Supplementary material

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

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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 μ 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 μ g/ml of MenC CPS conjugated or mixed to NadA-PhaC beads, PhaC wild type beads and NadA-His6 the rest of assay was performanced 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 4µg of MenC conjugated to NadA-PhaC beads; **b.** animals vaccinated with 4µg of MenC conjugated to PhaC wild type beads; **c.** animals vaccinated with 4µg of MenC conjugated to soluble NadA-His6; **d.** animals vaccinated with 4µg 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 4µg of MenC conjugated to NadA-PhaC beads; **b.** animals vaccinated with 4µg of MenC conjugated to soluble NadA-His6; **c.** animals vaccinated with 18µg of protein from NadA-PhaC beads; **d.** animals vaccinated with 18µg of protein from NadA-PhaC beads; **d.** animals vaccinated with 18µg of protein from NadA-PhaC beads; **d.** animals vaccinated with 18µg of protein from NadA-PhaC beads; **d.** animals vaccinated with 18µg of protein from NadA-PhaC beads; **d.** animals vaccinated with 18µg of protein from NadA-PhaC beads; **d.** animals vaccinated with 18µg of protein from NadA-PhaC beads without alum.

Supplementary Table 1: Strains, Plasmids and primers

Strains, plasmid	Features					
and primers						
	Strains					
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´proAB	Novagen				
	$lacl^{q}Z\Delta M15 Tn10 (Tet^{r})$	_				
Clear Coli	F- ompT hsdSB (rB - mB -) gal dcm lon λ (DE3 [lacI lacUV5-T7 gene 1	3				
	ind1 sam7 nin5]) msbA148 Δ gutQ Δ kdsD Δ lpxL Δ lpxM Δ pagP Δ lpxP					
	AeptA (LPS negative)	<u>L</u>				
pET-14b	Ap^{κ} and T7 promoter	Novagen				
pET-14b-phaC	pET-14b version, holding <i>phaC</i> gene fragment	4				
pUC57-nadA	pUC57 version, ColE1 origin, Ap ^{<i>R</i>} holding NdeI /nadA gene/SpeI.	GenScript				
pUC57-gna2091-	pUC57 version, ColE1 origin, Ap ^R holding NdeI/gna2091-fhbp-g1	GenScript				
fhbp-g1	gene/SpeI.					
pUC57-fhbp-g1-g1	pUC57 version, ColE1 origin, Ap ^R holding XhoI/fhbp-g1-g1gene/	GenScript				
	BamHI					
pMCS69	CmR; T7 promoter, pBBR1MCS derivative containing <i>phaA</i> and <i>phaB</i>	5				
	genes from Ralstonia eutropha co-downstream to lac promoter					
pET14b_NanA_Pha	Ampicillin resistance and T7 promotor, holding nana gene fused	6				
C (reversed)	translationally to 3' end of phaC gene					
pET-14b-nadA-phaC	pET-14b-phaC version, holding <i>nadA</i> gene fused translationally to 3' end	This study				
	of <i>phaC</i> gene					
pET-14b-gna2091-	pET-14b-phaC version, holding gna2091-fhbp-g1 gene fused	This study				
fhbp-phaC	translationally to 3' end of <i>phaC</i> gene					
pET-14b-phaC-fhbp-	pET-14b-phaC version, holding <i>fhbp-g1-g1</i> gene fused translationally to	This study				
g1-g1	5' end of <i>phaC</i> gene					
pET14b-nadA-hist6	Ampicillin resistance and T7 promotor, holding a Ndel/ nadA hist-tag	This study				
	/BamHI gene.					
pET14b-gna2091-	Ampicillin resistance and T7 promotor, holding Ndel/ gna2091-fhbp-g1-	This study				
fhbp-g1-hist6	his-tag/BamHI gene.					
	Primers					
nadA fwr (NdeI,	5'AAA <u>CATATG</u> GCTACCTCAGATGATGATGTCAAAAAGGCGG3'	This study				
underline restriction						
site)						
nadA rev (BamHI,	5'AAA <u>GGATCC</u> TCAGTGGTGGTGGTGGTGGTGCTTTCACTTT	This study				
underline restriction	TGCGTCCACATTTTGTTTGTTTTCATTCAC3'					
site)						
gna2091-fhbp-g1 fwr	5'AAA <u>CATATG</u> TGCGTTTCGGCGGTTATTGGCTCAGCGGCGGTT	This study				
(NdeI, underline	G3'					
restriction site)						
gna2091-fhbp-g1 rev	5'AAA <u>GGATCC</u> TCAGTGGTGGTGGTGGTGGTGCTGTTTTGCCGC	This study				
(BamHI, underline	CAGACCAATGTGACGAATACCGTTGACC 3'					
restriction site)						

