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1 **On the use of adenovirus dodecahedron as a carrier for glycoconjugate**

2 **vaccines**

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12 **Abstract**

13 Virus-Like Particles (VLPs) have been used as immunogenic molecules in numerous recombinant vaccines.
14 VLPs can also serve as vaccine platform to exogenous antigens, usually peptides incorporated within the protein
15 sequences which compose the VLPs or conjugated to them. We herein described the conjugation of a synthetic
16 tetrasaccharide mimicking the *Streptococcus pneumoniae* serotype 14 capsular polysaccharide to recombinant
17 adenoviral type 3 dodecahedron, formed by the self-assembling of twelve penton bases and investigated the
18 induced immune response when administered subcutaneously (*s.c.*). Whether formulated in the form of a
19 dodecahedron or disassembled, the glycoconjugate induced an anti-protein response after two and three
20 immunizations equivalent to that observed when the native dodecahedron was administered. On the other hand,
21 the glycoconjugate induced a weak anti-IgM response which diminishes after two doses but no IgM-to-IgG
22 switch was observed in mice against the serotype 14 capsular polysaccharide. In definitive, the whole
23 conjugation process preserved both particulate nature and immunogenicity of the adenoviral dodecahedron.
24 Further studies are needed to fully exploit adenoviral dodecahedron potential in terms of plasticity towards
25 sequence engineering and of its capacity to stimulate the immune system *via* the intranasal route of
26 administration as well as to shift the response to the carbohydrate antigen by playing both with the carbohydrate
27 to protein ratio and the length of the synthetic carbohydrate antigen.

28 **Keywords** Glycoconjugate vaccine, carbohydrate antigen, virus-like particle, adenovirus dodecahedron,
29 *Streptococcus pneumoniae*

30 **Introduction**

31 Polysaccharides as expressed at bacterial cell surfaces have been for long time recognized as potential targets for
32 humoral responses [1]. This has led to the advent of the polysaccharide vaccines and latter, to that of
33 glycoconjugate vaccines [2]. Glycoconjugate vaccines consist in the covalent linking of a carbohydrate antigen
34 to a protein. The protein referred to as carrier protein elicits a T-cell help which eventually evokes a
35 carbohydrate-specific memory response [3]. Although the carrier protein is essential in mounting the immune
36 response, it often appears as the neglected valency in comparison with the carbohydrate antigen [4],[5]. Only a
37 handful number of proteins have been approved for use as carriers in humans, typically bacterial toxoids such as
38 tetanus toxoid (150kDa), diphtheria toxoid or CRM₁₉₇ (62kDa) and *Haemophilus* protein D (42kDa) or the outer
39 membrane protein complex of serogroup B meningococcus (OMPC) (36kDa). Using proteins from a pathogen
40 playing the dual role of carrier as well as protective antigen has been envisaged to simplify multicomponent
41 vaccine formulations and to reduce cost associated with the development of a new carrier [6],[7]. However, such
42 strategy may result in suboptimal response due to carbohydrate and protein B cell epitope competition for
43 restricted T cell help [8],[9], an issue which mitigates its scope. To satisfy the need for novel carriers, we have
44 therefore thought to rely on VLPs as both an alternative and an attractive strategy. VLPs are self-assembled
45 structural viral proteins forming nanoparticles. Particulate systems are inherently more immunogenic than
46 soluble systems as nano- and microparticles mimics the size, geometry and properties that the immune system
47 recognizes [10]. In a way VLPs are very similar to live viruses since they can drain freely into the lymphatic
48 vessels, be taken up efficiently by the antigen-presenting cells and induce a strong immune response but they are
49 not infectious. Thanks to these properties several VLP-based vaccines have been licensed for the prevention of
50 viral diseases such as those caused by hepatitis B virus or papillomavirus [11],[12]. In addition, VLPs tolerate
51 genetic or chemical conjugation to give rise to multivalent nanoplatform for exogenous antigen delivery [12].
52 VLPs engineered with exogenous antigens are usually more immunogenic than the antigen itself as they present
53 a dense array of antigens which efficiently crosslinks B cell receptors thank to the VLPs' highly repetitive and
54 symmetrical nature [13],[14]. These unique features combined with their protein nature suitable to support a T
55 cell help design VLP as appealing platforms for glycoconjugate vaccines. However, few examples which make
56 use of a VLP as a carrier for carbohydrate antigen have been reported. Hence tumor-associated carbohydrate
57 antigen Tn [15], oligomannosides [16] or two synthetic tetrasaccharides representing one or two repeating units
58 of the polysaccharide capsules of *Streptococcus pneumoniae* serotype 14 or 3, respectively,[17] have been
59 chemically attached to the icosahedral capsid of bacteriophage Q β . All resulting glycoconjugates induced an

60 antibody response against the carbohydrate antigen. Of note, the pneumococcal glycoconjugate vaccines
61 involved glycopeptide-specific T cells and were protective in a passive infectious model in mice [17]. Keeping *S.*
62 *pneumoniae* as a model, we decided to investigate the human adenoviral type 3 dodecahedron (Dd) as a VLP-
63 based glycovaccine platform for its formidable capacity to infect human cells. Indeed, Dd is composed of twelve
64 pentameric penton bases, responsible for adenovirus cell penetration at an extent of 200,000 to 300,000 Dd/cell
65 in culture [18]. Such penetration efficiency is explain by the presence of an RGD motif within a loop which
66 favors its interaction with integrin receptor expressed at the target cell surfaces. In addition, Dd recognizes
67 heparan sulfate proteoglycans expressed on mammalian cells surfaces which form a second entry door [19],[20].
68 It has successfully been engineered to provide chimeric influenza [21],[22] or Chikungunya vaccines [23]
69 meaning that it can be used as a vaccine platform but its use as carbohydrate antigen carrier remains
70 unprecedented.

