Supporting Information

Deciphering Antigenic Determinants of *Streptococcus pneumoniae* Serotype 4 Capsular Polysaccharide using Synthetic Oligosaccharides

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1. Supporting experimental procedures

1.1. Glycan arrays

Glycans, proteins (CRM_{197} and a BSA-Spacer-Dimannose construct prepared the same way as CRM_{197} conjugates used in the study) and polysaccharide solutions (native polysaccharides from SSI Diagnostica, depyruvated ST4 prepared as explained below) at the concentrations (two concentrations per substance) shown in Supplementary Figure 2 were prepared in printing buffer (50 mM sodium phosphate, pH 8.5). Those solutions were spotted on CodeLink NHS activated glass slides (Surmodics) using a S3 piezoelectric microarray printer (Scienion) equipped with a type 4 coated nozzle. The spotting chamber was constantly kept at 65% relative humidity. Afterwards, the slides were incubated over night at room temperature in a humidity saturated chamber. Remaining reactive groups on the slide surface were quenched by incubation with 50 mM sodium phosphate, 100 mM ethanolamine pH 9.0 at room temperature for one hour. Slides were subsequently washed three times for 5 min with water, dried by centrifugation at 300 g for 5 min (CombiSlide system, Eppendorf) and stored at 4 °C until use.

Directly before the assay, the slides were blocked with a solution of 1% (w/v) BSA in PBS (BSA-PBS) for 30 min at RT, washed three times 5 min with PBS and dried by centrifugation. A 64 well incubation gasket (FlexWell 64 grid, Grace BioLabs) was attached to the slide and primary antibody dilutions in BSA-PBS were added in duplicates. In case of an inhibition assay, dilutions were made in BSA-PBS containing the appropriate inhibitors and incubated for 20 min at RT before application to the slides. After incubation for 1 h at RT, slides were washed three times for 5 min with PBS containing 0.1% (v/v) Tween-20 (PBST) by adding 50 µL to each well. Secondary antibody dilutions were prepared in BSA-PBS using the following antibodies and dilutions: goat anti-mouse IgG FITC (Sigma-Aldrich) 1:400, goat anti-mouse IgM AlexaFluor 594 (Dianova) 1:200, goat anti-human IgG Fc-specific AlexaFluor 488 (Dianova) 1:400, and goat anti-human IgM AlexaFluor 594 (Life Technologies) 1:400. In case the same secondary antibody solution was used for each well, the gasket was removed, the slide dried by centrifugation. The secondary antibody was then pipetted onto the complete glass slide and the slide covered using a cover slip. If different secondary antibody solutions were used, the dilutions were directly added to the wells of the gasket. After incubation at RT in the dark for 30 min, slides were washed twice for 5 min with PBST, once with PBS, rinsed with water and dried by centrifugation. Fluorescence was read out with a GenePix 4300A microarray scanner (Molecular Devices) and spots were analyzed using GenePix Pro 7 (Molecular Devices) using the mean fluorescence intensity of every spot subtracted by the local background. Spot diameter was kept identical for all substances. Usually, the mean of

four spots from duplicate wells was calculated, except when there were visibly unsuitable spots, those were left out of the calculation.

1.2. Synthesis and characterization of glycoconjugates

1.2.1. Conjugate of trisaccharide **3** for first immunization

Di-*N*-succinimidyl adipate (17.4 mg; 51.3 µmol) of was dissolved in 190 µL DMSO and 10 µL of triethylamine was added. To this solution, 2.8 mg (4.3 µmol) of saccharide **3** dissolved in 60 µL DMSO was pipetted slowly over 30 min under continuous stirring. After additional stirring for 1.5 h, 400 µL of 0.1 M sodium phosphate buffer (pH 7.4) was added and the unreacted linker was extracted twice with $CHCl_3$ by adding 12 mL of the solvent, mixing, and centrifuging at 4000 rpm for 4 min. The aqueous phase was transferred to a 1.5 mL reaction tube, centrifuged for 1 min to remove residual $CHCl_3$ and then added to a solution containing 1 mg CRM_{197} in 1 mL 0.1 M sodium phosphate buffer (pH 7.4). After stirring overnight at room temperature, the reaction mixture was transferred to an Amicon ultrafiltration device (MWCO 10 kDa, Merck Millipore) and desalted by repeatedly adding deionized water to the upper chamber.

1.2.2. Conjugate of trisaccharide **3** for second immunization

Di-*N*-succinimidyl adipate (16.6 mg; 49 µmol) was dissolved in 180 µL DMSO and 10 µL of triethylamine was added. To this solution, 3.0 mg (4.6 µmol) of saccharide **3** dissolved in 65 µL DMSO was pipetted slowly over 30 min under continuous stirring. After additional stirring for 1.5 h, 400 µL of 0.1 M sodium phosphate buffer (pH 7.4) was added and the unreacted linker was extracted twice with $CHCl_3$ by adding 12 mL of the solvent, mixing, and centrifuging at 4000 rpm for 4 min. The aqueous phase was transferred to a 1.5 mL reaction tube, centrifuged for 1 min to remove residual $CHCl_3$ and then added to a solution containing 1 mg CRM_{197} in 1 mL 0.1 M sodium phosphate buffer (pH 7.4). After stirring overnight at room temperature, the reaction mixture was transferred to an Amicon ultrafiltration device (MWCO 10 kDa, Merck Millipore) and desalted by repeatedly adding deionized water to the upper chamber.

1.2.3. Conjugate of tetrasaccharide **2**

Di-*N*-succinimidyl adipate (15.8 mg;46.4 µmol) was dissolved in 180 µL DMSO and 10 µL of triethylamine was added. To this solution, 3.3 mg (3.9 µmol) of saccharide **2** dissolved in 60 µL DMSO was pipetted slowly over 30 min under continuous stirring. After additional stirring for 1.5 h, 400 µL of 0.1 M sodium phosphate buffer (pH 7.4) was added and the unreacted linker was extracted twice with $CHCl_3$ by adding 12 mL of the solvent, mixing, and

centrifuging at 4000 rpm for 4 min. The aqueous phase was transferred to a 1.5 mL reaction tube, centrifuged for 1 min to remove residual $CHCl_3$ and then added to a solution containing 1 mg CRM_{197} in 1 mL 0.1 M sodium phosphate buffer (pH 7.4). After stirring overnight at room temperature, the reaction mixture was transferred to an Amicon ultrafiltration device (MWCO 10 kDa, Millipore) and desalted by repeatedly adding deionized water to the upper chamber.

1.2.4. Glycoconjugate characterization

Protein concentration after desalting was determined using the Micro BCA Protein Assay Kit (Thermo Scientific Pierce). Further characterization was carried out by sodium dodecyl sulfate – polyacrylamide gel electrophoresis. Sugar to protein ratio was determined by matrix assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry on an Ultraflex-II TOF/TOF instrument (Bruker Daltonics) equipped with a 200 Hz solid-state Smart beam laser using 1,6-dihydroxyacetophenone as matrix. The samples were measured in positive linear ion mode. FlexAnalysis was used for analysis of spectra.

1.3. Evaluation of immune response

Sera of individual mice at each time point were diluted in BSA-PBS. Starting dilution was 1 in 400 and, depending on time point, a number of further sequential one in four dilutions were prepared down to a dilution factor of 102,400 for weeks three and eight. Glycan array analysis was carried out as described above to obtain the immune response for single mice. The immune responses for the groups were calculated by using the mean and standard deviation of all mice in the groups. For trisaccharide **3** and preimmune serum to week three, the immune responses of both immunized groups, the first group and the second for preparation of monoclonal antibodies, were included in the calculation. Mann-Whitney Rank Sum Test was employed to compare immune responses of different groups.

1.4. Monoclonal antibody purification

Hybridoma were grown in standard tissue culture flasks (Corning) in ISF-1 serum free medium (Biochrom). Cells were removed by centrifugation at 300 g at RT. Supernatant was concentrated using Amicon centrifugal filter devices (MWCO 30,000; Merck-Millipore). Concentrated supernatant was applied to gravity flow columns (Biorad) containing 1 mL Protein A/G sepharose (BioVision) pre-equilibrated with PBS. Columns were closed and incubated under slow rotation over night at 4 °C. The flowthrough was collected for analysis and the column material was washed five times with 10 mL PBS. Each two additional wash steps of 5 mL were performed with 10 mM sodium phosphate, 1 M sodium chloride pH 7.4

and 100 mM sodium phosphate, 150 mM sodium chloride pH 5.5. Antibodies were eluted ten times with 1 mL of 100 mM citric acid containing 300 mM sodium chloride. Each elution fraction was immediately neutralized with 150 µL 1 M Tris pH 11. Elution fractions were pooled, buffer exchanged to PBS using Amicon centrifugal filter devices (MWCO 30,000; Merck-Millipore) and analyzed by SDS-PAGE and BCA protein determination (Thermo Scientific Pierce). Purified antibody was stored in small aliquots at -20°C until use. Column material was re-equilibrated with PBS and stored at 4 °C with the addition of 0.05% sodium azide until next purification of the same antibody.

