Title

Display of malaria transmission-blocking antigens on chimeric DHBV-derived virus-like particles produced in *Hansenula polymorpha*

Short title

Display of malaria TBV candidates on VLP

Authors

Wetzel, David[1, 2, *]; Chan, Jo-Anne[3]; Suckow, Manfred[1]; Barbian, Andreas[4]; Weniger, Michael[1]; Jenzelewski, Volker[1]; Reiling, Linda[3]; Richards, Jack S[3]; Anderson, David A[3]; Kouskousis, Betty[3]; Palmer, Catherine[3]; Hanssen, Eric[5]; Schembecker, Gerhard[2]; Merz, Juliane[6]; Beeson, James G[3,7,8]; Piontek, Michael[1]

Affiliations

[1] ARTES Biotechnology GmbH, Langenfeld, Germany

[2] Laboratory of Plant and Process Design, Technical University of Dortmund, Germany

[3] Burnet Institute for Medical Research and Public Health, Melbourne, Victoria, Australia

[4] Düsseldorf University Hospital, Institute for anatomy I, Düsseldorf, Germany

[5] The Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Victoria, Australia

[6] Evonik Technology & Infrastructure GmbH, Hanau, Germany

[7] Central Clinical School and Department of Microbiology, Monash University, Victoria, Australia

[8] Department of Medicine, Royal Melbourne Hospital, University of Melbourne, Victoria, Australia

* Corresponding author
E-Mail: d.wetzel@artes-biotechnology.com (DW)

1. Abstract

Background:

Malaria caused by *Plasmodium falciparum* is one of the major threats to human health globally. Despite huge efforts in malaria control and eradication, highly effective vaccines are urgently needed, including vaccines that can block malaria transmission. Chimeric virus-like particles (VLP) have emerged as a promising strategy to develop new malaria vaccine candidates.

Methods:

We developed yeast cell lines and processes for the expression of malaria transmission-blocking vaccine candidates Pfs25 and Pfs230 as VLP and VLP were analyzed for purity, size, protein incorporation rate and expression of malaria antigens.

Results:

In this study, a novel platform for the display of *Plasmodium falciparum* antigens on chimeric VLP is presented. Leading transmission-blocking vaccine candidates Pfs25 and Pfs230 were genetically fused to the small surface protein (dS) of the duck hepatitis B virus (DHBV). The resulting fusion proteins were co-expressed in recombinant *Hansenula polymorpha* (syn. *Pichia angusta, Ogataea polymorpha*) strains along with the wild-type dS as the VLP scaffold protein. Through this strategy, chimeric VLP containing Pfs25 or the Pfs230-derived fragments Pfs230c or Pfs230D1M were purified. Up to 100 mg chimeric VLP were isolated from 100 g dry cell weight with a maximum protein purity of 90 % on the protein level. Expression of the Pfs230D1M construct was more efficient than Pfs230c and enabled VLP with higher purity. VLP showed reactivity with transmission-blocking antibodies and supported the surface display of the malaria antigens on the native VLP.

Conclusion:

The incorporation of leading *Plasmodium falciparum* transmission-blocking antigens into the dS-based VLP scaffold is a promising novel strategy for their display on nano-scaled

63 particles. Competitive processes for efficient production and purification were established in
64 this study.

65 2. Background

66 Malaria is one of the world's deadliest human diseases with nearly half of the global
67 population living at risk. There were an estimated 216 million cases and 445,000 deaths due
68 to malaria in 2016 [1]. This life-threatening disease is caused by *Plasmodium* parasites and
69 is transmitted via the bite of infected female *Anopheles* mosquitoes. The majority of malaria
70 is caused by *P. falciparum*, with *P. vivax* being a second major cause of disease [1]. Despite
71 substantial financial investment, US$ 2.7 billion in 2016, and decades of intense research
72 and development, only one malaria vaccine has progressed through phase 3 clinical trials
73 and is now undergoing phase 4 implementation trials (RTS,S; Mosquirix™). However,
74 vaccine efficacy in phase III clinical trials was low in young children (up to 50% efficacy in the
75 first year, but waning over 18 months) [2]. The World Health Organization has set a strategic
76 goal of developing vaccines with at least 75% efficacy [3], including the development of
77 vaccines that block malaria transmission [1]. Various approaches are under investigation
78 including whole parasite vaccines and subunit vaccines that are composed of defined,
79 purified antigens or their sub-domains [4]. Subunit vaccines have the potential to use
80 established technologies and processes for low-cost production and distribution through
81 existing vaccine delivery mechanisms [5]. A variety of *Plasmodium* antigens are currently
82 under investigation as potential subunit vaccine components and can be classified into one
83 of the following groups based on *Plasmodium* lifecycle stages [6]: i) pre-erythrocytic antigens
84 (e.g. CSP [7]); ii) blood-stage antigens [8]; iii) transmission-stage antigens (e.g. Pfs25,
85 Pfs230 [9–11]).
86
87 Unfortunately, subunit vaccine candidates often suffer from weak immunogenicity that has to
88 be compensated by smart formulation and/or delivery strategies [12] such as virus-like
89 particles (VLP [13,14]). Since the 1980`s, VLP have been approved for use as safe and