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

Fusion proteins	Peptides fragment identified by MALDI-TOF/MS.*
NadA-PhaC	A11-K47, L134-K146, Q267-R309, A317-K341, V482-
	R498, I535-K552, F608-641, I657-K691, Y712-K874,
	L928-K979, R1025-R1035
GNA2091-fHbp-G1-PhaC	<u>T31-R45, G52-R64, Q78-105, V170-R180, D212-R224,</u>
	F259-R263, I315-R347, S388-R395, G405-R414, V641-
	R476, I503-R514, A531-R565, F586-R620, I635-R647,
	Y690-R745, E811-R826, H873-K938, F940-K958, A1004-
	R1023
PhaC-fHbp-G1-G1	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,</u>
	<u>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 \pm 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	NARALTELADAVEADAKTRQRIR	PhaC (64.2kDa)
PhaC		MATGKGAAASTOFGKSOPE
displayed on	TGKGAAASTOFGKSOPE+Carbami	KVTPGPFDPATWI FWSROW
PHR beads	domethyl (13) Formyl (3)	OGTEGNGHA A ASGIPGI DAL
(C)	AAIRAIEVARDISGODKINVL	AGVKIAPAOLGDIOORYMKD
(0)	RAIEPAPGRYVKAKA	FSALWOAMAEGKAEATGPL
		HDRRFAGDAWRTNLPYRFA
		AAFYLLNARALTELADAVEA
		DAKTRORIRFAISOWVDAMS
		PANFLATNPEAORLLIESGGE
		SLRAGVRNMMEDLTRGKISQ
		TDESAFEVGRNVAVTEGAVV
		FENEYFQLLQYKPLTDKVHA
		RPLLMVPPCINKYYILDLQPE
		SSLVRHVVEQGHTVFLVSWR
		NPDASMAGSTWDDYIEHAAI
		RAIEVARDISGQD K INVLGFC
		VGGTIVSTALAVLAARGEHP
		AASVTLLTTLLDFADTGILDV
		FVDEGHVQLREATLGGGAG
		APCALLRGLELANTFSFLRPN
		DLVWNYVVDNYLKGNTPVP
		FDLLFWNGDATNLPGPWYC
		WYLRHTYLQNELKVPGKLT
		VCGVPVDLASIDVPTYIYGSR
		EDHIVPWTAAYASTALLANK
		LRFVLGASGHIAGVINPPAKN
		KRSHWTNDALPESPQQWLA
		GAIEHHGSWWPDWTAWLAG
		QAGAKRAAPANYGNARYRA
		IEPAPGRYV K A K A

	Lysine residues (labelled bold) represent putative polysaccharide conjugations sites: K: 5, 15, 139, 312, 498, 586, 588
NadA-PhaC AAIRAIEVARDISGQDKINVL versus ATSDDDVKKAATVAI conjugated ATSDDDVKKAATVAIVAA NadA-PhaC DVEADDFKGL displayed on NARALTELADAVEADAK PHB beads NARALTELADAVEADAK (C) TKTVNENKQNVDAKVK	NadA (1-463)-PhaC (464-1051) fusion protein (112.8kDa) MATSDDDV KK AATVAIVAA YNNGQEINGFKAGETIYDIGE DGTITQKDATAADVEADDF K GLGLKKVVTNLT K TVNEN K QNVDA K V K AAESEIEKLTTK LADTDAALADTDAALDETTN ALNKLGENITTFAEETKTNIV KIDEKLEAVADTVDKHAEAF NDIADSLDETNTKADEAVKT ANEAKQTAEETKQNVDAKV KAAETAAGKAEAAAGTANT AADKAEAVAAKVTDIKADIA TNKADIAKNSARIDSLDKNV ANLRKETRQGLAEQAALSGL FQPYNVGRFNVTAAVGGYKS ESAVAIGTGFRFTENFAAKAG VAVGTSSGSSAAYHVGVNYE WKAGETIYDIGEDGTITQKD KAGETIYDIGEDGTITQKDKA GETIYDIGEDGTITQKDKK GETIYDIGEDGTITQKDKK AATVAIVT K TVNEN K QNVD A K V K ATSATGKGAAASTQEG KSQPFKVTPGPFDPATWLEW SRQWQGTEGNGHAAASGIPG LDALAGVKIAPAQLGDIQQR YMKDFSALWQAMAEGKAEA TGPLHDRRFAGDAWRTNLPY RFAAAFYLLNARALTELADA VEADA K TRQRIRFAISQWVD AMSPANFLATNPEAQRLLIES GGESLRAGVRNMMEDLTRG KISQTDESAFEVGRNVAVTE VSWRNPDASMAGSTWDDYI EHAAIRAIEVARDISGQD K IN VLGFCVGGTIVSTALAVLAA