1.5. ELISA

Immulon 4 HBX plates (Thermo Scientific) were coated with the natural polysaccharide (SSI Diagnostica, Denmark) by incubating with 100 µL of a 10 µg/mL solution in PBS at 4 °C for at least 16 h. The plates were washed twice with PBS + 0.05% Tween-20. After that, the plates were incubated with 100 µL of BSA-PBS per well for blocking and washed twice with PBS + 0.05% Tween-20. Then the primary antibody dilutions (pools of sera of three mice immunized with conjugate of **2** and two mice immunized with conjugate of **3** in BSA-PBS were added in triplicates. After incubation for 1 h at 37 °C, the plates were washed three times with PBS + 0.05% Tween-20. 100 µL of a 1 in 10000 dilution of HRP-conjugated goat-anti-mouse IgG (Dianova) were added and the plates were incubated for 1.5 h at 37 °C. After washing four times, plates were incubated at room temperature for 10 min with 50 µL of 1 Step Ultra TMB ELISA substrate (Thermo Scientific Pierce). Stop solution (2% sulfuric acid in water; 50 µL) was added and absorbance at 450 nm was read out using an Infinite M200 microplate reader (Tecan).

1.6. Immunofluorescence

UV inactivated TIGR4 pneumococci (7e8 cfu) in Tool-Hewitt medium containing 10 % FCS and 20 % glycerol were washed with PBS three times by resuspension and centrifugation (10 min, 7000 g). The sample was resuspended in PBS, split into nine wells in a V shaped 96 well plate (Greiner) and pelleted by centrifugation (3220 g, 10 min). Bacteria were resuspended in primary antibody dilutions in BSA-PBS (primary antibody: Prebleed, post boost and pre-fusion mouse sera at dilution 1 in 100, monoclonal antibodies B3 and H16 and a monoclonal antibody directed against PS-I of *Clostridium difficile (1)* as isotype control (concentrations: 5 µg/mL and 0.5 µg/mL) and *S. pneumoniae* serotype 4 typing serum (SSI Diagnostica, dilution 1 in 100) as positive control. The plate was incubated under shaking at 240 rpm for 1 h at RT. Bacteria were washed three times with BSA-PBS by resuspension in 200 µL and centrifugation and subsequently resuspended in secondary antibody dilution in

BSA-PBS (goat anti-mouse IgG FITC (Sigma) diluted 1 in 100; goat anti-rabbit IgG FITC (Abcam) 1 in 100 for the rabbit serum) also containing 1.5 nM 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) as nuclear stain. The plate was incubated under shaking at 240 rpm for 45 min at RT in the dark. Bacteria were washed three times with BSA-PBS and finally resuspended in 50 µL PBS for imaging on a LSM 700 (Carl Zeiss) confocal LASER-scanning microscope.

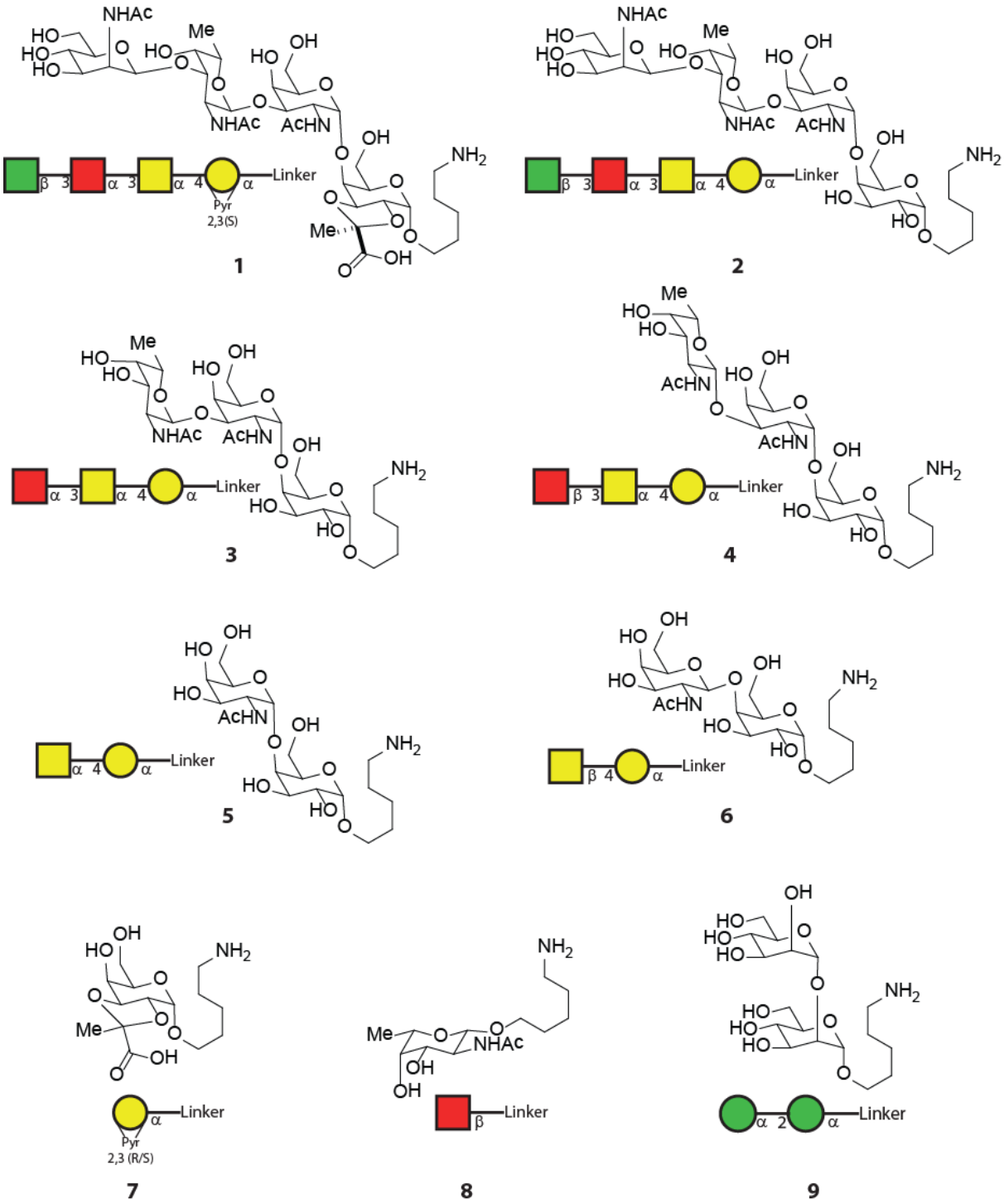
1.7. Depyruvation of ST4 CPS

Four polypropylene tubes (Eppendorf) each containing 1 mg of ST4 CPS (LGC standards / ATCC) in 990 µL water were heated to 98°C using an Eppendorf thermomixer. Diluted hydrochloric acid (1 N in water; 10 µL) was added and the samples were incubated at 98 °C for six minutes and subsequently quickly cooled on ice. Samples were pooled and solvent was exchanged to water using an Amicon centrifugal filter device (MWCO 30,000; Merck Millipore). The sample was lyophilized in aliquots and stored at -20 °C until use. For experiments, aliquots were dissolved in water at 5 mg/mL and diluted for the respective application. Aliquots that were dissolved were kept in that state at -20 °C. NMR characterization was performed on a Varian 600-MR in D_2O at 70 °C with 1 mg of sample. *(2)* Untreated polysaccharide was measured as reference under the same conditions.

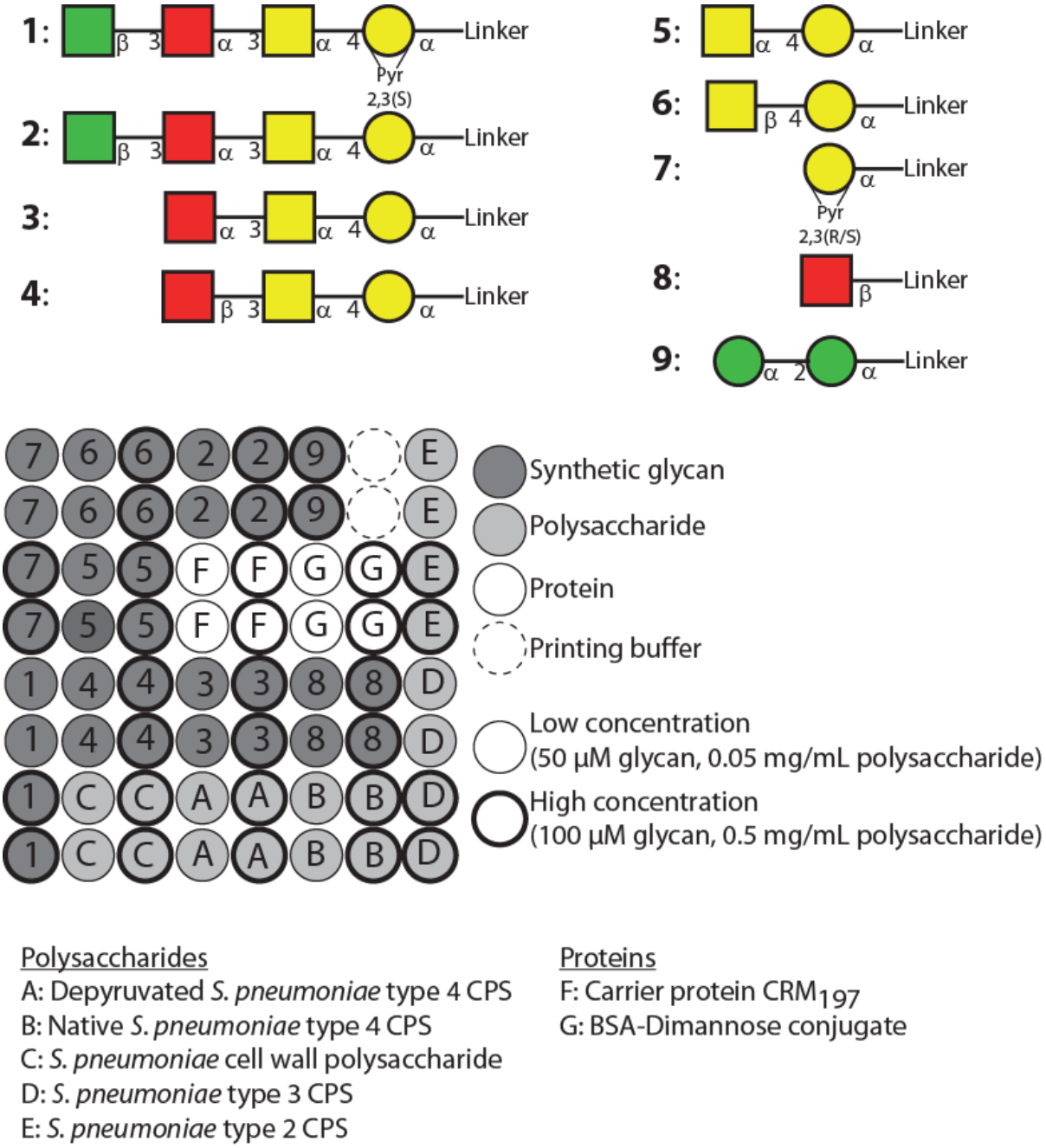
1.8. Surface plasmon resonance (SPR)