effective subunit vaccines against several pathogens [15]. They can also be used as a scaffold for the incorporation of antigens derived from foreign pathogens to enhance their immunogenic potential (chimeric VLP [16]). Accordingly, the RTS,S vaccine contains chimeric VLP with a truncated construct of CSP, the major surface antigen expressed on sporozoites during the pre-erythrocytic stage. However, its efficacy was low in young children; approaches are urgently needed to develop highly efficacious vaccines to improve malaria control and elimination [2,17], such as the inclusion of additional antigens.

Recently, there has been a renewed focus of malaria vaccine development on transmission stage antigens, and transmission-blocking activity is a stated priority in the WHO Malaria Vaccine Technology Roadmap [3]. Transmission-blocking vaccines (TBV) are thought to act by inhibiting the transmission of malaria from humans to the mosquito vector, largely through the action of antibodies taken up in the mosquito's blood-meal [18]. Leading vaccine candidates that are expressed during the transmission stages of *P. falciparum* include Pfs25 [9] and Pfs230 [10,19]. Both antigens have been shown to generate antibodies that are capable of blocking transmission through standard membrane feeding assays [20–22]. Pfs230 is expressed on gametocytes and gametes and is a target of naturally-acquired antibodies from malaria-exposed populations, whereas Pfs25 is only expressed by zytgotes and ookinetes in the mosquito stage and, therefore, naturally-acquired immunity is not generated [18]. However, the development of TBV remains challenging.

The recombinant production and folding of Pfs25 is difficult because it is cysteine-rich and contains four tandem epidermal growth factor (EGF)-like domains [21]. Nevertheless, success has been achieved with yeast-derived versions of Pfs25 that are emerging as prominent TBV candidates [23–25]. However, the immunogenicity of Pfs25 is weak [26], but can be enhanced by construction of fusion proteins [27–29] or by VLP or nanoparticle-based approaches [30–33]. The 363 kDa Pfs230 is a large and complex protein that is predicted to contain multiple cysteine-rich domains [34,35]. Its potential as a transmission-blocking

118 vaccine candidate was identified in the 1980s [10,19]. However, recombinant production of
119 full-length Pfs230 has not been accomplished to the present time. Therefore, research has
120 focused on truncated Pfs230 versions named Pfs230c [22] and Pfs230D1M [36]. These
121 variants were shown to retain the property to elicit transmission-blocking antibodies and can
122 be produced as recombinant antigens [22,36,37]. The Pfs230c construct contains the first
123 two protein domains. Expression studies of Pfs230 in yeast led to the development of the
124 shortened Pfs230D1M construct, which includes only the first domain, and could be
125 efficiently expressed in *Pichia pastoris* [36].
126
127 Despite progress that has been made towards effective malaria vaccines, novel protein
128 conjugation strategies and delivery platforms as well as the use of strong adjuvants may be
129 essential to meet the goals of the malaria control and eradication agenda set by the World
130 Health Organization [38]. Our present study introduces a new platform for the display of the
131 malaria transmission-blocking vaccine candidates Pfs25 [25], Pfs230c [22] and Pfs230D1M
132 [36] on the surface of chimeric VLP. The small surface protein (dS) of the duck hepatitis B
133 virus (DHBV) was used as VLP scaffold [39,40]. The *P. falciparum* transmission stage
134 antigens were genetically fused to the dS and the resulting fusion proteins were co-
135 expressed with non-fused wild-type dS in recombinant strains of the methylotrophic yeast
136 *Hansenula polymorpha* (*H. polymorpha*, syn. *Pichia angusta*, *Ogataea polymorpha*, [41])
137 which allowed the isolation of chimeric VLP composed of wild-type dS and the respective
138 fusion protein. In contrast to previous VLP platforms, the dS-based VLP scaffold allows the
139 stable incorporation of a variety of large molecular weight (MW) foreign antigens. In
140 combination with the yeast expression system, this technology is highly productive and not
141 limited to small scale fundamental research [42]. Thus, the key challenges in the field of
142 chimeric VLP development are met [13,14,43] which makes this platform an attractive and
143 competitive alternative to previously described VLP platforms [30–33]. Furthermore,
144 expression of transmission-blocking antigens as VLP may enable the future co-formulation of
145 these with RTS,S in multistage vaccines.