71 **Material and methods**

72 **Reagents**

73 Anhydrous solvents CH₂Cl₂, EtOH and MeOH were purchased from Acros Organics. Anhydrous sodium sulfate,
74 sodium borohydride, boric acid and sulfuric acid were from VWR, nickel (II) chloride (NiCl₂.6H₂O) was from
75 Acros Organics, 25% sodium methoxide solution in MeOH , ethylene diamine, glutaraldehyde (25% in H₂O),
76 amberlite IR120(H⁺) and anthrone were purchased from Sigma-Aldrich.

77 **Expression and purification of Dd**

78 Dd was expressed in FlashBacGold (Oxford Expression Technologies) baculovirus expression system following
79 the manufacturer's protocol. Virus stocks were prepared by infection of *Spodoptera frugiperda* 21 monolayer.
80 For Dd expression, *Trichoplusia ni* (HighFive) cells (2×10^6 cells/ml) grown in suspension were infected with
81 the recombinant baculovirus at multiplicity of infection 4 and cultured for 48 h. Expressing cells were lysed and
82 Dd was purified from the supernatant by size exclusion chromatography followed by anion exchange
83 chromatography, as described previously [24]. Dd preparation was concentrated using Amicon Ultra
84 concentrators (Merck Millipore), protein concentration was determined by NanoDrop ND-200 UV-Vis
85 Spectrophotometer (Thermo Scientific). The homogeneity of preparations were analyzed by SDS-PAGE 12%.
86 The assembly status of purified Dd was analyzed by electron microscopy.

87 **Tetrasaccharide synthesis (Pn14TS)**

88 To a solution of known 2-azidoethyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-(2,3,6-tri-*O*-acetyl-β-
89 D-galactopyranosyl)-(1→6)-[(2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyl)-(1→4)]-2-deoxy-2-phtalimido-β-D-

90 glucopyranoside [25] (29.6 mg, 0.023 mmol) in a mixture of CH₂Cl₂/EtOH (1.69:0.36 mL) were successively
91 added NaBH₄ (1.84 mg, 2.1 equiv) and a catalytic amount of NiCl₂·6H₂O at room temperature. The reaction
92 mixture was stirred for 1h and then concentrated under reduced pressure. The crude residue was diluted in
93 CH₂Cl₂ and washed with H₂O (twice) and brine. The organic layer was dried over Na₂SO₄, filtered and
94 concentrated under reduced pressure to afford a peracetylated intermediate. To a solution of this intermediate (29
95 mg) in MeOH were added ethylene diamine (18 μL, 12 equiv) and MeONa in 25% solution in MeOH (10 μL).
96 The reaction mixture was stirred for 3 hour at room temperature, neutralized to pH 7 (paper) using Amberlite
97 IR120(H⁺), filtered and concentrated under reduced pressure to provide 2-aminoethyl (β-D-galactopyranosyl)-
98 (1→4)-(β-D-galactopyranosyl)-(1→6)-[(β-D-galactopyranosyl)-(1→4)]-2-deoxy-2-acetamido-β-D-
99 glucopyranoside (Pn14TS) (17 mg, quantitative); HR-ESI-MS: *m/z* Calcd for C₂₈H₅₀N₂O₂₁ [M + H]⁺ 751.2984,
100 found 751.2949.

101 **Conjugate Synthesis**

102 In a typical experiment, to a solution of Pn14TS (100 μg) in 100 μl of borate buffer (0.1M sodium borate, 150
103 mM NaCl, pH 9.6) was added 30 μl of glutaraldehyde (25% solution in H₂O), then the solution is stirred for 4
104 min at 4 °C. Next, 1 ml of Dd solution at 1 mg / ml in 20 mM HEPES buffer (containing EDTA 2 mM, NaCl
105 150 mM pH 7.5) is added, and the reaction mixture is further stirred for 1 hour at 4 °C. Finally, NaBH₄ (450 μg)
106 is added and the reaction mixture stirred for 15 min at 4 °C. The solution was then dialyzed against 20 mM Tris,
107 pH 7.5, containing 2 mM EDTA, 5% glycerol using Pierce Slide-A-Lyzer cassette (molecular weight cutoff,
108 20K) and stored at 4 °C. Bradford assay (BioRad protein assay) was used to determine the concentration of the
109 protein. The anthrone/sulphuric acid method [26] was used for the quantification of the sugar content using
110 Pn14TS as a standard, as described previously [27]. The carbohydrate antigen protein ratio was determined by
111 mass spectrometry.

112 **Mass spectrometry**

113 High-resolution mass spectrum was recorded in positive mode on Waters SYNAPT G2-Si HDMS with detection
114 with a hybrid quadrupole time of flight (Q-TOF) detector. The compounds was dissolved in MeOH at a
115 concentration of 1 mg/ml and then infused into the electrospray ion source at a flow rate of 10 μl·min⁻¹ at 100
116 °C. The mass spectrometer was operated at 3 kV while scanning the magnet at a typical range of 4000–100 Da.
117 The mass spectrum was collected as continuum profile data. An accurate mass measurement was achieved based
118 on every five-second auto-calibration using leucine-enkephalin ([M + H]⁺ = 556.2771 *m/z*) as an internal
119 standard.