		GILDVFVDEGHVOLREATLG
		GGAGAPCALLRGLELANTES
		FI RPNDI VWNYVVDNYI KG
		NTPVPEDI I EWNGDATNI PG
		PWVCWVI RHTVI ONEI KVP
		CKI TVCCVPVDI ASIDVPTVI
		UKLIVCUVFVDLASIDVFIII VCSDEDHIVDWTAAVASTAI
		LAINKLERFVLGASGHIAGVINP
		PAKNKKSHW INDALPESPQQ WI AGAIEHHGSWWDDWTA
		WI ACOACAKDAADANVCNA
		RYRAIEPAPGRYVKAKA
		Lysine residues (labelled in bold)
		represent putative polysaccharide
		conjugations sites:
		$\mathbf{K} \cdot 9 = 10 60 73 79 85 87 429$
		A35 AA1 AA3 AA6 A52 A58
		453, 441, 443, 440, 452, 450, 460, 601 (130 in PhaC) 774 (312)
		(139 III I IIaC), 774 (312)
		Italics: Conjugation sites not
		found in soluble NadA
		Toulid in soluble Madry
NadA-PhaC	AATVAIVAAYNNGOEINGFK+Dea	NadA (1-463)-PhaC (464-1051)
versus	midated (11) Deamidated (12)	fusion protein (112.8kDa)
conjugated	AATVAIVAAYNNGOEINGFK+Dea	F
NadA-PhaC	midated (12)	MATSDDDVKKAATVAIVAA
displayed on	AATVAIVAAYNNGOEINGFK+Dea	YNNGOEINGF K AGETIYDIGE
PHB beads	midated (12) Deamidated (17)	DGTITOKDATAADVEADDFK
(T)	ASMAGSTWDDYIEHAAIR	GLGLKKVVTNLTKTVNENK
	CVGGTIVSTALAVLAAR+Carbami	ONVDAKVKAAESEIEKLTTK
	domethyl (1)	LADTDAALADTDAALDETTN
	DASMAGSTWDDYIEHAAIR	ALNKI GENITTFAEETKTNIV
	DESALWOAMAEGK	KIDEKLEAVADTVDKHAEAF
	DESALWOAMAEGK+Carbamidome	NDIADSI DETNT K ADEAVKT
	thyl (13)	ANEAKOTAEETKONVDAKV
	DESALWOAMAEGKAEATGPLHD	KAAETAAGKAEAAAGTANT
	R	AADKAFAVAAKVTDIKADIA
	FDHIVPWTAAYASTALLAN	TNKADIAKNSARIDSI DKNV
	FDHIVPWTAAYASTALLANK	ANI RKETROGI AFOAAI SGI
	FDHIVPWTAAYASTALLANKI R	FOPYNVGRENVTAAVGGYKS
	EDHIVPWTAAYASTALLANKIR+	FSAVAIGTGERFTENFAAKAG
	Carbamidomethyl (20)	VAVGTSSGSSAAYHVGVNYF
	FAISOWVDAMSPANELATNPEAO	WKAGETIVDIGEDGTITOKD
	R+Oxidation (6)	KAGETIYDIGEDGTITOKDKA
	FAISOWVDAMSPANELATNPEAO	GETIYDIGEDGTITOKDTSDD
	R+Oxidation (6) Oxidation (10)	DVKKAATVAIVTSDDDVKK
	GLELANTESELR	AATVAIVTKTVNENKONVDA
	GVPVDI ASIDVPTVIVGSR	KVKATKTVNENKONVDAKV
		KATSATGKGAAASTOEGKSO
	K	PFK VTPGPFDPATWI FWSRO
	K	PFKVTPGPFDPATWLEWSRQ