SPR measurements using an antibody capture system were performed as described previously on a GE Healthcare Biacore T100 instrument at 25 °C using PBS as running buffer.*(3)* Goat anti-mouse IgG was immobilized in flow path 2 or 4 of a CM5 chip (GE Healthcare) using the mouse antibody capture kit and the amine coupling kit (both GE Healthcare) according to the manufacturer's instructions. Immobilization level of capture antibody was between 12,000 and 14,000 response units (RU). Blank immobilization was used for respective reference flow cell (1 or 3). Monoclonal antibodies were captured at a flow rate of 10 µL/min in PBS at a concentration to achieve a capture level between 500 and 1,000 RU. Sugars were flowed over the surface at a flow rate of 30 µL/min in running buffer during association phase. Regeneration was performed with 10 mM glycine/HCl pH 1.7 for 30 s. Double referenced sensorgrams (signals of both of reference cell and blank buffer injection subtracted) were evaluated with the Biacore T100 Evaluation Software (GE Healthcare) using the one-to-one kinetic model.

2. Supplementary Figures



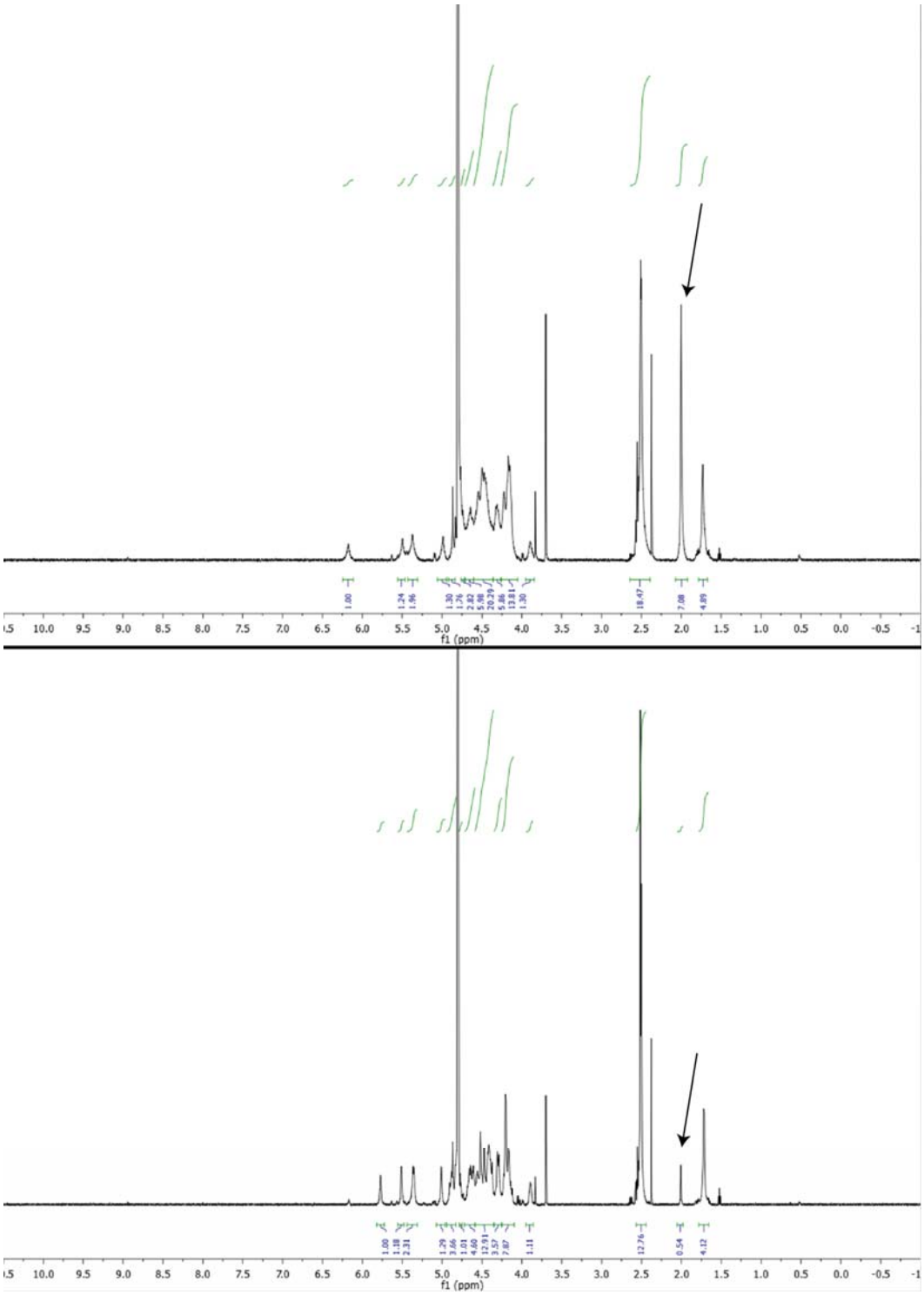
Supplementary Figure 1: Complete chemical structures of synthetic glycans used in the study and their symbol representations. Synthesis was described by Pereira et al.*(4)*



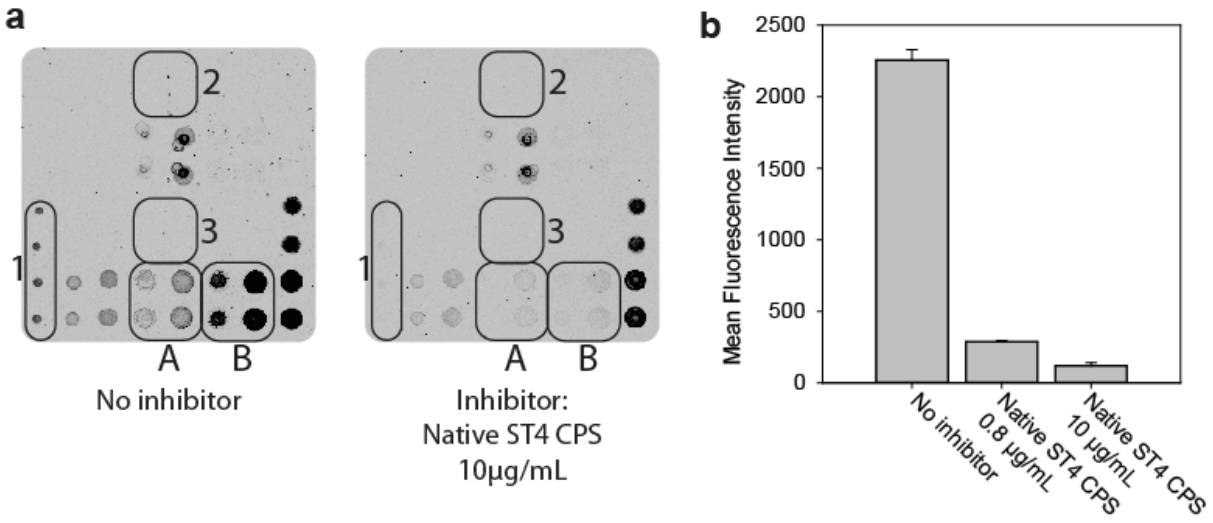
Polysaccharides
A: Depyruvated *S. pneumoniae* type 4 CPS
B: Native *S. pneumoniae* type 4 CPS
C: *S. pneumoniae* cell wall polysaccharide
D: *S. pneumoniae* type 3 CPS
E: *S. pneumoniae* type 2 CPS