146 # 3. Materials and methods

147 ## 3.1. Genes, plasmids and strains

148 Open reading frames (ORF) encoding the designed fusion proteins were synthesized by
149 GeneArt/Life Technologies (Regensburg, Germany). They were codon-optimized for
150 heterologous expression in *H. polymorpha* and flanked by *Eco*RI and *Bam*HI restriction sites.
151 Synthesized ORF were inserted between the *Eco*RI and *Bam*HI sites of a derivative of the
152 *H. polymorpha* expression plasmid pFPMT121 [44] which carried the *LEU2* instead *URA3*
153 gene for selection in yeast. The sequences of the ORF post subcloning were confirmed by
154 sequencing prior to yeast transformation. Cloning was done in bacterial strain
155 *Escherichia coli* NEB® 10-beta (New England Biolabs, Frankfurt a. M., Germany) grown at
156 37 °C in lysogeny broth [45] supplemented with 60 mg L^{-1} ampicillin (Applichem, Darmstadt
157 Germany).
158 The auxotrophic *H. polymorpha* strain ALU3 (relevant genotype: *ade1*, *leu2*, *ura3*) [46]
159 derived from wild type strain ATCC® 34438™ (CBS 4732, IFO 1476, JCM 3621, NBRC 1476,
160 NCYC 1457, NRRL Y-5445) was used as expression host. Recombinant yeast cell lines
161 were generated by electroporation [47] and a subsequent strain generation and isolation
162 protocol [48]. Thereby, the expression plasmids integrated genomically stable in different
163 copy numbers into the host genome. Heterologous yeast strains were stored as glycerol
164 stocks at − 80 °C.
165 Recombinant *H. polymorpha* strains co-producing the dS and a fusion protein were
166 generated by the “staggered transformation approach” and screened as previously described
167 [42].
168
169
170

171 ## 3.2. Yeast cell mass generation

172 ### 3.2.1. Shake flask

173 VLP composed of Pfs230D1M-dS and dS were purified from cell mass of strain Ko#119,
174 grown in 2 L baffled shake flasks filled with 200 mL YPG medium containing 20 g L^{-1} glycerol
175 (AppliChem, Darmstadt, Germany) as carbon source and 0.1 g L^{-1} adenine (AppliChem,
176 Darmstadt, Germany). A pre-culture grown in YPD medium to stationary phase was used as
177 inoculum. The main cultures were incubated at 37 °C and 130 rpm with 5 cm throw. After
178 56 h of derepression, 1 % (v/v) methanol was added to the cultures for induction of target
179 gene expression. After 72 h total cultivation time, cells were harvested by centrifugation
180 (6,000*g*, 15 min, 4 °C), washed once with wash buffer (50 mM Na-phosphate buffer, 2 mM
181 EDTA, pH 8.0) and stored at -20 °C.
182

183 ### 3.2.2. Bioreactor

184 VLP containing the fusion proteins Pfs25-dS or Pfs230c-dS were purified from cell mass of
185 strain RK#097 or RK#114, respectively. Strains were grown in a 2.5 L scale stirred tank
186 bioreactor (Labfors 5, Infors, Bottmingen, Switzerland). It was sterilized by autoclaving after
187 filling with 2.5 L animal component free complex medium containing 20 g L^{-1} yeast extract
188 (BD Biosciences, Heidelberg, Germany), 40 g L^{-1} peptone from soymeal (Applichem,
189 Darmstadt Germany), 10 g L^{-1} glycerol, 11 g L^{-1} glucose-monohydrate, and 0.1 g L^{-1} adenine.
190 Aqueous solutions of NH_3 (12.5 % (w/w), sterile filtered) and H_3PO_4 (28 % (w/w), Merck,
191 Darmstadt, Germany) were used as corrective media to keep pH constant (set point 6.5)
192 throughout fermentation and Struktol J 673 (10 % (v/v) aqueous solution, Schill+Seilacher,
193 Hamburg, Germany) was utilized as antifoam agent. Aeration was adjusted to 1 vvm
194 (2.5 NL min^{-1}) and the medium was inoculated to an optical density (OD_{600}) of 0.6 using
195 shake flask pre-cultures.