	LADTDAALADTDAALDETTNALN	WQGTEGNGHAAASGIPGLDA
	K+Deamidated (23)	LAGVKIAPAOLGDIOORYMK
	LDETNTKADEAVKTANEAK+Carb	DFSALWOAMAEG K AEATGP
	amidomethyl (13) Deamidated (5)	LHDRRFAGDAWRTNLPYRFA
	Formyl (19)	AAFYLLNARALTELADAVEA
	NMMEDLTRGKISOTDESAFEVGR	DAKTRORIRFAISOWVDAMS
	PATWLEWSR	PANFLATNPEAORLLIESGGE
	PDASMAGSTWDDYIEHAAIR	SLRAGVRNMMEDL TRGKISO
	PNDLVWNYVVDNYLK	TDESAFEVGRNVAVTEGAVV
	TDAALADTDAALDETTNALNK	FENEYFOLLOYKPLTDKVHA
	VTPGPFDPATWI FWSR	RPI I MVPPCINKYYII DI OPF
	VTPGPEDPATWLEWSR VTPGPEDPATWLEWSR	SSI VRHVVFOGHTVFI VSWR
	(11)	NPDASMAGSTWDDYIFHAAI
		RAIEVARDISGODKINVI GEC
		VGGTIVSTALAVI AARGEHP
		A SVTLI TTLI DEA DTCIL DV
		ADCALLDCLELANTESELDDN
		DI WWNVWDNVI KONTDUD
		FDLLF WNODAINLFOF WIC
		L DEVI CASCILIA CVINIDDA VN
		CALEHICSWWDDWTAWLAC
		GAIEHHGSW WPDW IAWLAG
		QAGAKKAAPAN I GNAK I KA
		IEPAPGKI VKAKA
		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), 8/5 (413 in PhaC), 960
		(498 in PhaC)
Soluble	AAETAAGKAEAAAGTAN	NadA-His6: (49.2 kDa)
NadA versus	AAETAAGKAEAAAGTANTAADK	MATSDDDV KK AATVAIVAA
conjugated	AEAVAAK	YNNGQEINGFKAGETIYDIGE
soluble NadA	AATVAIVAAYNNGQEINGFK	DGTITQKDATAADVEADDFK
(T)	AATVAIVAAYNNGQEINGFK+Dea	GLGLKKVVTNLTKTVNENK
	midated (11)	QNVDA K VKAAESEIEKLTTK
	AATVAIVAAYNNGQEINGFK+Dea	LADTDAALADTDAALDETTN
	midated (12) Deamidated (17)	ALN K LGENITTFAEET K TNIV
	AATVAIVAAYNNGQEINGFK+Dea	K IDEKLEAVADTVD K HAEAF
	midated(14)	NDIADSLDETNT K ADEAV K T
		ANEA K QTAEETKQNVDAKV

AGETIYDIGEDGTITOKDTSDDDV	KAAETAAG K AEAAAGTANT
КК	AADKAEAVAAKVTDIKADIA
AGETIYDIGEDGTITQKDTSDDDV	TNKADIAKNSARIDSLDKNV
KK+Deamidated (16)	ANLRKETRQGLAEQAALSGL
AVGTSSGSSAAYHVGVNYEWK	FQPYNVGRFNVTAAVGGYKS
HAEAFNDIADSLDETNTK	ESAVAIGTGFRFTENFAAKAG
HAEAFNDIADSLDETNTKADEAV	VAVGTSSGSSAAYHVGVNYE
K+Deamidated (16)	W K AGETIYDIGEDGTITQ K D
HAEAFNDIADSLDETNTKADEAV	KAGETIYDIGEDGTITQ K DKA
K+Deamidated (6)	GETIYDIGEDGTITQ K DTSDD
LADTDAALADTDAALDETTNALN	DV KK AATVAIVTSDDDVKK
К	AATVAIVT K TVNEN K QNVD
LADTDAALADTDAALDETTNALN	AKVKATKTVNENKQNVDAK
K+Deamidated (20)	VKAHHHHHH
LDETNTKADEAVKTANEAK+Carb	
amidomethyl (13) Deamidated (5)	Lysine residues (labelled in bold)
Formyl (19)	represent putative polysaccharide
LEAVADTVDKHAEAFNDIADSLD	conjugations sites:
ETNTK	K: 9, 10, 30, 47, 79, 85, 123 , 136,
LGENITTFAEE	141, 155, 173 , 179, 185 , 208, 223,
LGENITTFAEETKTNIVK	341, 358, 377, 396, 404, 405, 429 ,
NITTFAEETK	435 , 441 (bold number show sites
SAAYHVGVNYEWK	identified in the NadA when fused
SGSSAAYHVGVNYEWK	to PhaC)
SSGSSAAYHVGVNYEWK	
TDAALADTDAALDETTNALNK	
TIYDIGEDGTITQK	
TVAIVAAYNNGQEINGFK+	
Deamidated (10) Deamidated (15)	
TVAIVTKTVNENKQNVDAK	

*, Samples were prepared as previously described⁷

Supplementary Table 7:

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

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

Supplementary Table 8

Size distribution of PHB beads in vaccine formulations (µm) as measured my 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
	D x (10)	Dx (50)	Dx (90)	Dx (10)	D x (50)	Dx (90)
NadA-PhaC	0.6	6 5 5	60.0	0.832	7 29	50.4
beads	0.0	0.55	00.9	0.852	7.30	30.4
GNA2091-fHbp-	2.0	0.5	69.6	27	0.8	70.6
G1-PhaC beads	5.8	9.5	08.0	5.7	9.8	/0.0
PhaC-fHbp-G1-	0.4	2 07	41.0	0.429	4.22	596
G1 beads	0.4	5.87	41.9	0.438	4.22	38.0
PhaC wild type	0.4	1 2	6.00	0.434	2 77	19.4
bead	0.4	1.2	0.99	0.434	2.11	10.4

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