Proteins
F: Carrier protein CRM_{197}
G: BSA-Dimannose conjugate

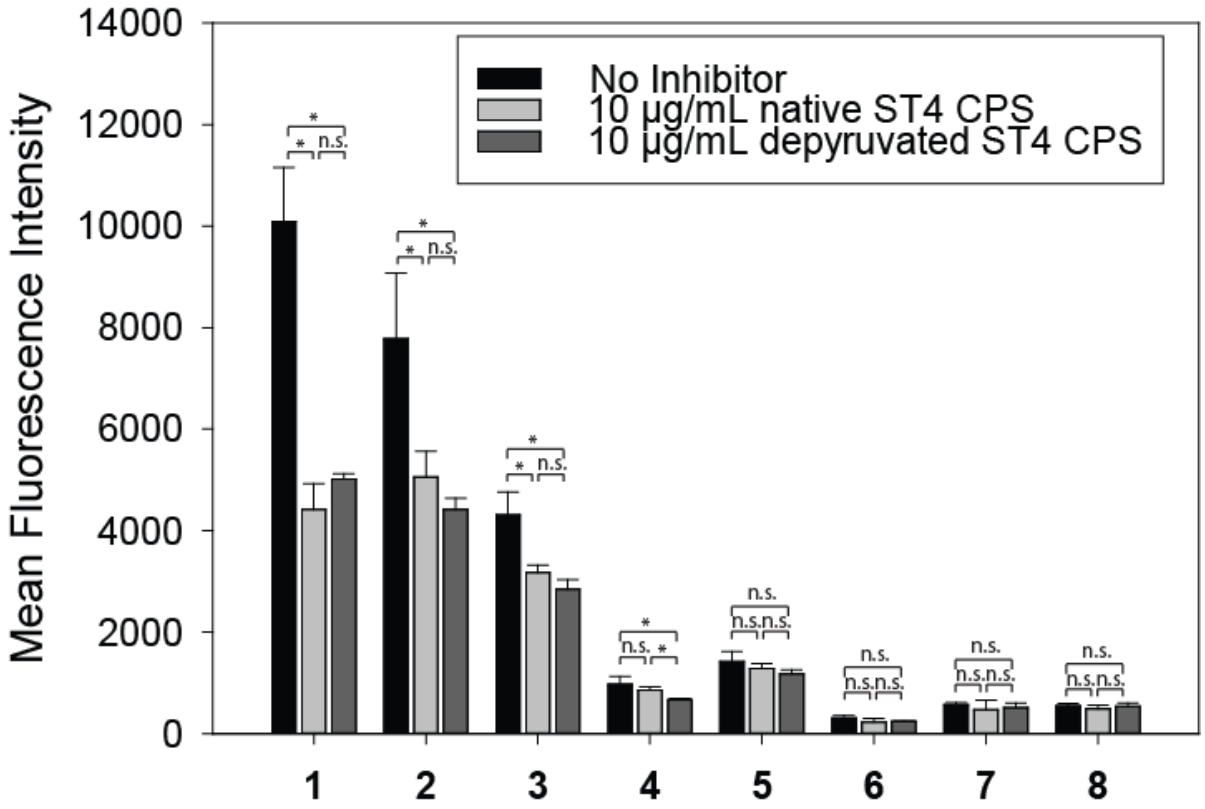
Supplementary Figure 2: Detailed printing pattern of glycan microarrays. Dimannoside **9** and its BSA conjugate have been described previously. *(5)*



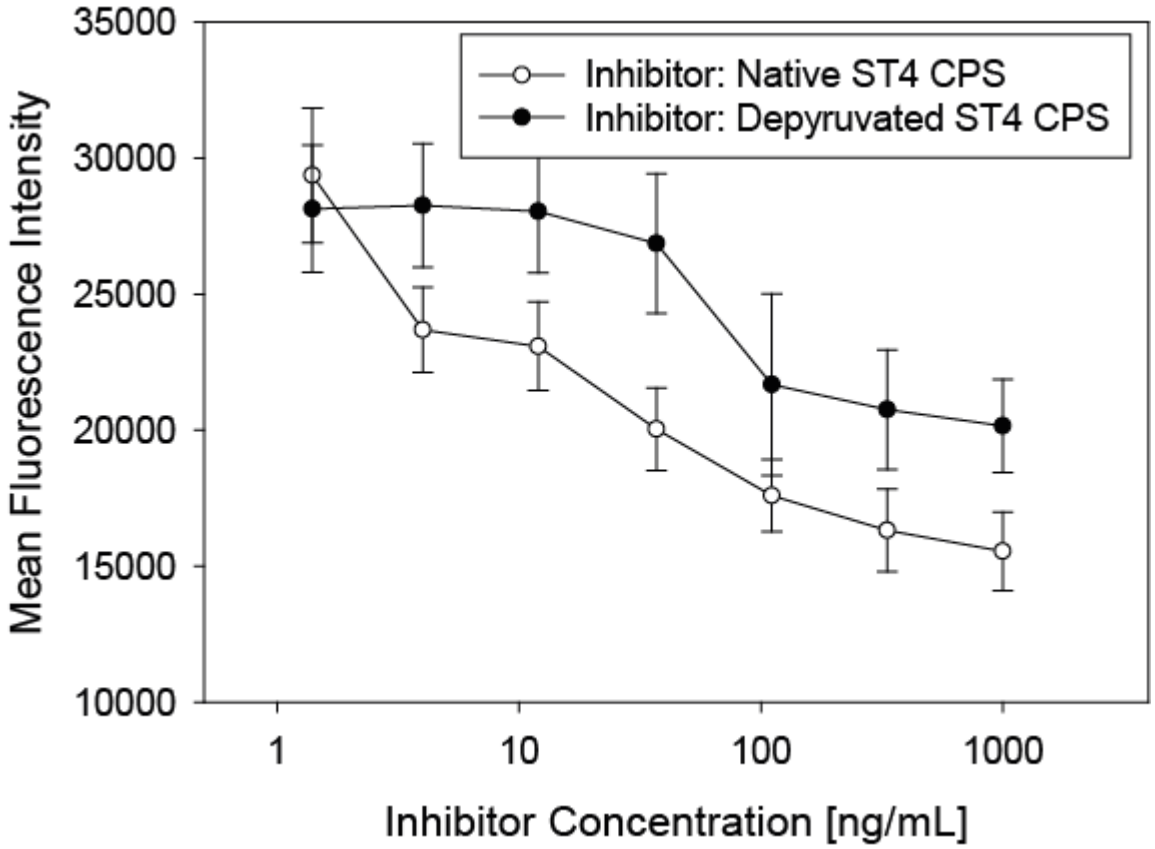
Supplementary Figure 3: ^{1}H-NMR comparison of native (upper panel) and depyruvated (lower panel) capsular polysaccharide from *S. pneumoniae* serotype 4 (ST4 CPS). The peak corresponding to the resonance of the pyruvate methyl group protons is strongly diminished after the depyruvation procedure (marked with arrow in both spectra).



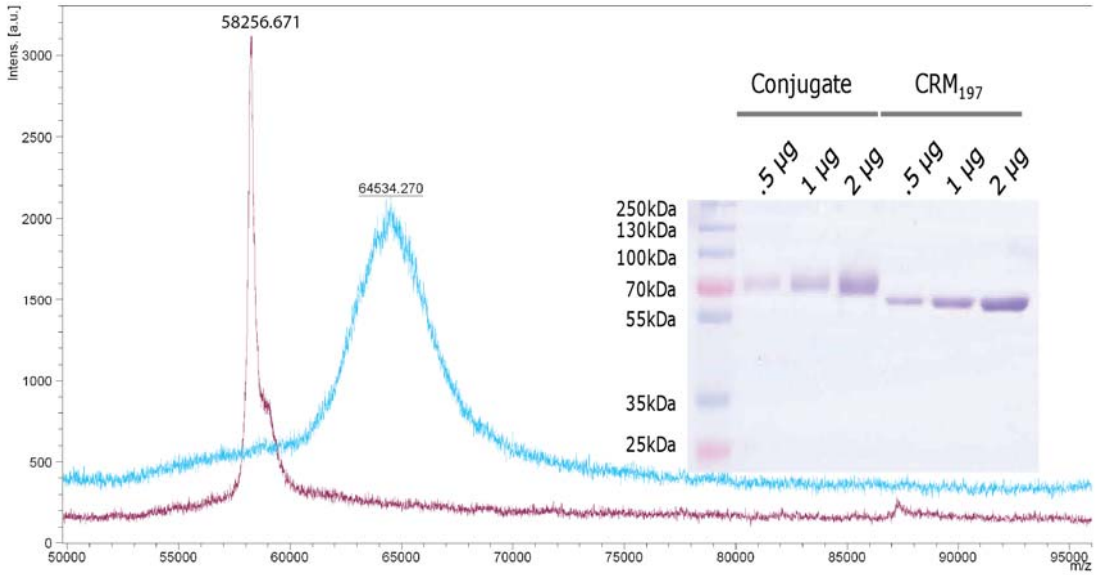
Supplementary Figure 4: Glycan array analysis of pooled serum (diluted 1 in 200) from mice immunized with the commercial pneumococcal conjugate vaccine Prevenar-13. As seen for rabbit ST4 typing serum before,*(4)* of all synthetic structures the pyruvated tetrasaccharide **1** is exclusively bound and binding can be inhibited by adding ST4 CPS to the sample prior to microarray incubation. Of the native capsular polysaccharides, ST3 and ST4 are detected, but not ST2 as this one is not included in Prevenar-13. a) Representative glycan array images. b) Inhibition efficiency by comparison of fluorescence intensities with and without native ST4 CPS as inhibitor.