120 The samples were analyzed by matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF) MS. A
121 sinapinic acid solution at 10 mg.mL⁻¹ in H₂O-CH₃CN-TFA (50:50:0.3) was used as the MALDI matrix. The
122 samples (75 pmol in 1 µL) were deposited and then covered by the matrix (1 µL) on a polished steel MALDI
123 target plate. MALDI measurements were then performed on a rapifleX MALDI-TOF spectrometer (Bruker
124 Daltonics, Bremen, Germany) equipped with a Smartbeam 3D laser (355 nm, 10000 Hz) and controlled using the
125 Flex Control 4.0 software package. The mass spectrometer was operated with positive polarity in linear mode.
126 Spectra were acquired in the range of 15000–100000 *m/z*. The mass spectrometer was calibrated between 20 kDa
127 and 70 kDa with the Protein Standard II (Bruker Daltonics, Bremen, Germany).

128 **Electron microscopy**

129 Negative Stain Mica-carbon Flotation Technique (MFT) was applied. Samples were absorbed to the clean side of
130 a carbon film on mica, stained and transferred to a 400-mesh copper grid. The images were taken under low dose
131 conditions (<10 e⁻/Å²) with defocus values between 1.2 and 2.5 µm on a Tecnai 12 LaB6 electron microscope at
132 120 kV accelerating voltage using CCD Camera Gatan Orius 1000.

133 **Immunization**

134 The Dd and Pn14TS-Dd formulations were dialyzed against 10 mM PBS using Pierce Slide-A-Lyzer cassette
135 (molecular weight cutoff, 10K). One batch of Pn14-Dd was freeze-dried in absence of cryo-protectant and then
136 reconstituted with H₂O. Actual concentration in protein of every formulation was about 400 µg/mL. The
137 assembly status of the different formulation was analyzed by electron microscopy.

138 Groups of 5 male C57/BL6J (5 week old) mice were injected *s.c.* with PBS, Dd (64 µg protein/dose), Pn14TS-
139 Dd conjugate or disassembled Pn14TS-Dd conjugate (64 µg protein/dose–1.9 µg tetrasaccharide/dose) diluted
140 with 160 µL of RIBI adjuvant (Sigma) in PBS. The mice were immunized at day 0, 14, and 28. The sera were
141 collected on days 21 and 35. Sera were stored at –80 °C.

142 **ELISA**

143 The Ab responses induced upon immunizations were assessed 1 week after the second and the third injections by
144 ELISA. Dd and capsular polysaccharide serotype 14 (CP14) (Alliance Bio Expertise), were used as coated
145 antigens to define the anti-Dd or anti-CP14 Ab titers. Dd (0.1 µg/well) in 10 mM PBS, pH 7.3 (100 µL/well),
146 was coated on 96 wells microtiter plates Nunc Maxisorp (ThermoFisher Scientific) plates overnight at 4 °C.
147 CP14 (1 µg/well) was coated for 48 h at 4 °C in 10 mM PBS, pH 7.3 (100 µL/well). Plates were then washed
148 with PBS 0.05% Tween 20 (3 × 200 µL), saturated using PBS containing 10% skimmed milk at 37 °C for 2 h,
149 then washed using PBS Tween 20 (PBST, 50 mM Tris, 150 mM NaCl, 0,1% Tween 20) (3 × 200 µL). Series of

150 dilution of sera in PBS containing 10% skimmed milk (100 μ L/well), were incubated at 37 °C for 2 h. Plates
151 were then washed with PBST (3 \times 200 μ L) and then incubated with goat anti-mouse IgG(H+L)-horse radish
152 peroxidase-labeled conjugate (CliniSciences) used as secondary Ab at a dilution of 1/6,000, for 1 h at 37 °C and
153 further washed with PBST (5 \times 200 μ L). The enzyme substrate, o-phenylenediamine dihydrochloride (100 μ L at
154 0.4 mg mL⁻¹) in 0.1 M sodium citrate (pH 5.2), containing 0.02% hydrogen peroxide, was added to each well
155 and the plate incubated for 20 min at room temperature in the dark. The reaction was terminated by adding 3 M
156 HCl (1,000 μ L per well), and the A492 was read in an Infinite M1000 spectrophotometer (TECAN). The Ab titer
157 was defined as the dilution of immune serum that gave an OD (405 nm) at least twice that observed with pre-
158 immune serum.

159 **Results and discussion**

160 **Conjugate synthesis**

161 We envisioned to use synthetic 2-aminoethyl (β -D-galactopyranosyl)-(1 \rightarrow 4)-(β -D-galactopyranosyl)-(1 \rightarrow 6)-[(β -
162 D-galactopyranosyl)-(1 \rightarrow 4)]-2-deoxy-2-acetamido- β -D-glucopyranoside tetrasaccharide (Pn14TS, Scheme 1) as
163 a model carbohydrate antigen to test Dd as a carrier protein in glycoconjugate vaccine formulation. Indeed,
164 Pn14TS is readily accessible by chemical synthesis from common sugars. Moreover, this tetrasaccharide was
165 previously selected by Polonskaya *et al.* to develop Q β -based VLP glycoconjugates [17] and Pn14TS-based
166 semi-synthetic glyconjugates proved to be immunogen in mice in several laboratories including ours
167 [28],[29],[25]. Both carbohydrate antigen size/length and carbohydrate antigen/carrier protein ratio impact the
168 anti-carbohydrate antigen response. Increasing the ratio usually counteracts the lower immunogenicity associated
169 with short carbohydrate antigens compared to longer ones [30],[31]. This is also observed for Pn14TS-
170 conjugates [32],[9]. Considering the huge molecular weight difference between Pn14TS (<1000 Da) and Dd
171 (3,800,000 Da), we therefore elected to use homobifunctional glutaraldehyde as crosslinking reagent to attach
172 the tetrasaccharide to the protein since we experienced that the actual loading was superior to other conjugation
173 techniques likewise thiol/maleimide conjugation [33].