196

197 After a batch phase of 8 h, strain RK#114 was fed continuously with 275 mL of derepression
198 solution (750 g L^{-1} glycerol) over 31 h. Formation of product was then induced by pulse-wise
199 addition of 100 mL induction solution (285 g L^{-1} glycerol and 715 g L^{-1} methanol). Cells were
200 harvested by centrifugation (6000*g*, 15 min, 4 °C) after 72 h total cultivation time, washed
201 with wash buffer (25 mM Na-phosphate buffer, 2 mM EDTA, pH 8.0) and stored at -20 °C
202 until further processing.

203

204 Strain RK#097 was fed with 160 mL of derepression solution over 36.5 h after the batch
205 phase. 90 mL induction solution were added pulse-wise and fermentation was stopped after
206 65.2 h total cultivation time. Cells were harvested and stored as described before.
207 The dry cell weight (DCW) was quantified using a moisture analyzer (MLS 50-3 HA250, Kern
208 & Sohn, Balingen, Germany). OD_{600} of cell suspensions was determined with a
209 spectrophotometer (DU 640 Beckman Coulter, Brea, California, USA).

210

211 3.3. Purification of VLP

212 All VLP preparations were formulated in desalting buffer (8 mM Na-phosphate buffer pH 7,
213 154 mM NaCl) at concentrations in the range of mg mL^{-1}. However, the purification protocols
214 steps were adjusted for the different chimeric VLP.

215

216 3.3.1. Purification of Pfs25-dS/dS VLP

217 Pfs25-dS/dS VLP were purified from strain RK#097 in preparative manner as described
218 before [42,49]. Briefly, cells were disrupted by six cycles of high pressure homogenization
219 (~1500 bar, APV 2000, SPX Flow Technology, Unna, Germany) and the cell homogenate
220 was adjusted to 4.5 % (w/w) PEG_{6000} and 0.45 M NaCl. After incubation over-night at 4 °C
221 and subsequent centrifugation (17,000*g*, 30 min, 4 °C), the product was adsorbed to fumed

222 silica matrix Aerosil (type 380 V, Evonik, Essen, Germany). The matrix was washed once
223 with 77 mM NaCl aqueous solution. Desorption buffer (10 mM di-sodium tetraborate
224 decahydrate, 2 mM EDTA, 6 mM deoxycholic acid sodium salt, pH 9.1) was used to remove
225 the product from the Aerosil (1 h, 25 °C). The desorbed material was applied to ion exchange
226 chromatography (Mustang Q XT, PALL Life Sciences, Port Washington, New York, United
227 States). Product containing fractions were pooled and concentrated by ultrafiltration
228 (Minimate™ TFF tangential flow filtration Capsule Omega 100 k Membrane, PALL, Port
229 Washington, New York, United States) prior to CsCl density gradient ultracentrifugation
230 (1.5 M CsCl) in Optima™ L90K centrifuge (rotor type: 70.1 Ti, tubes: 16 * 76 mm, Beckman
231 Coulter, Brea, California, USA) for 65 h at 48,400 rpm and 4 °C. Product containing fractions
232 were pooled, desalted by dialysis (Slyde-A-Lyzer™ dialysis cassettes, MWCO 20 kDa,
233 Thermo Fisher Scientific, Waltham, USA) against desalting buffer (8 mM Na-phosphate
234 buffer pH 7, 154 mM NaCl, AppliChem, Darmstadt, Germany) and 0.45 µm filtered (Filtropur
235 S 0.45 filters, Sarstedt, Nümbrecht, Germany).
236

