

Supplementary material

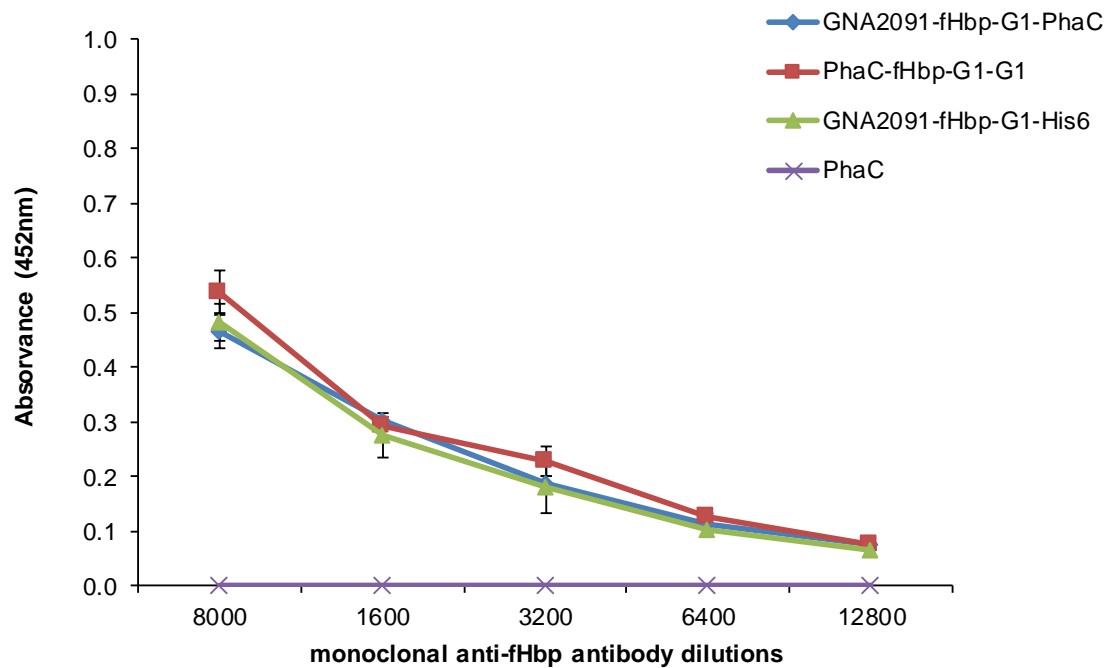
Bioengineered polyester beads co-displaying protein and carbohydrate-based antigens induce protective immunity against bacterial infection

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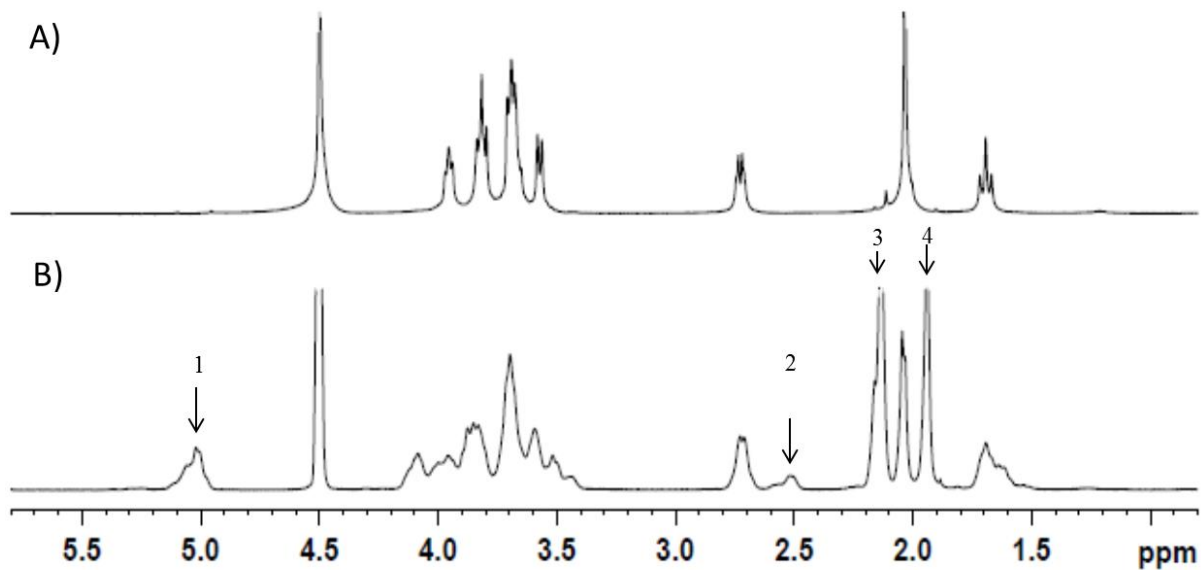
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Supplementary Figure 1:

FHbp confirmation of molecular identity and bead display by ELISA using a commercial monoclonal anti-fHbp antibody (JAR4, NIBCS, UK).

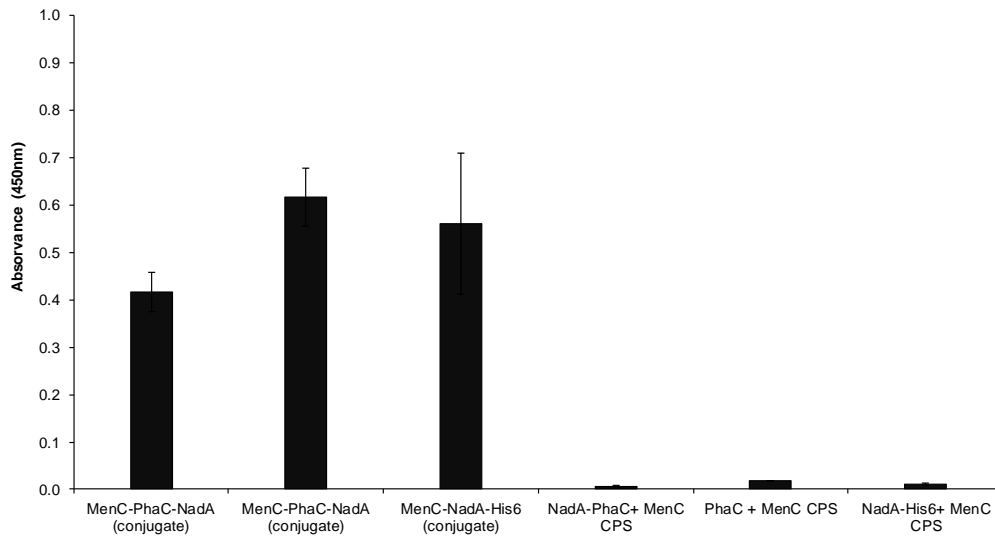
Maxisorp 96-well plates (NUNC) were coated using 0.5 $\mu\text{g/ml}$ of each protein and the rest of assay was performed as mentioned above¹. The results are expressed as the mean \pm SD of two replicas per sample.



Supplementary Figure 2:

¹H NMR monodimensional spectra of CPS.