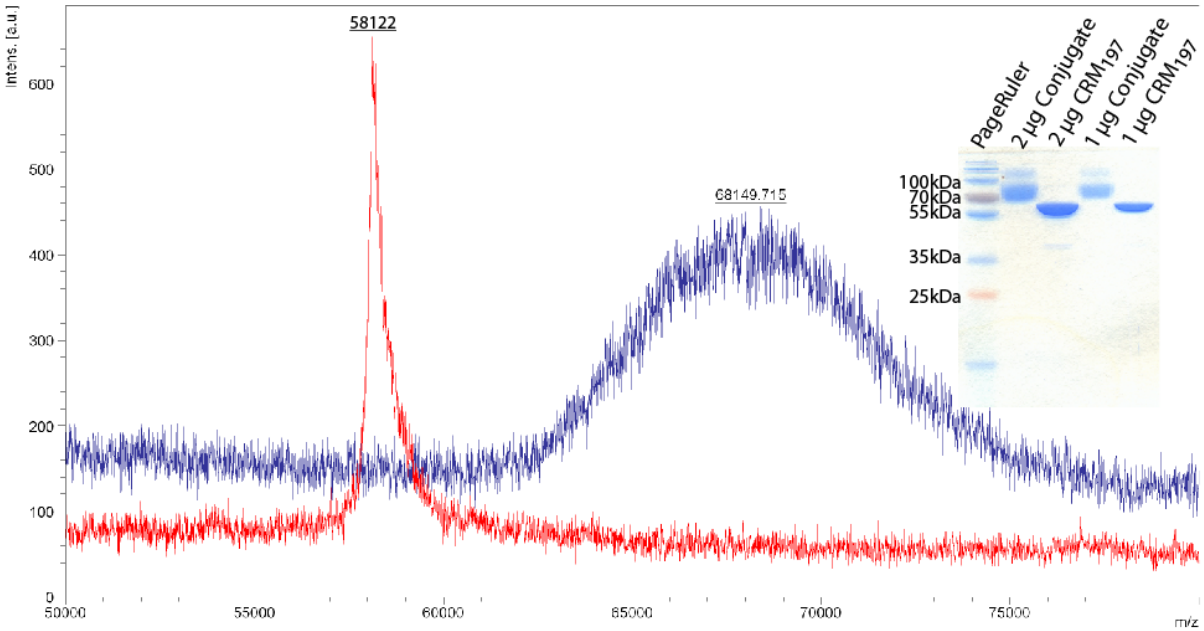
Supplementary Figure 5: IgG levels in human reference serum 007sp towards synthetic ST4 CPS oligosaccharides. The reference serum was diluted 1:and incubated on the slide in the presence or absence of native or depyruvated ST4 CPS to sequester antibodies specific for the polysaccharides. Each bar is the mean of four spots with the error bar giving the standard deviation. Significance levels (Mann-Whitney rank sum test): *: $p < 0.05$; n.s.: $p > 0.05$



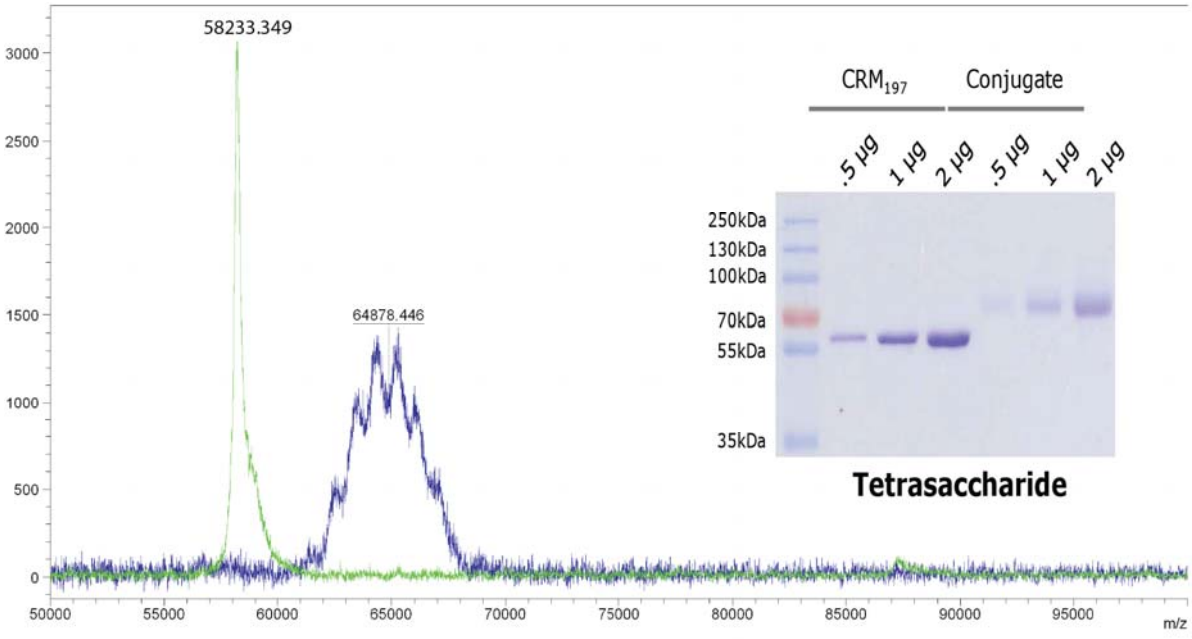
Supplementary Figure 6: Comparison of inhibition efficiency towards pyruvated tetrasaccharide **1** between native and depyruvated ST4 CPS. As expected, the native ST4 CPS is the better inhibitor and most of the inhibition ability of the depyruvated ST4 CPS can probably be attributed to the few remaining pyruvyl groups (see Supplementary Figure 3).



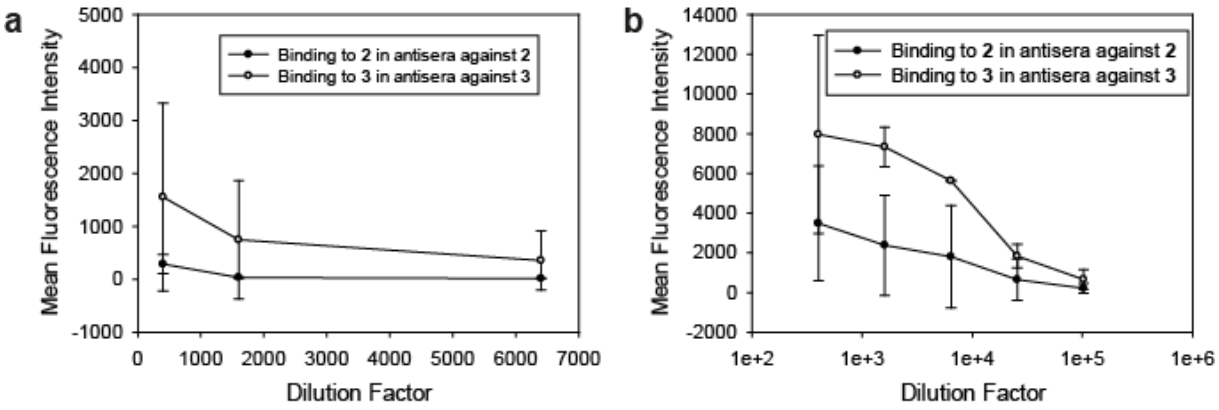
Supplementary Figure 7: Characterization of conjugate of carrier protein CRM_{197} and depyruvated trisaccharide **3** (used for first immunization). The difference in molecular weight as measured by MALDI-TOF MS compared to the unconjugated CRM_{197} is 6300 Da corresponding to an average of eight saccharides bound to each protein molecule. In the inset, a mass shift is clearly visible on SDS PAGE.



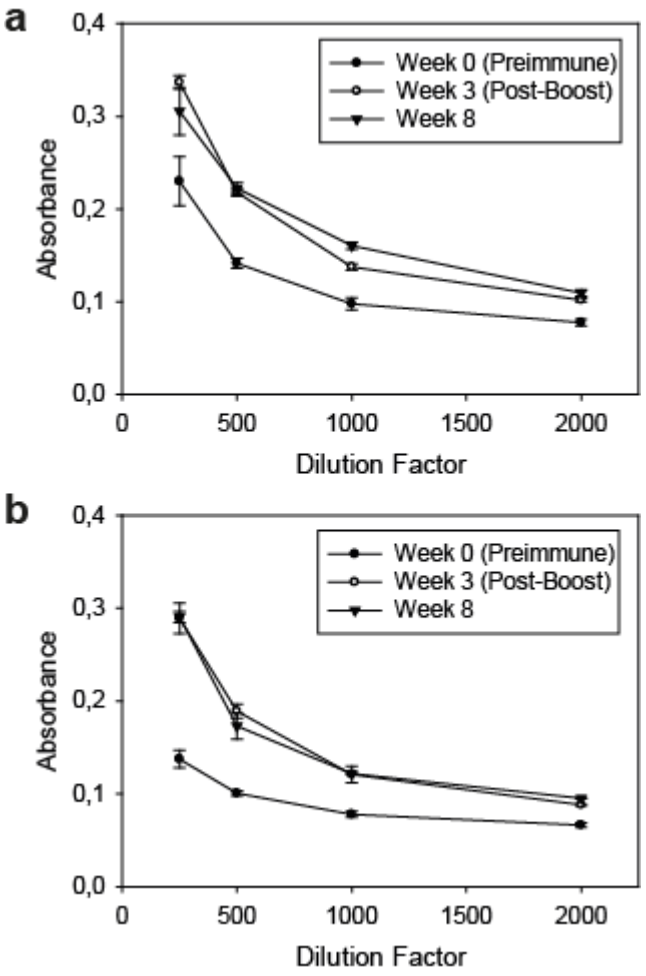
Supplementary Figure 8: Characterization of conjugate of carrier protein CRM_{197} and depyruvated trisaccharide **3** (used for second immunization in order to develop monoclonal antibodies) The difference in molecular weight as measured by MALDI-TOF MS compared to the unconjugated CRM_{197} is 10000 Da corresponding to an average of 13 saccharides bound to each protein molecule. In the inset, a mass shift is clearly visible on SDS PAGE.