237 ### 3.3.2. Purification of Pfs230c-dS/dS VLP

238 The following adjustments were made for the purification of Pfs230c-dS/dS VLP from strain
239 RK#114: 2.5 % (w/w) PEG_{6000} and 0.25 M NaCl were used for clarification of the crude cell
240 lysate after high pressure homogenization. Following the Aerosil batch adsorption step, the
241 silica matrix was washed twice applying aqueous solution of 77 mM NaCl and 2.5 mM
242 deoxycholic acid sodium salt or aqueous solution of 77 mM NaCl. The desorbed material
243 was then subjected two consecutive times to Capto Core 700 chromatography matrix (GE
244 Healthcare, Amersham, UK) applying 5 mg protein per ml resin. The unbound product
245 fraction was concentrated by ultrafiltration and the retentate was applied to CsCl density
246 gradient ultracentrifugation as described for the Pfs25-dS/dS VLP purification. Product
247 containing fractions were then pooled and dialyzed against desalting buffer in two steps: first,
248 the CsCl concentration was reduced to 0.5 M CsCl. Then, 0.05 % (w/v) SDS were added

249 before the dialysis against desalting buffer was continued. The dialyzed sample was 0.45 µm
250 filtered.
251

252 3.3.3. Purification of Pfs230D1M-dS/dS VLP

253 The protocol for purifying Pfs230D1M-dS/dS VLP from strain Ko#119 was modified
254 compared to the purification of Pfs230c-dS/dS VLP from strain RK#114. A 100 mM Na-
255 carbonate/bicarbonate buffer (pH 9.2) with 1.2 M urea [50] was used for desorption of the
256 product from the fumed silica matrix Aerosil. The desorbate was subjected to only one run of
257 Capto Core 700 chromatography. Ultrafiltration, CsCl density gradient ultracentrifugation,
258 dialysis and filtration were performed as described for Pfs25-dS/dS VLP purification from
259 strain RK#097.
260

261 3.4. Protein, lipid and VLP analysis

262 Protein concentrations were determined with the Pierce™ BCA protein Assay kit (Thermo
263 Fisher Scientific, Waltham). Lipid content of VLP preparations was determined based on
264 sulfo-phospho-vanillin reaction [51] with refined soya oil (Caesar & Loretz GmbH, Hilden,
265 Germany) used as standard.
266 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western
267 blotting were performed as previously described [42]. In short: The Criterion™ system from
268 BioRad (München, Germany) was used to run SDS-PAGE. Cellulose nitrate membranes
269 (Sartorius Stedim Biotech, Göttingen, Germany) were used for semi dry Western blotting of
270 the proteins. Membranes were subsequently blocked by 3 % powdered milk in PBS
271 containing 0.05 % Tween 20 (Pfs230-related Western blots) or Roti®-Block (Carl Roth GmbH,
272 Karlsruhe, Germany, Pfs25-related Western blots). Primary antibodies used for
273 immunolabelling (Table ***1***) were utilized in combination with appropriate secondary antibodies

274 purchased from BioRad (München, Germany) and BCIP-NBT (VWR international, Radnor,
275 USA) or HRP substrate (Thermo Fisher Scientific, Waltham, USA).
276 Coomassie staining of polyacrylamide (PAA) gels were also done as previously described
277 [52].
278 *N*-Glycosylation of the heterologous target proteins was analyzed by treatment with an
279 endoglycosidase H (EndoH) prior to SDS-PAGE [42].
280 Host cell proteins were quantified by anti-HCP enzyme-linked immunosorbent assay (ELISA)
281 as described previously [42].
282 Analyses by ELISA were performed in Nunc MaxiSorp™ flat-bottom 96 well ELISA plate
283 (Thermo Fisher Scientific, Waltham). For specific detection of *P. falciparum* antigen Pfs25,
284 the wells were coated over-night at 4 °C with 1 µg mL^{-1} (50 µL per well) of the respective VLP
285 in PBS and blocked with 1 % (w/v) BSA in PBS for 1 - 2 h at RT before polyclonal mouse
286 anti-Pfs25 antibodies were applied as primary immunoreagents for 2 h. The plate was
287 washed thrice using PBST in between antibody incubation steps. Secondary polyclonal goat
288 anti-mouse IgG HRP-conjugated antibody was used to detect antibody binding. Color
289 detection was developed using ABTS liquid substrate (Sigma-Aldrich) which was
290 subsequently stopped with 1 % SDS. The level of antibody binding was measured as optical
291 density in a GENios Microplate Reader (Tecan, Männedorf, Switzerland) at 405 nm.
292 For specific detection of *P. falciparum* antigen Pfs230, plates were coated with indicated
293 concentrations of VLP (4°C, over-night) and subsequently blocked with 1% casein in PBS
294 (Sigma-Aldrich) for 2h at 37°C before primary antibodies were added (polyclonal mouse anti-
295 Pfs230 or monoclonal 1B3 antibody, 10 µg mL^{-1}). Secondary HRP-conjugated antibodies
296 (polyclonal goat anti-mouse IgG at 1/1000 from Millipore) were used to detect antibody
297 binding. Color detection was developed using ABTS liquid substrate (Sigma-Aldrich), which
298 was subsequently stopped using 1 % SDS. PBS was used as a negative control and plates
299 were washed thrice using PBS with 0.05 % Tween in between antibody incubation steps.
300 The level of antibody binding was measured as absorption at 405nm (A_{405nm}).