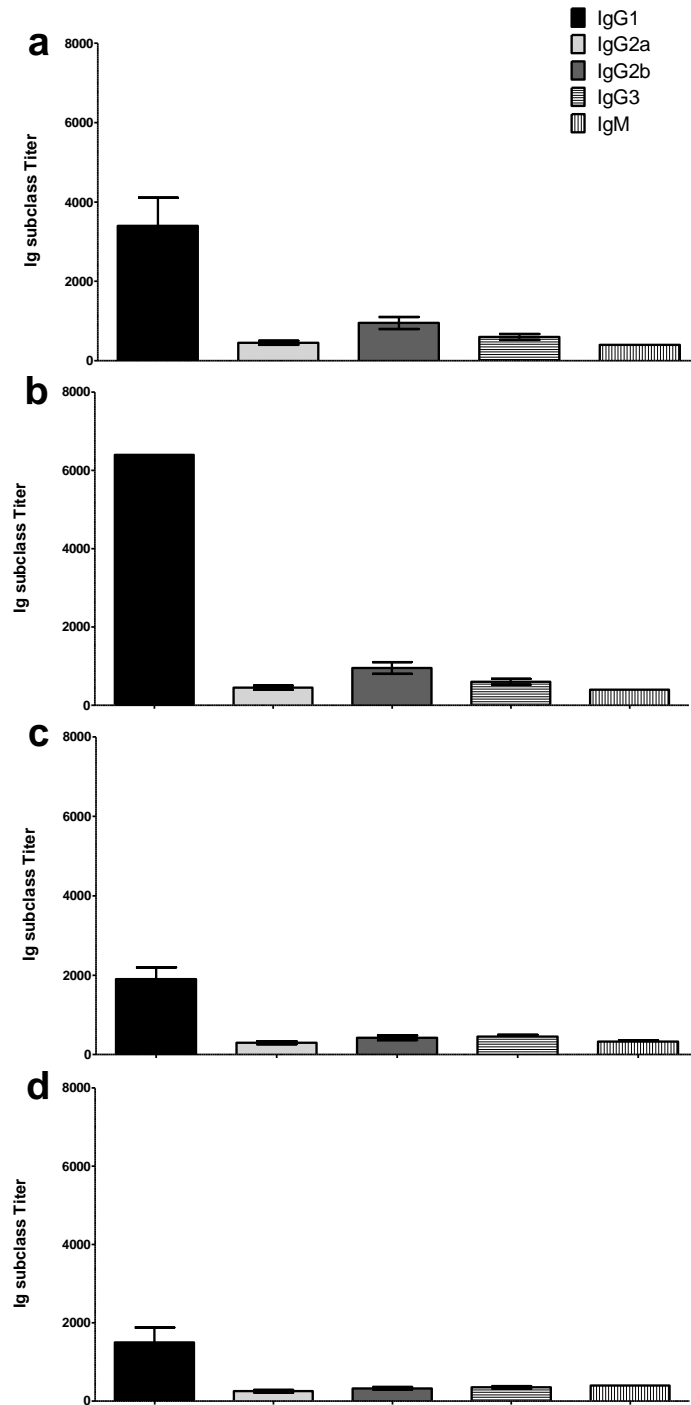
A) Activated polysaccharide (APS) and B) Capsular polysaccharide (CPS), both from *Neisseria meningitidis* Serogroup C. The peaks 1,2,3, and 4 are missing in the Aps spectrum because the O-Acetyl groups were removed during activation reaction, but the rest of the chemical structure are conserved after activation as mentioned above ².



Supplementary Figure 3:

MenC confirmation of molecular identity by ELISA using a commercial anti-CPS (MenC) monoclonal antibody (NIBS, UK).

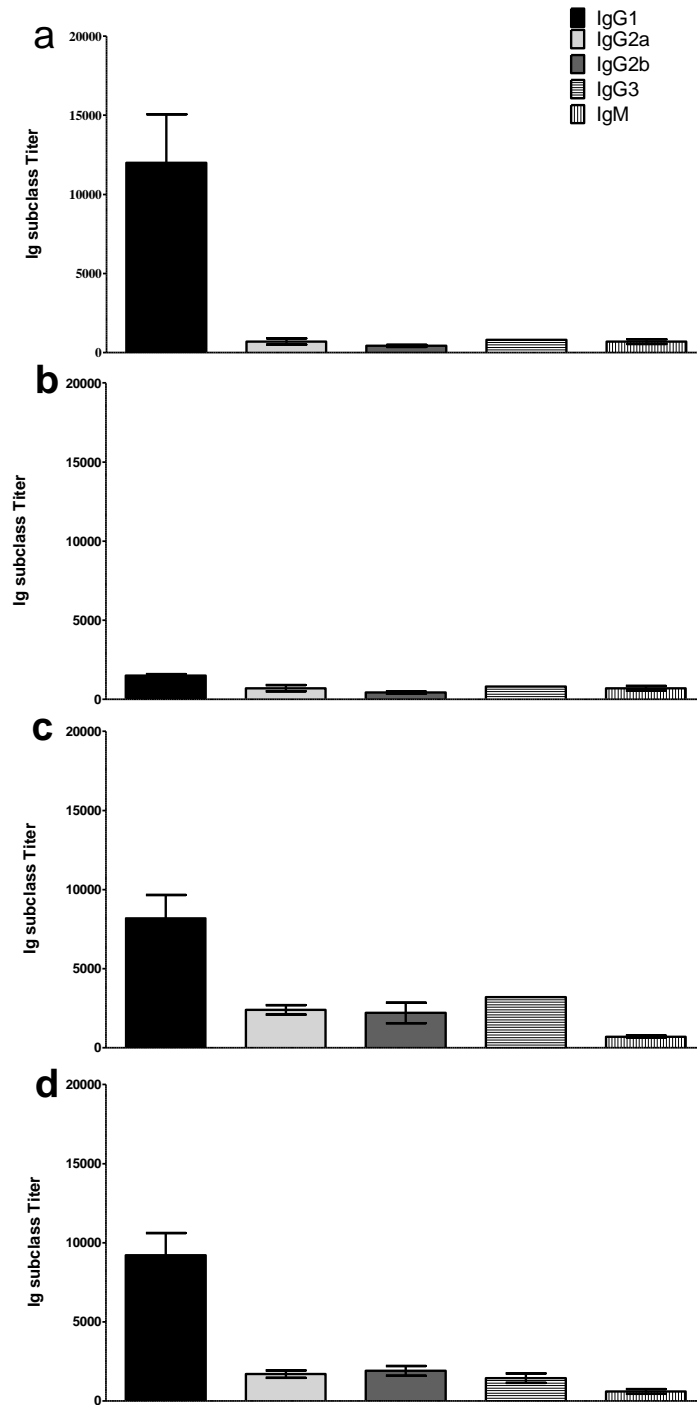
Maxisorp 96-well plates (NUNC) were coated using 5 $\mu\text{g/ml}$ of MenC CPS conjugated or mixed to NadA-PhaC beads, PhaC wild type beads and NadA-His6 the rest of assay was performed as mentioned above¹. The results are presented as the mean \pm SD of two replicas. The results are expressed as the mean \pm SD of two replicas per sample.