174 Pn14TS was obtained from a 2-azidoethyl-functionalized peracetylated precursor [25] in two steps comprising
175 the nickel boride reduction [34] of the azide into amine followed by Zemplén deprotection of the acetates
176 (Scheme 1). Then, Pn14TS was first reacted shortly with glutaraldehyde, and the resulting mixture further
177 condensed with Dd at a 400:1 tetrasaccharide protein ratio, followed by sodium borohydride reduction of the
178 imine linkages into amines to stabilize the conjugates.

179 **Conjugate characterization**

180 After dialysis the conjugates were analyzed for their protein and sugar contents according to colorimetric
181 Bradford and anthrone/sulfuric acid method, respectively. Protein recovery was estimated equal to 70% while
182 total sugar molar content was 180 times that of the protein. The MALDI-TOF mass spectrum of the conjugate
183 preparation was next compared with that of Dd to determine the extent of derivatization (Fig. 1). The analysis
184 can be performed on the penton base, the monomeric unit which composes Dd. The molecular weight of the
185 penton base and the conjugated penton base differs from 2,086 mass unit which corresponds to 2.5 conjugated
186 tetrasaccharides on average. Consequently a loading of 150 Pn14TS was achieved per particle on average. The
187 penton base contains 25 lysine residues within its sequence among with K152, K166, K305, K306, K348, K349,
188 K358, K361 and K390 appear readily surface accessible for conjugation as determined from adenovirus
189 dodecahedron X-ray crystallography structure [35],[36]. Vicinity of most accessible lysine side-chain amines
190 might contribute to mitigate the number of conjugated Pn14TS due to steric hindrance caused by the attachment
191 of a first tetrasaccharide unit (Fig. 2).

192 The assembly status of Dd and Pn14TS-Dd was analyzed by electron microscopy (EM) prior to immunization
193 (Fig. 3). Both Dd (Fig. 3a) and Pn14TS-Dd (Fig. 3b) formulations contain homogeneous particles meaning that
194 neither sample preparation nor conjugation affect the particle assembly. Alternatively, in order to evaluate
195 whether the nanoparticle nature of the glycoconjugate influences the level or the nature of the immune response,
196 a batch of the conjugate was freeze-dried in absence of cryo-protectant and then reformulated in PBS. As seen in
197 Fig. 3c, dodecahedra were not detected by EM suggesting that the freeze-drying step has led to the complete
198 disruption of the complexes as expected. Finally, antigenicity of Pn14TS-Dd was assessed by Western-Blot
199 experiment using polyclonal Abs contained in a hyperimmune mouse sera raised against a Pn14TS-based
200 glycoconjugate [25] and goat anti-mouse IgG(H+L)-Alexa680-labeled conjugate as secondary Ab. Neither
201 conjugation and purification steps interfered with accessibility of Pn14TS which retained its antigenicity in
202 comparison with purified serotype 14 capsular polysaccharide used as a positive control and PBS or native Dd
203 (negative controls) (data not shown).

204 **Immunization of mice and humoral response evaluation**

205 Groups of C57BL/6 mice were immunized thrice *s.c.* at two weeks interval with Dd, the Pn14TS-Dd conjugate
206 in both native (particle), and disassembled form and PBS as a negative control. Antibody responses against
207 purified *S. pneumoniae* serotype 14 polysaccharide capsule (CP) and Dd was assessed one week after the second
208 (D21) and the third (D35), immunizations.

209 The titers of anti-Dd IgG were first measured using Dd as a coating antigen (Fig. 4A and 4B). Dd as well as both
210 formulations of Pn14TS conjugates were able to induce anti-Dd IgGs while such response was not observed for
211 PBS control. High level of Abs was detected after the second immunization (Day 28) (Fig. 4A) and maintained
212 after the boost (Day 35), in the group of mice immunized with disassembled Pn14TS-Dd and increased for the
213 groups immunized with either Dd or Pn14TS-Dd. Observed increase was only statistically significant ($P < 0.05$),
214 between the group immunized with Dd and that immunized with disassembled Pn14TS-Dd. The later conjugate
215 corresponds to a conjugate whereby the tetrasaccharide has been conjugated to free adenoviral type 3 penton
216 base. Recombinant penton base proteins are easily expressed in *Escherichia coli* and have sometimes been
217 proposed as efficient surrogates for live-attenuated adenoviral vaccine [37]. However, true comparison with
218 dodecahedra has not yet been reported to our knowledge. Of note, penton bases have been shown to be 1000
219 times less efficient than dodecahedra to infect cells [38], a feature which might explain observed differences in
220 the IgG response between the two groups. In the present study, IgGs titers induced in mice by the Pn14TS-Dd
221 conjugate is comprised between those induced by Dd and those induced by disassembled Pn14TS-Dd used as
222 immunogens. Therefore, one cannot exclude that important B epitopes are masked by the attached
223 tetrasaccharide, despite the low extent of derivatization and/or a response against unraveled ones, not detected by
224 native Dd used as the coated antigen for ELISA assays.