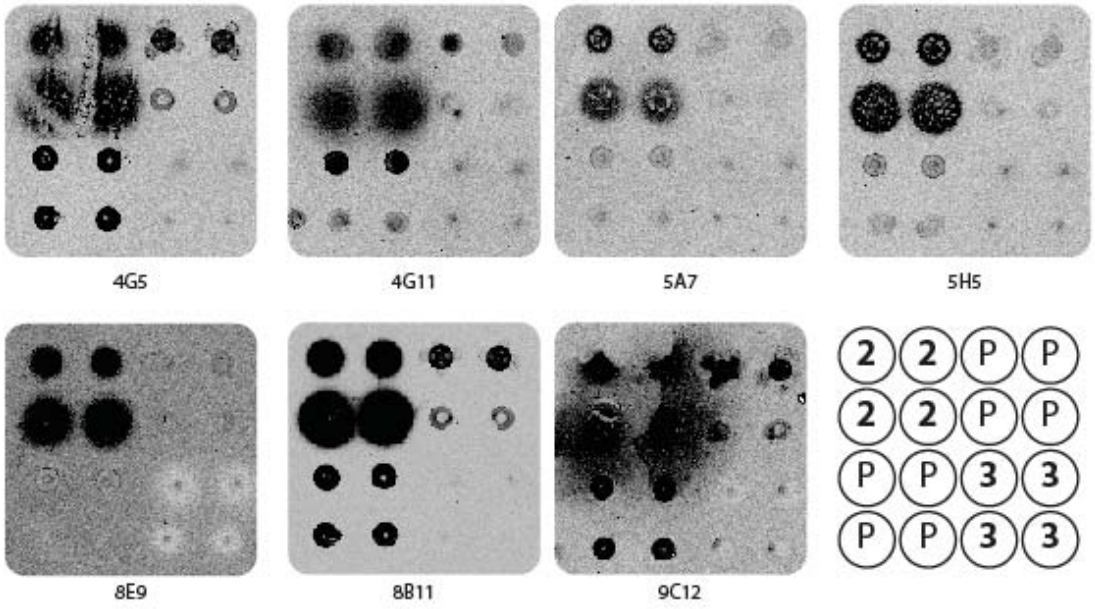
Supplementary Figure 9: Characterization of conjugate of carrier protein CRM_{197} and depyruvated tetrasaccharide **2**. A clear bandshift on SDS PAGE (insert) to higher molecular weights is visible for the conjugate compared to native CRM_{197}. Difference in molecular weight as measured by MALDI-TOF MS is 6650 Da corresponding to an average of seven bound glycans per CRM_{197} molecule.



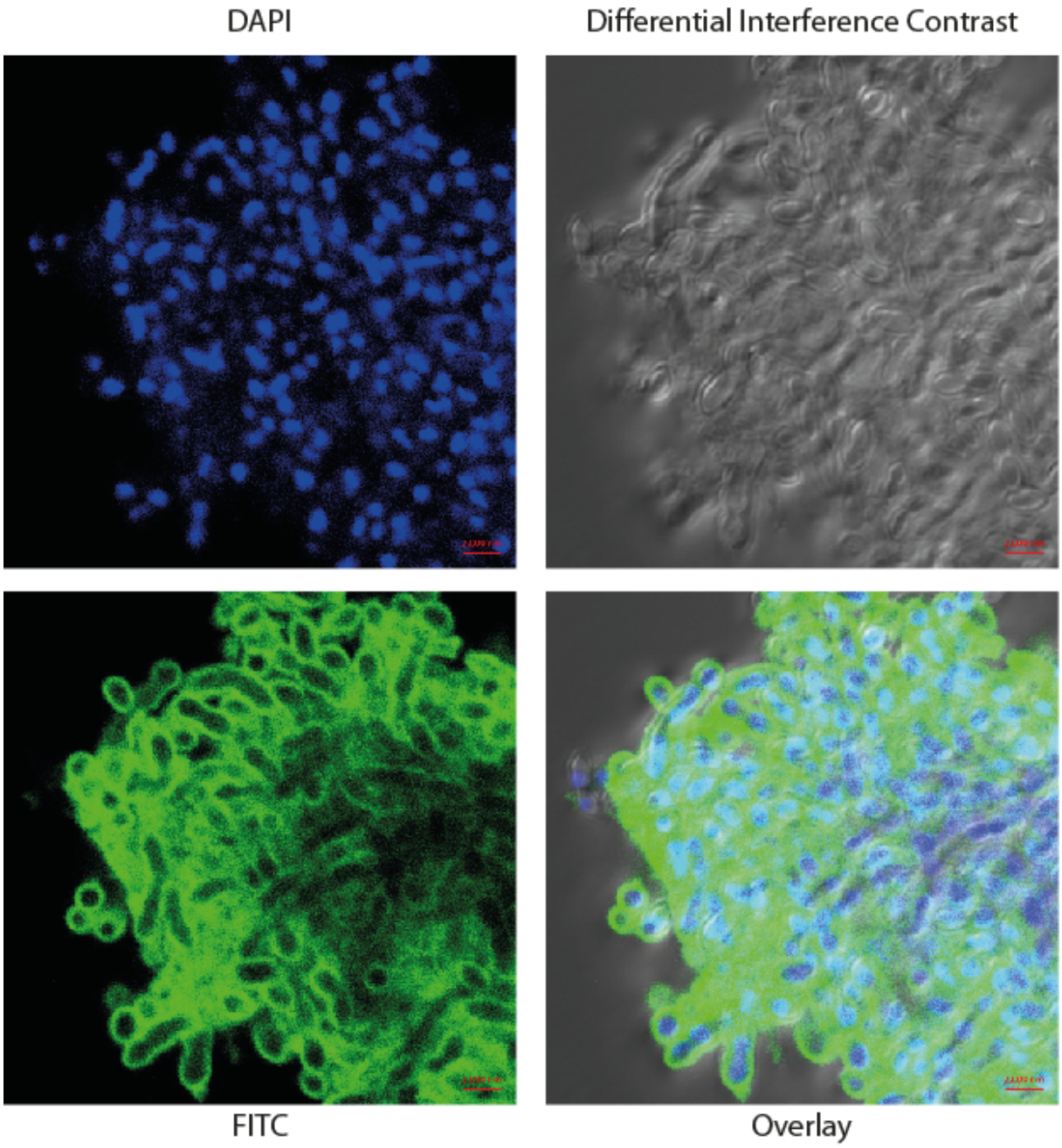
Supplementary Figure 10: Antibody titers in mice immunized with glycoconjugates of tetrasaccharide **2** and trisaccharide **3** two weeks post priming (a, primary response) and at week 8 (b).