Supplementary Figure 4:

Assessment of IgG subclass binding to MenC evaluated by ELISA.

Results are expressed as the mean \pm SEM of 8 animals and the titer was calculate as mentioned above. **a.** animals vaccinated with 4 μ g of MenC conjugated to NadA-PhaC beads; **b.** animals vaccinated with 4 μ g of MenC conjugated to PhaC wild type beads; **c.** animals vaccinated with 4 μ g of MenC conjugated to soluble NadA-His6; **d.** animals vaccinated with 4 μ g of MenC conjugated to DT.



Supplementary Figure 5:

Assessment of IgG subclass binding to NadA protein evaluated by ELISA.

Results are expressed as the mean \pm SEM of 8 animals and the titer was calculate as mentioned above. **a.** animals vaccinated with 4 μ g of MenC conjugated to NadA-PhaC beads; **b.** animals vaccinated with 4 μ g of MenC conjugated to soluble NadA-His6; **c.** animals vaccinated with 18 μ g of protein from NadA-PhaC beads; **d.** animals vaccinated with 18 μ g of protein from NadA-PhaC beads without alum.

Supplementary Table 2: Identification of fusion proteins by peptide fingerprinting analysis (MALDI-TOF/MS)

| Fusion proteins | Peptides fragment identified by MALDI-TOF/MS.* |
|------------------------------|---|
| <u>NadA</u> -PhaC | <u>A11-K47, L134-K146, Q267-R309, A317-K341</u> , V482-R498, I535-K552, F608-641, I657-K691, Y712-K874, L928-K979, R1025-R1035 |
| <u>GNA2091-fHbp-G1</u> -PhaC | <u>T31-R45, G52-R64, Q78-105, V170-R180, D212-R224, F259-R263, I315-R347, S388-R395, G405-R414</u> , V641-R476, I503-R514, A531-R565, F586-R620, I635-R647, Y690-R745, E811-R826, H873-K938, F940-K958, A1004-R1023 |
| PhaC- <u>fHbp-G1-G1</u> | A2-W31, A59-Y65, L161-L171, T191-T218, T232-L240, V262-F273, I305-L316, A333-L346, R386-W404, L546-W554, R575-Y584, <u>A592-I638, T643-Y665, T725-M731, R738-Y77, K793-Y822</u> |

* Red bold, identified peptides belonging to the respective neisserial antigen.

Supplementary Table 3:**Correlation between Zeta potential and pH of various PHB beads.**

| PHB beads | Theoretical pI of the respective protein* | Zeta potential (mV) pH (3.5) ** | Zeta potential (mV) pH (5.5) ** | Zeta potential (mV) pH (7.5) ** |
|--|--|--|--|--|
| NadA-PhaC on PHB beads | 5.01 | 5.20 ± 0.9 | -13.3 ± 2.2 | -20.4 ± 3.2 |
| GNA2091-fHbp-G1-PhaC on PHB beads | 6.77 | 16.2 ± 1.4 | -8.9 ± 0.9 | -16.5 ± 2.5 |
| PhaC-fHbp-G1-G1 on PHB beads | 6.21 | 11.1 ± 2 | -8.4 ± 0.7 | -17.9 ± 2 |
| PhaC, (non-antigen displaying PHB beads) | 6.08 | 14.4 ± 1.8 | -5.7 ± 0.9 | -16.8 ± 0.35 |

* [ExPASy - Compute pI/Mw tool](#) (theoretical isoelectric point (pI)); ** mean of 3 replicates ± SD.