225 We next turned our attention to the anti-CP response. The IgG response raised against CP by the conjugates was
226 very low and not significant compared to Dd or the negative control group (data not shown) [39]. This response
227 was equally low when we used Pn14TS as the coating antigen suggesting that the observed results were not due
228 to a lack of recognition of CP by anti-Pn14TS Abs. Anti-CP IgM response was also determined after the second
229 and the third immunizations. Significant titers of anti-CP IgM Abs could be measured in the sera of mice
230 immunized with either native or disassembled Pn14TS-Dd in comparison with both Dd and PBS for which no
231 anti-CP response was observed (Fig. 4C). Anti-CP response was however absent in third sera (data not shown).
232 Since the IgM response is not sustained between the second and the third immunization, this
233 hyporesponsiveness, in particular the absence of IgM-to-IgG switch, unlikely derived from the presence of free
234 Pn14TS (16%), which contaminates the formulations as suggested by the comparison of total sugar *versus*
235 covalently bound sugar content as determined by colorimetric method and mass spectrometry (*vide supra*).

236 Along this line, to our knowledge, acceptable amount of free polysaccharide present in polysaccharide–protein
237 conjugate vaccines is 20% of the total carbohydrate antigen content. Moreover suppression of the T-dependent
238 immune response could not be determined for pneumococcal conjugates, which typically contain 10% of free

239 polysaccharide [40],[41]. On the other hand, Dd MHCII epitopes are functional since a strong anti-carrier IgG
240 response is observed (Fig. 4A). This observation suggests that anti-CP IgG is delayed (in their study Polonskaya
241 *et al.* carried out two immunizations at 6 weeks interval and analyzed the sera at weeks 5 and 8 [17] while we
242 carried out analyses at weeks 3 and 5), that Dd B cell epitopes are immunodominant compared to Pn14TS
243 antigenic determinants, that conjugation took place too far from Dd MHC II epitopes to benefit of T cell help or
244 to recruit Tcarb [42],[9],[3] or that Pn14TS loading or density is too weak [13]. Anti-Pn14TS response strongly
245 depends on both dose and Pn14TS/carrier protein ratio. For example, it has been reported that levels of anti-
246 Pn14TS and anti-CRM₁₉₇ (used as a carrier) IgG correlate positively and negatively, respectively, with Pn14TS
247 loading at a 2.5 µg Pn14TS/mouse/dose [32]. Significant IgG level were raised against CRM₁₉₇ and, conversely
248 moderate anti-Pn14TS IgG response, even at a 5:1 Pn14TS/CRM₁₉₇ ratio whilst CRM₁₉₇ is known to induce low
249 anti-carrier Abs and is therefore considered as the best protein carrier for carbohydrate antigens to date [43]. This
250 situation should be stressed herein whereby Pn14TS has been administered at a 1.9 µg dose at a 2.5:1
251 Pn14TS/Penton base (which has same molecular weight as CRM₁₉₇) molar ratio or at a 150:1 Pn14TS/Dd molar
252 ratio which corresponds to a 1.9/64 µg ratio (0.03 weight ratio). In the past, IgG responses have been observed at
253 Pn14TS dose of 1.1 µg [29] and we obtained a significant response using as less as 0.5 µg of Pn14TS with
254 pneumococcal surface protein A as the carrier protein (unpublished results) but at higher 5.4:1 Pn14TS/carrier
255 protein ratio. In their study, Polonskaya *et al.* used a different VLP carrier, bacteriophage Qβ [17]. Mice were
256 immunized with 100 µg of conjugates bearing on average 20, 40, 80 or 200 Pn14TS per particle *i.e.* roughly 1.4,
257 2.7, 5.4 and 13.4 µg of Pn14TS, respectively. The herein described Pn14TS-Dd conjugate is similar to the 20:1
258 (0.01 weight ratio) and 40:1 (0.03 weight ratio) Pn14TS-Qβ conjugates. Noticeably, anti-Pn14TS IgG response
259 was not elicited by the former conjugate while the later elicited some variability in both the primary and the
260 secondary responses, observations which are in agreement with our own results. It is thus possible that the
261 threshold required to induce a strong anti-Pn14TS was not reached by Pn14TS-Dd.

262 **Conclusion**

263 Glycoconjugate vaccines are very efficient and well tolerated in humans. Future glycoconjugate vaccine
264 improvements consist in increasing the number of carrier protein available to avoid carrier epitope suppression
265 phenomena as well as in mobilizing broader immune cell population subsets such as T follicular helper-like cells
266 [44]. For example, it has been shown that recruitment of T follicular helper cells strongly correlates with
267 enhanced opsonophagocytic antibody response induced by conjugated pneumococcal vaccines in young
268 compare with elderly population. This observation suggests that elicitation and boosting of T follicular helper