301 Analysis of VLP were performed as essentially described previously described [42] by
302 dynamic light scattering (DLS), super-resolution microscopy (N-SIM; structured illumination
303 microscopy) and transmission electron microscopy (TEM). As primary anti-Pfs230 antibodies,
304 polyclonal mouse antibodies were applied that were described to have transmission-blocking
305 activity [53]. Cross-reactivity with plain dS VLP without a fusion protein was checked
306 carefully.

307

308 **Table 1 List of immunoreagents used for specific detection of the target proteins**

Antigen	Primary antibody	Source	Reference
dS	7C12[(a)]	BioGenes GmbH (Berlin, Germany)	[54,55]
Pfs25	32F81[(b)]	National Institutes of Health (NIH, Bethesda, Maryland, USA)	[9,56]
	4B7[(b)]	National Institutes of Health (NIH, Bethesda, Maryland, USA)	[23,57]
Pfs230	mouse polyclonal[(c)]	National Institutes of Health (NIH, Bethesda, Maryland, USA)	[53]
	1B3	National Institutes of Health (NIH, Bethesda, Maryland, USA)	[10]

309 (a) detects the wild-type dS and the dS domain of each fusion protein
310 (b) Monoclonal antibodies 32F81 and 4B7 were kindly provided by PATH Malaria Vaccine Initiative and BEI
311 Resources NIAID, NIH.
312 (c) Polyclonal mouse antibody was kindly provided by Carole Long and Kazutoyo Miura, NIH.
313

314 4. Results

315 4.1. Design of fusion proteins

316 In this study, three different types of chimeric VLP each displaying a different foreign antigen
317 derived from *P. falciparum* were developed. The incorporation of *P. falciparum* transmission
318 stage antigens into the dS-based VLP scaffold was realized by the design of fusion proteins
319 each containing one of the malaria antigens *N*-terminally fused to the dS. The formation of
320 VLP was allowed by interaction of dS subunits or dS domains of fusion proteins, respectively.
321 Literature was screened for promising targets and the following antigens were chosen to be

322 displayed on the surface of the membranous VLP. Additional details on the fusion protein
323 construction are given in Table 2.
324 The fusion protein Pfs25-dS comprised amino acids (aa) 23-193 of the cysteine-rich
325 zygote/ookinete surface protein Pfs25 of *P. falciparum* fused to the dS. The Pfs25 part
326 contained the four EGF-like domains of the naive antigen but missed the *N*-terminal signal
327 sequence and the hydrophobic C-terminus [24,25,53]. Pfs230c-dS was constructed by fusing
328 a 630 aa fragment (Pfs230c, [22]) of the *P. falciparum* transmission stage antigen Pfs230 to
329 the dS. Due to its size, this fragment was challenging for a chimeric VLP-based approach.
330 Thus, a shorter variant thereof (Pfs230D1M, aa 542-736 according to MacDonald et al. [36])
331 was introduced in the third fusion protein construct, Pfs230D1M-dS; this construct has been
332 effectively expressed as a monomeric protein in *Pichia pastoris* and is a TBV candidate in
333 clinical development [36]. All fusion protein encoding genes were inserted into a pFPMT121-
334 based plasmid [44] which carried the *LEU2* gene for selection of transformed yeast strains.
335
336

337 **Table 2 Summary of fusion proteins constructed and recombinantly produced in *H. polymorpha***

Fusion protein	Number of aa[(a)]	MW [kDa]	Genbank reference of CDS	*P. falciparum* donor protein (Genbank reference)	Donor protein domain fused to dS	aa linker between *P. falciparum* antigen and dS
Pfs25-dS	341	37.0	MH142260	zygote/ookinete surface protein Pfs25 (XP_001347587.1)	aa 23-193 [25]	GAGA
Pfs230c-dS	861	98.0	MH142261	gametocyte surface protein Pfs230 (XP_001349600.1)	aa 443-1132 [22]	GAGA
Pfs230D1M-dS	366	40.3	MH142262	gametocyte surface protein Pfs230 (XP_001349600.1)	aa 542-736[(b)] [36]	GAGA