Supplementary Table 4:**Amount of neisserial antigen attached to PHB beads and immunization doses.**

| PHB beads | neisserial antigen/ wet Bead (µg/mg) | neisserial antigen immunization dose (µg) | PHB beads immunization dose (mg) |
|--------------------------------|---|--|---|
| NadA-PhaC | 0.254 | 2 | 10 |
| GNA2091-fHbp-G1-PhaC | 0.677 | 7 | 10 |
| PhaC-fHbp-G1-G1 | 0.110 | 1 | 10 |
| PHB beads | PhaC/ wet Bead (µg/mg) | PhaC immunization dose (µg) | PHB beads immunization dose (mg) |
| PhaC, (non-antigen displaying) | 0.977 | 7 | 7 |

Supplementary Table 5:

Carbohydrate/protein ratios and carbohydrate yield after conjugation and purification.

| Conjugated | Carbohydrate/protein ratio (mg/mg) | Carbohydrate yield (%) |
|----------------------|---|-------------------------------|
| MenC-NadA-PhaC beads | 0.20/1 | 4.0 |
| MenC-PhaC beads | 0.27/1 | 4.4 |
| MenC-NadA-His6 | 2.4/1 | 30.2 |
| MenC-DT | 2.5/1 | 36.0 |

Supplementary Table 6

Conjugation site analysis results by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS). Tryptic and chymotryptic digests of non-conjugated and conjugated proteins either soluble or attached to PHB beads were analysed by liquid chromatography-coupled LTQ-Orbitrap tandem mass spectrometry. Significant differences in peptide abundances between the non-conjugated and conjugated proteins were identified by comparing precursor peak intensities using the SIEVE software (ThermoScientific).

| Samples | Chymotryptic (C)/tryptic (T) peptides >3-fold more abundant in non-conjugated sample versus conjugated sample based on LC/MS-MS analysis* | Amino acid sequence of targeted protein showing putative conjugations sites in bold |
|--|--|--|
| PhaC versus conjugated PhaC displayed on PHB beads (C) | <p>NARALTELADAVEADAKTRQRIR F GNARYRAIEPAPGRYVKAKA TGKGAAASTQEGKSQPF+Carbami domethyl (13) Formyl (3) AAIRAIEVARDISGQDKINVL RAIEPAPGRYVKAKA</p> | <p>PhaC (64.2kDa)</p> <p>MATGKGAAASTQEGKSQPF KVTPGPFDPATWLEWSRQW QGTEGNGHAAASGIPGLDAL AGVKIAPAQLGDIQQRYMKD FSALWQAMAEGKAEATGPL HRRRFAGDAWRTNLPYRFA AAFYLLNARALTELADAVEA DAKTRQRIRFAISQWVDAMS PANFLATNPEAQRLLIESGGE SLRAGVRNMEDLTRGKISQ TDESAFEVGRNVAVTEGAVV FENEYFQLLQYKPLTDKVHA RPLLMVPPCINKYYILDLQPE SSLVRHVVEQGHTVFLVSWR NPDASMAGSTWDDYIEHAAI RAIEVARDISGQDKINVLGFC VGGTIVSTALAVLAARGEHP AASVTLTLLDFADTGILDV FVDEGHVQLREATLGGGAG APCALLRGGLELANTFSFLRPN DLVWNYVVDNYLKGNTPV FDLLFWNGDATNLPGPWYC WYLRHTYLQNELKVPGKLT VCGVPVDLASIDVPTYIYGSR EDHIVPWTAAYASTALLANK LRFVLGASGHIAGVINPPAKN KRSHWTNDALPESPQQWLA GAIEHHGSWWPDWTAWLAG QAGAKRAAPANYGNARYRA IEPAPGRYVKAKA</p> |