269 cells among the later population is a valuable strategy for the generation of durable antibody responses to
270 optimize current glycoconjugate vaccines [45]. Virus-like nanoparticles, due to their large surface area to volume
271 ratio and multivalency which resembles the natural features of viruses can fulfil these goals: B cells specific for
272 Q β -derived VLP used as antigen are sufficient to induce T follicular helper cell development in absence of
273 antigen presenting cells [46]. Therefore, VLPs are expected to favor follicular lymph nodes processing of
274 glycoconjugates. Along this line, adenoviral type 3 Dd has been herein proposed as a novel nanoparticle carrier
275 for glycoconjugate vaccine using a synthetic tetrasaccharide mimic of the *S. pneumoniae* serotype 14 capsular
276 polysaccharide. Resulting VLP-based glycoconjugate proved robust to sequential synthetic and purification steps
277 and easily characterized. Noticeably, conjugation of the synthetic tetrasaccharide to Dd particles was carried out
278 without impacting their assembly. Glutaraldehyde was selected as bifunctional immuno-silent linker for the
279 conjugation step as it was anticipated that a high carbohydrate antigen/carrier ratio would be necessary to induce
280 an anti-carbohydrate antigen response. Despite a 150:1 ratio, a weak, early anti-carbohydrate antigen IgM and no
281 IgG response have been determined in mice following *s.c.* immunizations. Contrasting with these observations
282 the Dd carrier was strongly immunogenic and induced an anti-Dd IgG response. Further studies will thus be
283 needed to validate adenoviral type 3 Dd as a carrier. Efforts will focus on increasing length of the linker to make
284 the carbohydrate antigen more accessible to the immune system, or increasing the length of carbohydrate antigen
285 itself and reaching higher extent of derivatization while exploiting its tolerance towards sequence engineering,
286 the opportunity to encapsulate molecules *e.g.* Toll-like receptor agonists and exploring mucosal administration,
287 the natural route of infection of adenoviruses,

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301 **Compliance with ethical standards**

302 **Conflicts of interest** The authors declare that they have no conflicts of interest.

303 **Ethical approval** The animal study was reviewed and approved by ethical permit number from Comité
304 d’Ethique en Expérimentation Animale (CEEA): 7897.

305 **Data availability** The datasets generated during and/or analysed during the current study are available from the
306 corresponding author on reasonable request.

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442 FIGURE LEGENDS

443 **Scheme 1** Synthesis of the conjugates. Preparation of Pn14TS from peracetylated precursor followed by
444 conjugation with Dd (represented using PyMol from pdb structure 2C9G [47])using glutaraldehyde to give
445 Pn14TS-Dd.

446
447 **Figure 1** MALDI-TOF-MS spectra of Dd and of Pn14TS-Dd. Zoom of the m/z 55 000-73 000 region (Insert
448 panel). Penton base protein of Dd (Top) and penton base protein of Dd conjugated to Pn14TS (Bottom); 60
449 copies of penton base protein make up 1 Dd.

450
451 **Figure 2** Lysine residues (salmon) with amine side chain groups (green) exposed on the surface of the Dd
452 structure (PDB ID: 4AQQ), as seen in penton base protein monomer (a), pentameric penton base (blue) in the
453 context of whole dodecahedron (gray) (b), and dodecahedron (c). The K337 and K339 are present in the part of
454 RGD loop (from L315-A344) invisible in Dd crystallographic structure (indicated by arrows). Structures
455 rendered in Chimera software.[35]

456

457 **Figure 3** Negative-stain electron microscopy of Dd (a) Pn14TS-Dd conjugate (b) and disassembled Pn14TS-Dd

458 conjugate (c). Stain Acetate d'uranyl (AcUr) $\text{UO}_2(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ at 2% in distilled water (pH4,2-4,5)

459 Magnification $\times 23,000$; bar represents 100 nm.

460

461 **Figure 4** Titers of anti-Dd (coated on microtiter plates) IgG Abs of mice immunized with Dd, Pn14TS-Dd,

462 disassembled Pn14TS-Dd and PBS after the second (aA) and the third immunization (bB); Titers of anti-CP

463 (coated on microtiter plates) IgM Abs of mice immunized with Dd, Pn14TS-Dd, disassembled Pn14TS-Dd and

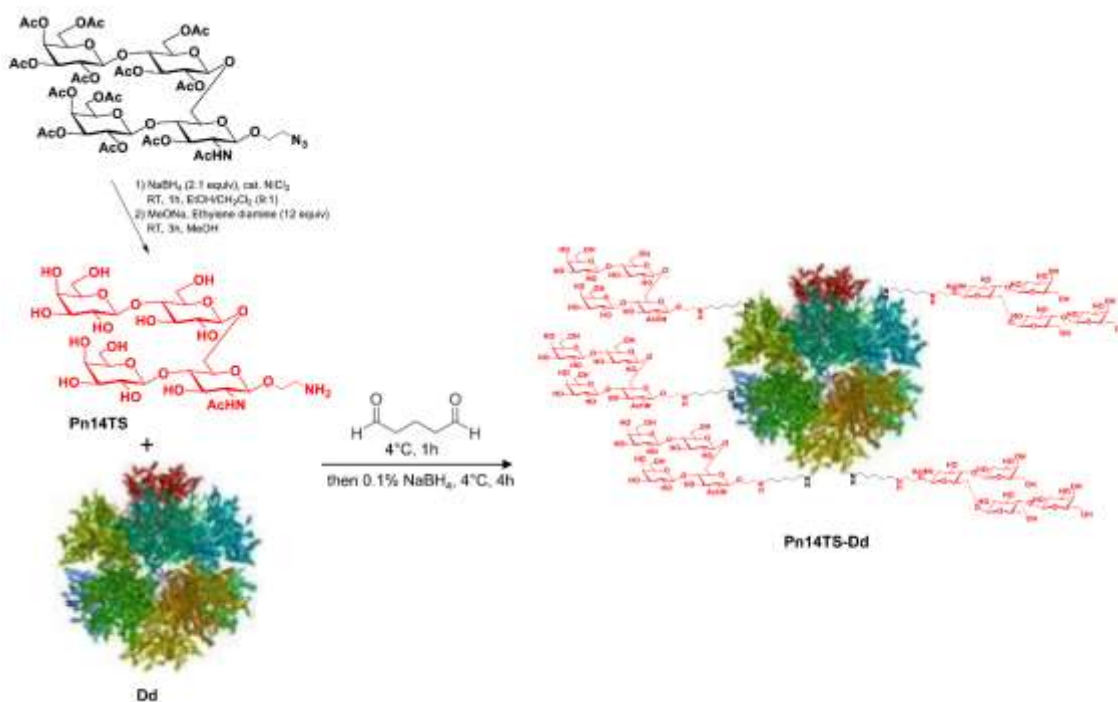
464 PBS after the second immunization (cC). The serum samples data presented as geometric mean titer of five mice

465 per group. Statistical analysis was performed using one-way ANOVA with Tukey analysis for multiple

466 comparisons. Statistical difference between the groups is *P < 0.05, **P<0.01, ***P<0.001.