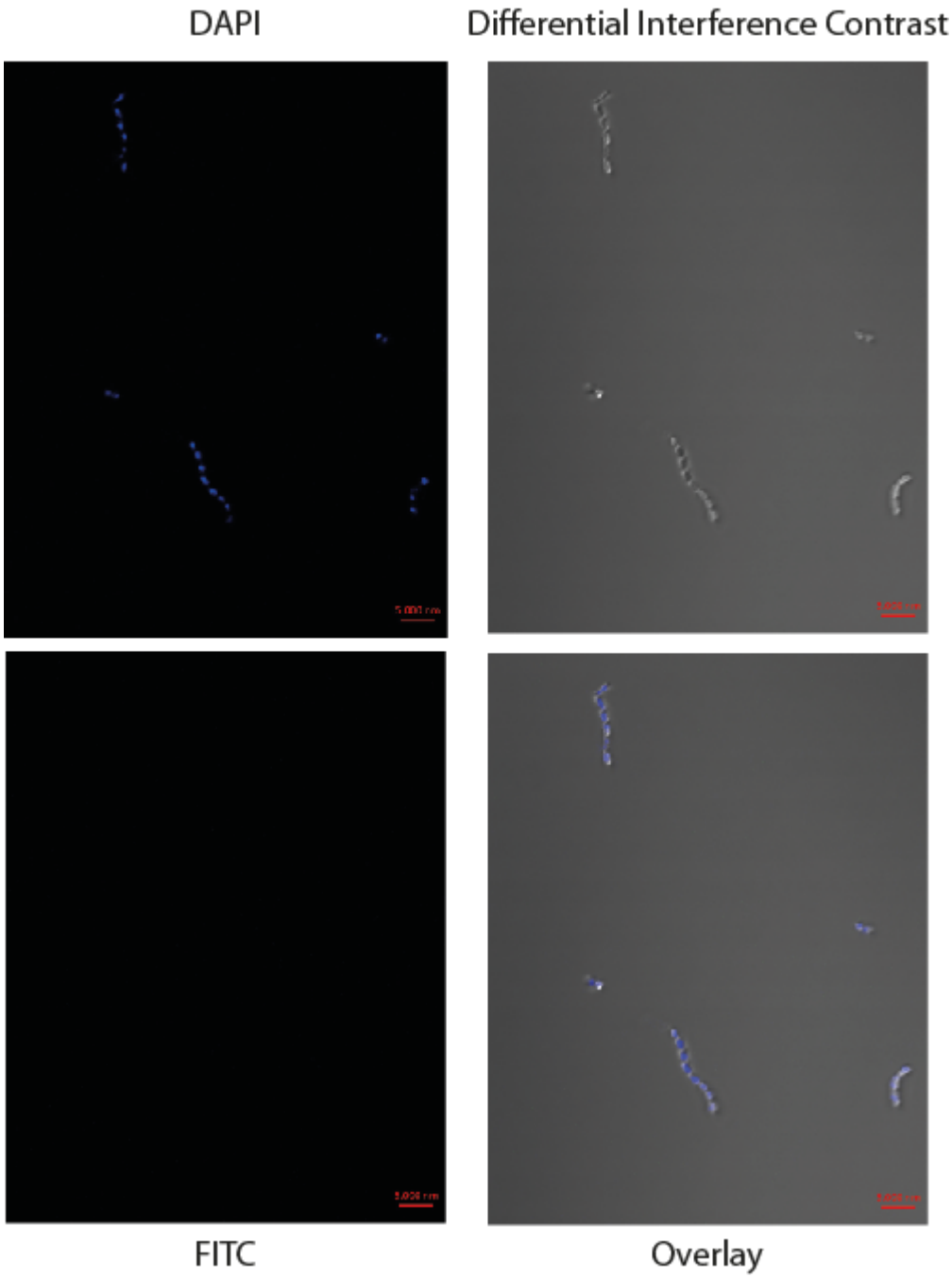
Supporting Figure 11: Raise in antibodies levels towards the native ST4 CPS in mice determined by ELISA (pooled serum, mean of absorbance of three wells, error bars represent standard deviation) immunized with glycoconjugates of the depyruvated trisaccharide **3** (b) or the depyruvated tetrasaccharide **2** (a). For both constructs, only a slight increase in titers is seen between post-boost sera (week 3) and preimmune sera (week 0). Antibody levels stay constant afterwards while strong titer increases are still seen towards the synthetic structures, probably due to affinity maturation that does not influence binding to native CPS (Figure 3F and G).



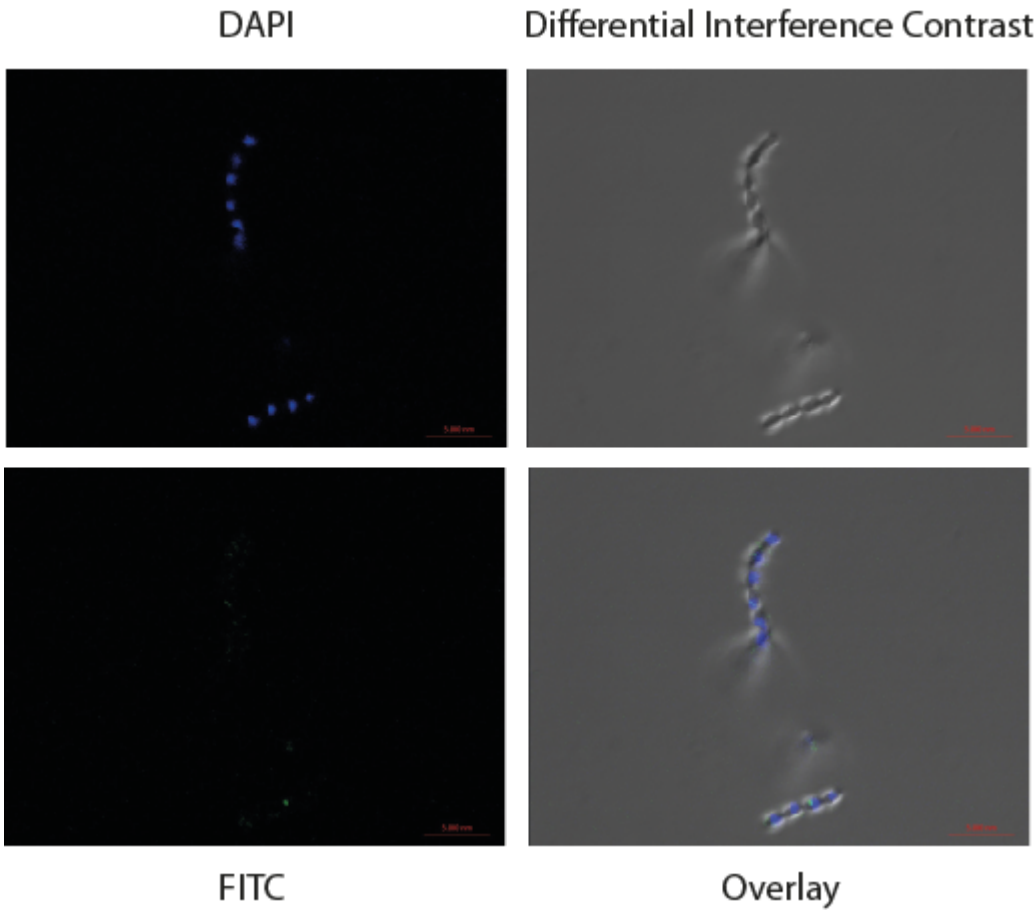
Supplementary Figure 12: Selected wells of glycan array analysis of supernatants from fusion plates (well positions indicated) for development of hybridomas secreting monoclonal antibodies against tetrasaccharide **2**. Glycan array design differs from those used in other experiments of this study and is outlined in the bottom right corner. (P: Spots printed with protein control constructs)



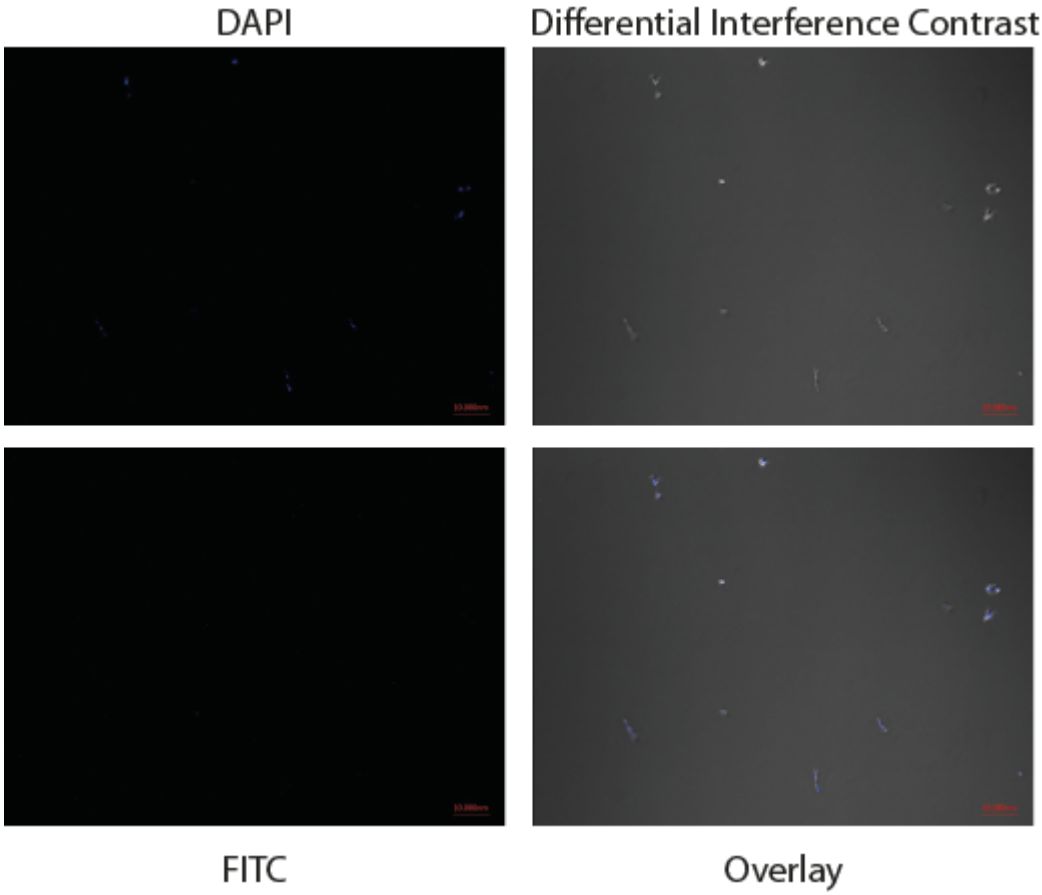
Supplementary Figure 13: Positive control of immunofluorescence staining of UV inactivated TIGR4 pneumococci. In contrast to sera of mice immunized with neoglycoconjugates of depyruvated ST4 repeating unit based glycans (Supplementary Figures 14 and 15) and monoclonal antibodies against trisaccharide **3** (Supplementary Figure 16), the rabbit ST4 typing sera shows agglutination of bacteria and a strong staining of the capsule (FITC channel).