338 [(a)] Including an artificial start-methionine and aa 2-167 of the dS at the C-terminus
339 [(b)] Including N585Q aa exchange, elimination of a potential *N*-glycosylation site
340

341 ## 4.2. Isolation of recombinant *H. polymorpha* production
342 strains

343 Typically, co-production of dS and a fusion protein composed of a foreign antigen fused to
344 dS allows formation of chimeric "antigen-dS/dS" VLP [39,40]. For the generation of the three
345 different types of VLP each displaying one of the *P. falciparum*-derived antigens, three
346 recombinant *H. polymorpha* cell lines needed to be isolated that co-produce the scaffold
347 protein dS and Pfs25-dS, Pfs230c-dS or Pfs230D1M-dS, respectively. To generate such
348 strains, the dS-producing cell line A#299 [42] was super-transformed with an expression
349 plasmid encoding the respective fusion protein. From each of the transformations, one strain
350 co-producing the dS and the respective fusion protein was selected from the resulting
351 transformants and used for production of chimeric VLP containing dS in combination with
352 Pfs25-dS, Pfs230c-dS or Pfs230D1M-dS, respectively. The recombinant *H. polymorpha*
353 strains that were used for production of VLP are indicated in Table 3.

354

355 **Table 3 Recombinant *H. polymorpha* production strains used for the generation of**
356 **VLP.**

Yeast strain designation	Transformed host strain	Produced recombinant protein(s)	Relevant genotype
RK#097	A#299[(a)]	dS and Pfs25-dS	*URA3, LEU2, ade1*
RK#114	A#299[(a)]	dS and Pfs230c-dS	*URA3, LEU2, ade1*
Ko#119	A#299[(a)]	dS and Pfs230D1M-dS	*URA3, LEU2, ade1*

357 [(a)] Recombinant dS-producing *H. polymorpha* strain previously described [42]