467

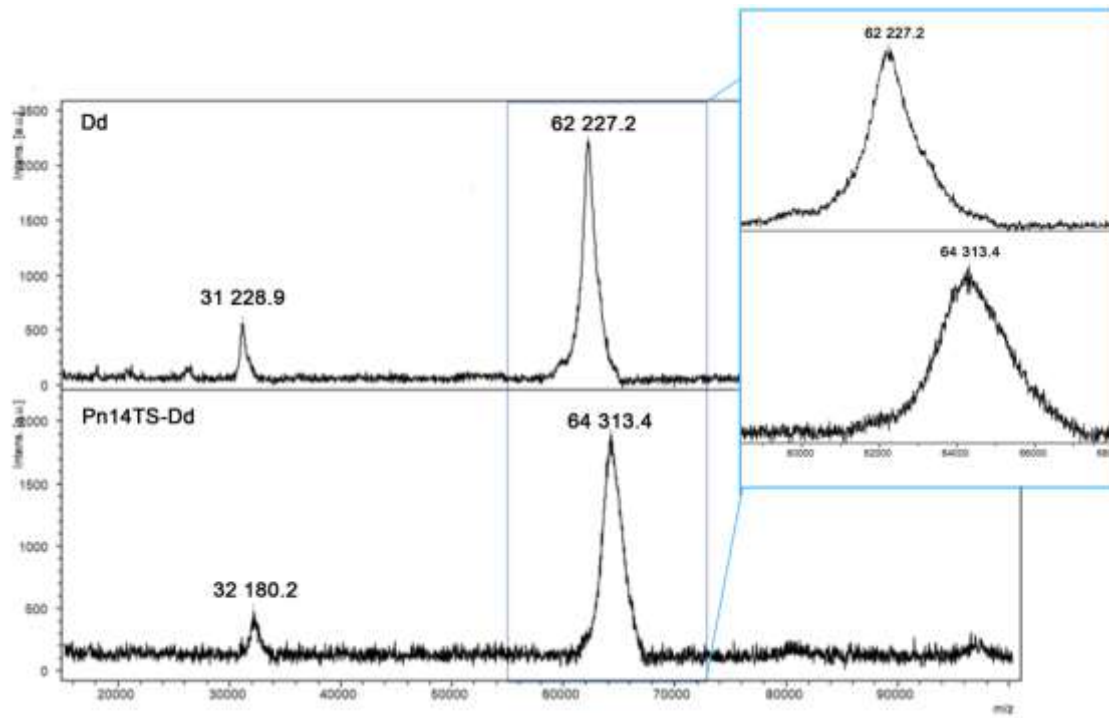
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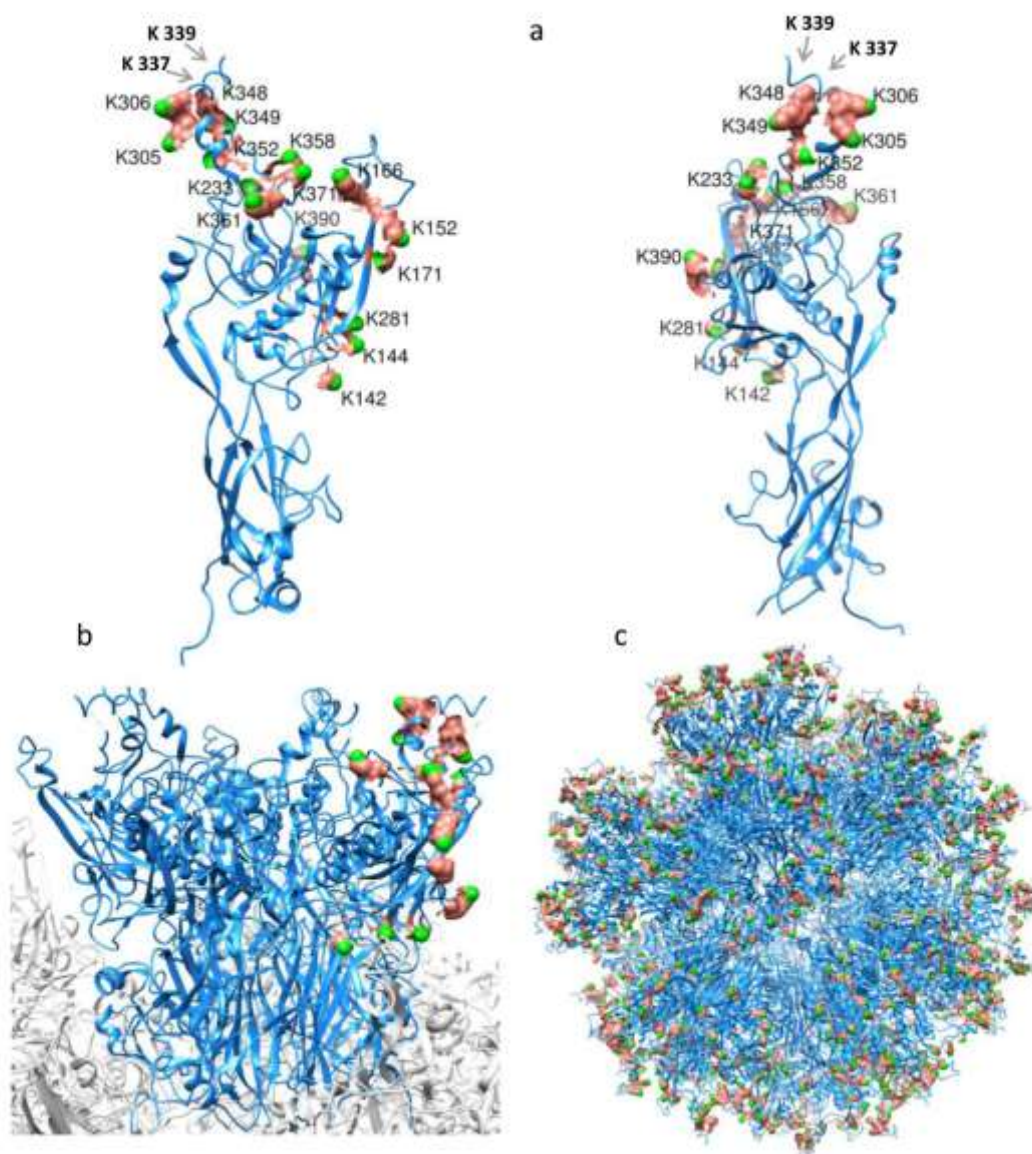
470 **Scheme 1**