| | | |
|--|---|---|
| | | Lysine residues (labelled bold) represent putative polysaccharide conjugations sites: K : 5, 15, 139, 312, 498, 586, 588 |
| NadA-PhaC versus conjugated NadA-PhaC displayed on PHB beads (C) | AAIRAIEVARDISGQDKINVL ATSDDDVKKKAATVAI ATSDDDVKKKAATVAIVAA DVEADDFKGL NARALTELADAVEADAK NARALTELADAVEADAKTR TKTVNENKQNVDKVK | NadA (1-463)-PhaC (464-1051) fusion protein (112.8kDa) MATSDDDDV K KAATVAIVAA YNNQGEINGFKAGETIYDIGE DGTITQKDATAADVEADDF K GLGLKKVVTNLT K TVNEN K QNVD A K V K AAESEIEKLTTK LADTDAALADTDAALDETTN ALNKLGENITTFAEETKTNIV KIDEKLEAVADTVDKHAEAF NDIADSLDETNTKADEAVKT ANEAKQTAETKQNVD A KV KAAETAAGKAEAAAGTANT AADKAEVA A KVTDIKADIA TNKADIAKNSARIDSLDKNV ANLRKETRQGLAEQAALSGL FQPYNVGRFNVTA A VGGYKS ESAVAIGTGFRFTENFA A KAG VAVGTSSGSS A YHVG V NYE WKAGETIYDIGEDGTITQKD KAGETIYDIGEDGTITQK D KA GETIYDIGEDGTITQK D TSDD DV K KAATVAIVTSDDDDV K AATVAIVT K TVNEN K QNVD A K V K AT K TVNEN K QNVD A K V K ATSATGKGAAASTQEG KSQPFKVTPGPFDPATWLEW SRQWQGTEGNGHAAASGIPG LDALAGVKIAPAQLGDIQQR YMKDFSALWQAMAEGKAE A TGPLHDRRFAGDAWR T NLPY RFA A AFYLLNARALTELADA VEAD A K TRQRIRFAISQWVD AMSPANFLATNPE A Q R LLIES GGESLRAGVRNM M EDLTRG KISQTDESAFEVGRNV V TE GAVVFENEYFQL L QYKPLTD KVHARPL L MVPPCINKYYILD LQPESSLVRHVVEQGH T VFL VSWRNPDAS M AGSTWDDYI EHAAIRAIEVARDISGQD K IN VLGFCVGGTIVSTAL A V L AA RGEHPAASV T LL T LL D FADT |

| | | |
|---|---|---|
| | | <p>GILDVVFVDEGHVQLREATLG GGAGAPCALLRGLELANTFS FLRPNDLVWNYVVDNYLKG NTPVPDFLLFWNGDATNLPG PWYCWYLRHTYLQNELKVP GKLTVCVGPVDLASIDVPTYI YGSREDHIVPWTAAYASTAL LANKLRFVLGASGHIAGVINP PAKNKRSHWTNDALPESPQQ WLAGAIEHHGSSWWPDWTA WLAGQAGAKRAAPANYGNA RYRAIEPAPGRYVKAKA</p> <p>Lysine residues (labelled in bold) represent putative polysaccharide conjugations sites: K: 9, 10, 60, 73, 79, 85, 87, 429, 435, 441, 443, 446, 452, 458, 460, 601 (139 in PhaC), 774 (312 in PhaC) Italics: Conjugation sites not found in soluble NadA</p> |
| <p>NadA-PhaC versus conjugated NadA-PhaC displayed on PHB beads (T)</p> | <p>AATVAIVAAYNNGQEINGFK+Deamidated (11) Deamidated (12) AATVAIVAAYNNGQEINGFK+Deamidated (12) AATVAIVAAYNNGQEINGFK+Deamidated (12) Deamidated (17) ASMAGSTWDDYIEHAIR CVGGTIVSTALAVLAAR+Carbamidomethyl (1) DASMAGSTWDDYIEHAIR DFSALWQAMAEGK DFSALWQAMAEGK+Carbamidomethyl (13) DFSALWQAMAEGKAEATGPLHDR EDHIVPWTAAYASTALLAN EDHIVPWTAAYASTALLANK EDHIVPWTAAYASTALLANKLR EDHIVPWTAAYASTALLANKLR+Carbamidomethyl (20) FAISQWVDAMSPANFLATNPEAQR+Oxidation (6) FAISQWVDAMSPANFLATNPEAQR+Oxidation (6) Oxidation (10) GLELANTFSFLR GVPVDLASIDVPTYIYGSRLADTDAALADTDAALDETTNALNK</p> | <p>NadA (1-463)-PhaC (464-1051) fusion protein (112.8kDa)</p> <p>MATSDDDDVKKAAATVAIVAAYNNGQEINGFKAGETIYDIGEDGTITQKDATAADVEADDFKGLGLKKVVTNLTKTVNENKQNVDAKVKAASEIEKLTTKLADTDAALADTDAALDETTNALNKLGENITTFEETKTNIVKIDEKLEAVADTVDKHAEAFNDIADSLDETNTKKADEAVKTANEAKQTAEEKQNVDAKVKAAETAAGKAEAAAGTANTAADKAEAVAAKVTDIKADIA TNKADIAKNSARIDSLDKNVANLRKETRQGLAEQAALSGLFQPYNVGRFNVTAAVGGYKSESAVAIGTGFRFTENFAAKAGVAVGTSSGSSAAYHVGVNYESWKAGETIYDIGEDGTITQDKAGETIYDIGEDGTITQKDKAGETIYDIGEDGTITQKDTSSDDVKKAAATVAIVTSSDDDVKKAAATVAIVTKTVNENKQNVDAKV KATKTVNENKQNVDAKVKATSATGKGAAASTQEGKSQPFKVTPGPFDPATWLEWSRQ</p> |