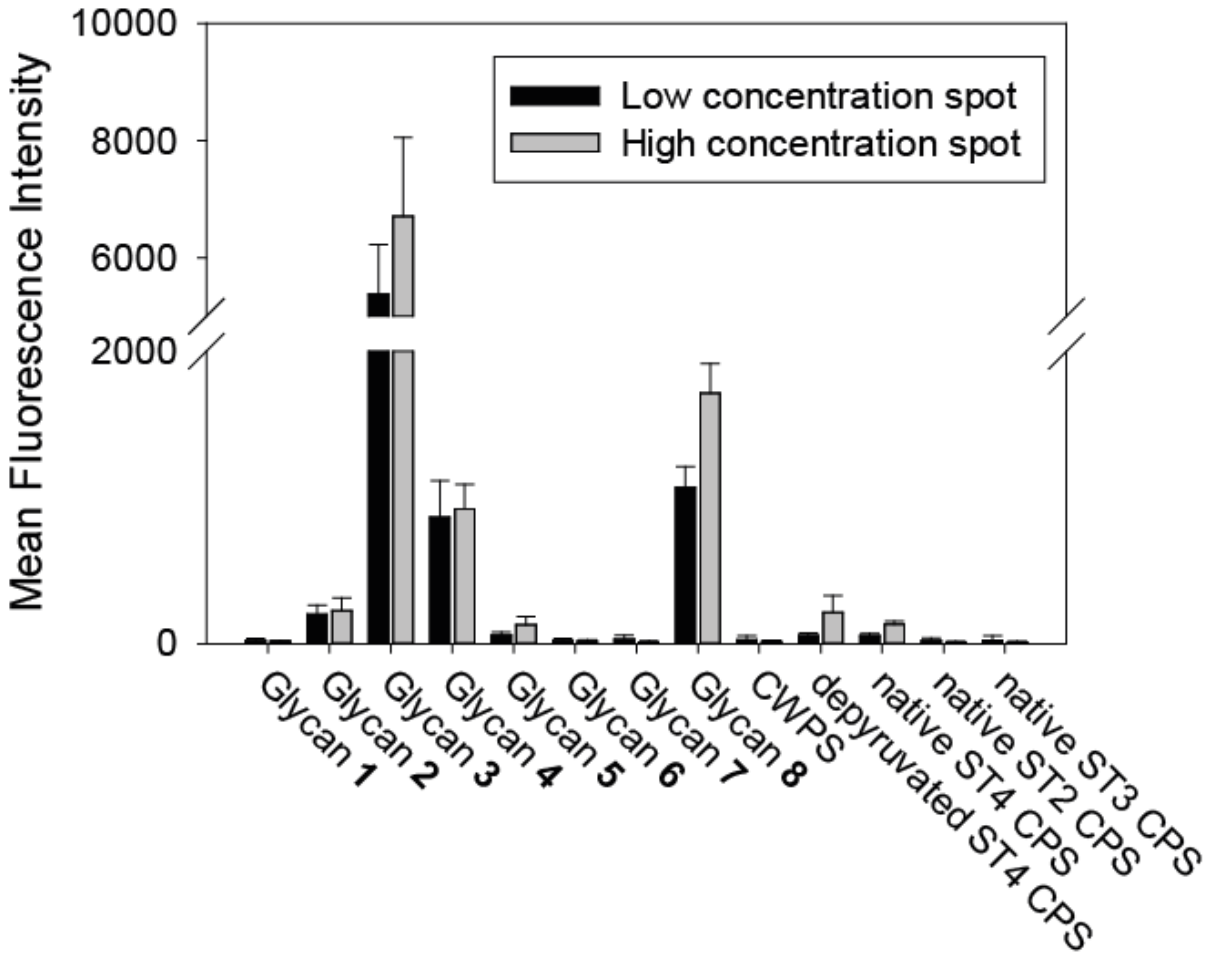
Supplementary Figure 14: Immunofluorescence staining of TIGR4 pneumococci with antiserum against tetrasaccharide **2**. No agglutination of bacteria and no staining of the capsule (FITC channel) is visible.



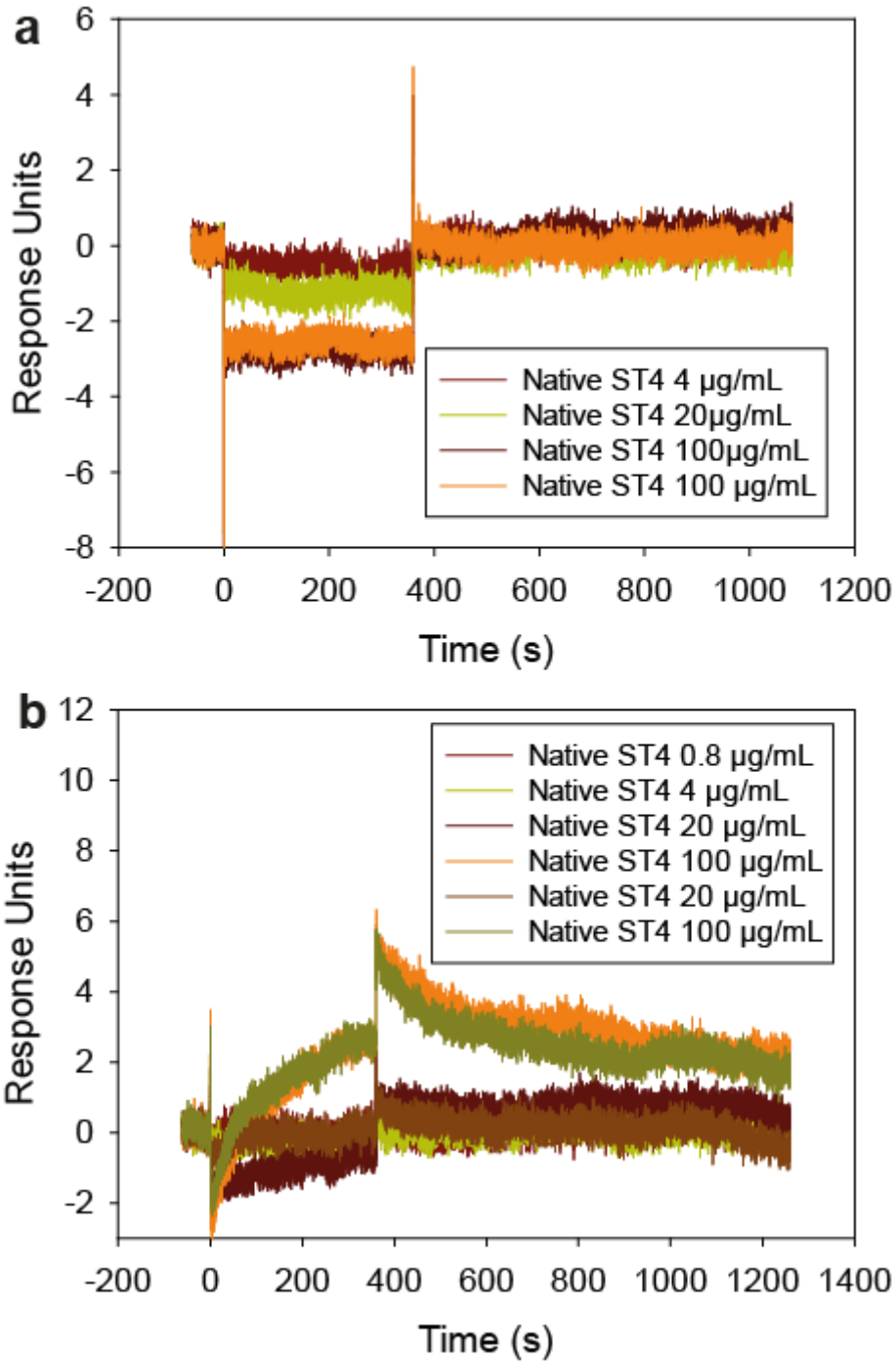
Supplementary Figure 15: Immunofluorescence staining of TIGR4 pneumococci with antiserum against trisaccharide **3**. No agglutination of bacteria and no staining of the capsule (FITC channel) is visible.



Supplementary Figure 16: Immunofluorescence staining of TIGR4 pneumococci with purified monoclonal antibody H16 (5 µg/mL) against trisaccharide **3**. No agglutination of bacteria and no staining of the capsule (FITC channel) is visible.



Supplementary Figure 17: Binding profile of monoclonal antibody B3 raised against synthetic trisaccharide **3** towards the set of synthetic glycans and isolated polysaccharide on glycan array. Mean of four spots per value with error bars giving standard deviation.



Supplementary Figure 18: SPR analysis of monoclonal antibody binding to ST4 CPS a) Less affine monoclonal antibody B3 shows no binding to isolated ST4 CPS even at a high concentration of 100 µg/mL.b) Higher affine monoclonal antibody H16 shows binding to ST4 CPS in solution phase at high concentrations. However, response is very low compared to trisaccharide **3** that the antibody is directed against while in case of strong binding the much larger CPS should result in a much higher response. Due to the high number of possible epitopes on the repetitive polysaccharide and the resulting multivalence, a quantitative evaluation of the curves is not possible.

3. References

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