471



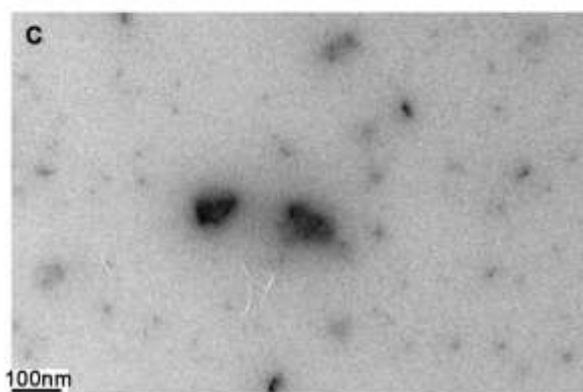
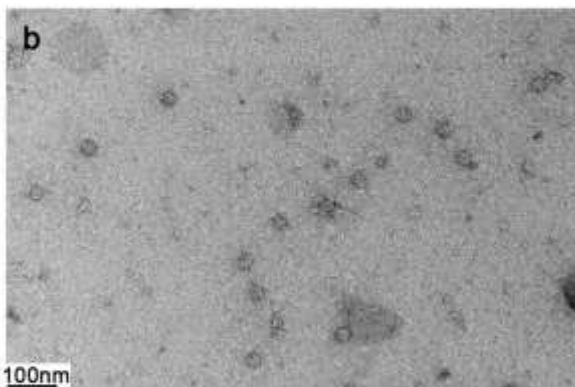
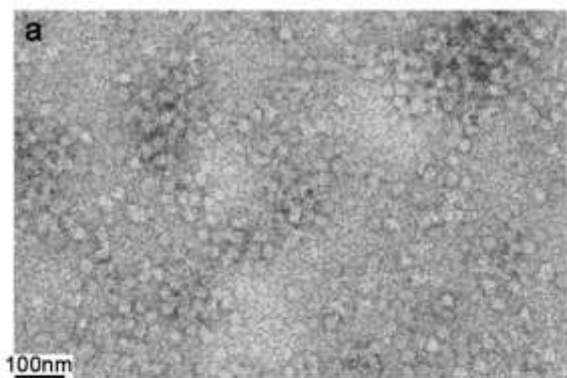
472

473 **Figure 1**



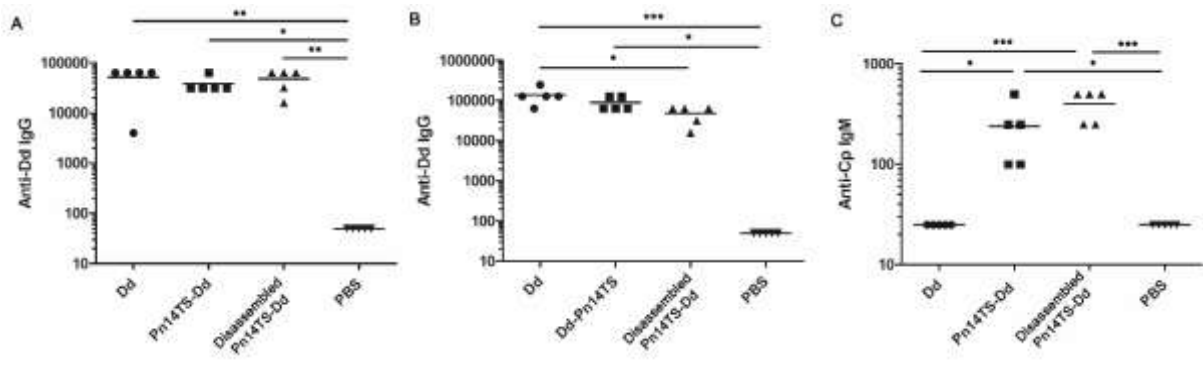
474

475 **Figure 2**



476

477 **Figure 3**



478

479 **Figure 4**