| | | |
|--|--|--|
| | <p>LADTDAALADTDAALDETTNALN K+Deamidated (23) LDETNTKADEAVKTANEAK+Carb amidomethyl (13) Deamidated (5) Formyl (19) NMEDLTRGKISQTDESAFEVGR PATWLEWSR PDASMAGSTWDDYIEHAAIR PNDLVWNYVVDNYLK TDAALADTDAALDETTNALNK VTPGPFDPATWLEWSR VTPGPFDPATWLEWSR+Oxidation (11)</p> | <p>WQGTEGNGHAAASGIPGLDA LAGVKIAPAQLGDIQQRYSK DFSALWQAMAEGKAEATGP LHDRRFAGDAWRTNLPYRFA AAFYLLNARALTELDADAVEA DAKTRQRIRFAISQWVDAMS PANFLATNPEAQRLLESNGE SLRAGVRNMEDLTRGKISQ TDESAFEVGRNVAVTEGAVV FENEYFQLLQYKPLTDKVHA RPLLMVPPCINKYYILDQPE SSLVRHVVEQGHVFLVSWR NPDASMAGSTWDDYIEHAAI RAIEVARDISGQDKINVLGFC VGGTIVSTALAVLAARGEHP AASVTLTLLDFADTGILDV FVDEGHVQLREATLGGGAG APCALLRGLANTFSFLRPN DLVWNYVVDNYLKGNTPVP FDLLFWNGDATNLPGPWYC WYLRHTYLQNELKVPGKLT VCGVPVDLASIDVPTYIYGSR EDHIVPWTAAAYASTALLANK LRFVLGASGHIAGVINPPAKN KRSHWTNDALPESPQQWLA GAIEHHGSWWPDWTAWLAG QAGAKRAAPANYGNARYRA IEPAPGRYVKAKA Red: Potential polysaccharide conjugation sites sterically impacting on trypsin digest (sites are not part of identified peptide) K: 482, 601, 774, 913, 917 Lysine residues (labelled in bold) represent putative polysaccharide conjugations sites: K: 30, 123, 173, 185, 552 (90 in PhaC), 875 (413 in PhaC), 960 (498 in PhaC)</p> |
| <p>Soluble NadA versus conjugated soluble NadA (T)</p> | <p>AAETAAGKAEAAAGTAN AAETAAGKAEAAAGTANTAADK AEAVAAK AATVAIVAAAYNNGQEINGFK AATVAIVAAAYNNGQEINGFK+Dea midated (11) AATVAIVAAAYNNGQEINGFK+Dea midated (12) Deamidated (17) AATVAIVAAAYNNGQEINGFK+Dea midated(14)</p> | <p>NadA-His6: (49.2 kDa) MATSDDDVKKAAATVAIVAA YNNNGQEINGFKAGETIYDIGE DGTITQKDATAADVEADDFK GLGLKKVVVTLNLTKTVNENK QNVDAKVKAAESEIEKLTTK LADTDAALADTDAALDETTN ALNKLGENTTFAEETKTNIV KIDEKLEAVADTVDKHAEAF NDIADSLDETNTKADEAVKT ANEAKQTAETKQNVDAKV</p> |

