEtn amino when ratios of blocking agent and substrate were carefully controlled. The remaining amino functionality was now uniquely available for reaction with the linker molecule, and the blocking group was then subsequently removed, thus directing the location of conjugation between the carbohydrate and carrier protein. This elegant chemical methodology has enabled us to construct conjugates with high carbohydrate loading whilst maintaining conjugation via the lipid A region of the molecule and without destroying the ring integrity of the reducing end glucosamine residue. Clearly in this study using the same linker chemistries with maleimide and thiol groups we struggled to attach 2 carbohydrates per CRM_{197} targeting lysine residues, whereas 12–18 carbohydrates were attached targeting carboxyl groups. The high loading achieved with this methodology is a significant improvement over previous conjugate preparations where evidence of perhaps at best 2–3 carbohydrate molecules per carrier protein was obtained. This improvement is evidently due to targeting of carboxyl groups rather than amino functionalities on the carrier protein. The carrier protein used in these studies, CRM_{197}, contains 63 carboxyl and 39 amino functionalities, so although there are more carboxyl moieties available, it is unlikely that the increase in loading is simply due to the number of functionalities available, but perhaps the accessibility of the targeted functionalities. The loading achieved here can be qualitatively and quantitatively characterised by SDS-PAGE / Western and MALDI MS techniques, respectively, and can also be controlled by variation in the amount of activated carbohydrate utilised in the conjugation reaction, which will permit us to explore the impact that carbohydrate loading has on the immunogenicity of the conjugates, and this is currently under investigation.

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