| | | |
|--|---|--|
| | <p>AGETIYDIGEDGTITQKDTSDDDV KK AGETIYDIGEDGTITQKDTSDDDV KK+Deamidated (16) AVGTSSGSSAAYHVG VNYEWK HAEAFNDIADSLDETNTK HAEAFNDIADSLDETNTKADEAV K+Deamidated (16) HAEAFNDIADSLDETNTKADEAV K+Deamidated (6) LADTDAALADTDAALDETTNALN K LADTDAALADTDAALDETTNALN K+Deamidated (20) LDETNTKADEAVKTANEAK+Carb amidomethyl (13) Deamidated (5) Formyl (19) LEAVADTVDKHAEAFNDIADSLD ETNTK LGENITTFAE LGENITTFAEETKTNIVK NITTFAEETK SAAYHVG VNYEWK SGSSAAYHVG VNYEWK SSGSSAAYHVG VNYEWK TDAALADTDAALDETTNALNK TIYDIGEDGTITQK TVAIVAAAYNNGQEINGFK+ Deamidated (10) Deamidated (15) TVAIVTKTVNENKQNVDK</p> | <p>KAAETAAGKAEEAAAGTANT AADKAEEVAAKVTDIKADIA TNKADIAKNSARIDSLDKNV ANLRKETRQGLAEQAALSGL FQPYNVGRFNVTAAVGGYKS ESAVAIGTGFRFTENFAAKAG VAVGTSSGSSAAYHVG VNYE WKAGETIYDIGEDGTITQKD KAGETIYDIGEDGTITQKDKA GETIYDIGEDGTITQKDTSD DVKKAATVAIVTSDDDDVK AATVAIVTKTVNENKQNVD AKVKATKTVNENKQNVDA VKAHHHHHH</p> <p>Lysine residues (labelled in bold) represent putative polysaccharide conjugations sites: K: 9, 10, 30, 47, 79, 85, 123, 136, 141, 155, 173, 179, 185, 208, 223, 341, 358, 377, 396, 404, 405, 429, 435, 441 (bold number show sites identified in the NadA when fused to PhaC)</p> |
|--|---|--|

*, Samples were prepared as previously described⁷

Supplementary Table 7:

IgG/IgM ratio after first (1D) and third (3D) blood collection assayed against MenC.

| Vaccinated groups | Mean IgG (1D) | Mean IgG (3D) | Mean IgM (1D) | Mean IgM (3D) | Ratio IgG/IgM (1D) | Ratio IgG/IgM (3D) |
|--------------------------|----------------------|----------------------|----------------------|----------------------|---------------------------|---------------------------|
| MenC-PhaC-NadA | 231 | 3400 | 325 | 400 | 0.7 | 8.5 |
| MenC-PhaC | 200 | 5600 | 400 | 400 | 0.5 | 14.0 |
| MenC-NadA-His6 | 125 | 2400 | 500 | 325 | 0.25 | 7.3 |
| MenC-DT | 100 | 1800 | 400 | 400 | 0.25 | 4.5 |

Supplementary Table 8

Size distribution of PHB beads in vaccine formulations (μm) as measured by dynamic laser scattering. Dx represents the particle diameter corresponding to X% cumulative particle size distribution.

| Samples | PHB bead in PBS 1X | | | PHB bead in Al (OH) ₃ | | |
|----------------------------|--------------------|---------|---------|----------------------------------|---------|---------|
| | Dx (10) | Dx (50) | Dx (90) | Dx (10) | Dx (50) | Dx (90) |
| NadA-PhaC beads | 0.6 | 6.55 | 60.9 | 0.832 | 7.38 | 50.4 |
| GNA2091-fHbp-G1-PhaC beads | 3.8 | 9.5 | 68.6 | 3.7 | 9.8 | 70.6 |
| PhaC-fHbp-G1-G1 beads | 0.4 | 3.87 | 41.9 | 0.438 | 4.22 | 58.6 |
| PhaC wild type bead | 0.4 | 1.2 | 6.99 | 0.434 | 2.77 | 18.4 |

References

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