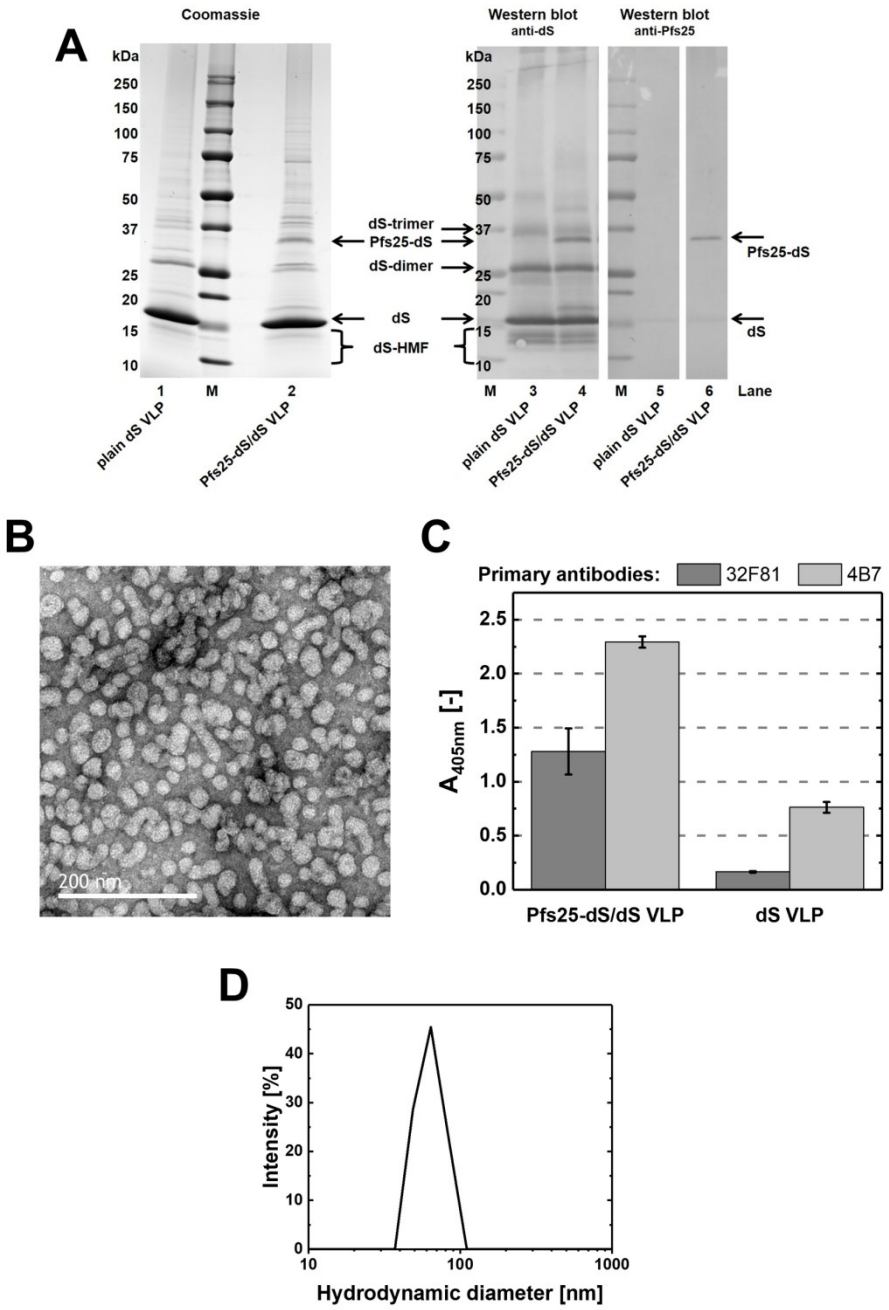
358

359 ## 4.3. Production of chimeric Pfs25-dS/dS VLP

360 Chimeric Pfs25-dS/dS VLP composed of wild-type dS and the fusion protein Pfs25-dS were
361 isolated from cell paste of strain RK#097. A total of 97.6±10.3 mg chimeric VLP could be
362 isolated from 97±3 g DCW that were processed (1.0±0.1 mg g^{-1}). In denaturing assays (SDS-
363 PAGE, Western blot, Fig 1 A), the final sample was compared to plain dS VLP containing the
364 dS without fusion protein [42] and thus lacks the fusion protein-specific signals. Apart from

365 that, similar protein signal patterns were observed for the chimeric Pfs25-dS/dS VLP
366 preparation in comparison to the plain dS VLP. Analysis of the Coomassie stained PAA gel
367 by densitometry (lane 2) indicated 90 % Pfs25-dS/dS purity on the protein level and about
368 3 % fusion protein content. The estimated purity was in good correlation with anti-HCP
369 ELISA results showing 8±1 % HCP content (data not shown). Anti-dS and anti-Pfs25
370 Western blots (lanes 4 and 6) were used to identify the VLP forming proteins. The apparent
371 MW of Pfs25-dS (~33 kDa) and dS (~15 kDa) were slightly below their theoretical MW of
372 37 kDa or 18.2 kDa, respectively. For both VLP preparations additional signals were
373 detected in the anti-dS Western blot that likely correspond to either oligomeric forms (dimers,
374 trimers, etc.) of the dS or authentic forms of higher mobility (dS-HMF, [42]). The Pfs25-dS
375 was reactive with transmission-blocking mAb 32F81 but non-specific cross reactivity with the
376 dS was observed (lanes 5 and 6) applying Roti®-Block (Carl Roth GmbH) as blocking
377 reagent. Cross reactivity was not observed if 3 % powdered milk in PBS containing 0.05 %
378 Tween 20 was applied as blocking reagent (Fig S1 in the supplementary material).
379
380 ELISA was used to detect expression of Pfs25 on the surface of native VLP. Reactivity of the
381 Pfs25-dS/dS VLP preparation was demonstrated with two Pfs25-specific antibodies 32F81
382 [9] and 4B7 [23] having transmission-blocking activity (Fig 1 C). Just as in the anti-Pfs25
383 Western blot, cross reactivity to the dS was observed. However, the Pfs25-dS/dS VLP were
384 substantially more reactive.
385
386 Analysis by negative staining TEM and DLS (Fig 1 B and D) confirmed the formation of
387 homogeneous particles. TEM imaging indicated particles of predominantly 20-40 nm
388 according to manual evaluation. DLS showed a monomodal size distribution and a
389 monodisperse particle population characterized by a hydrodynamic diameter of 64 nm
390 (PDI 0.11). A summary of the production process and the composition of the final Pfs25-
391 dS/dS VLP preparation can be found in Table 4.
392

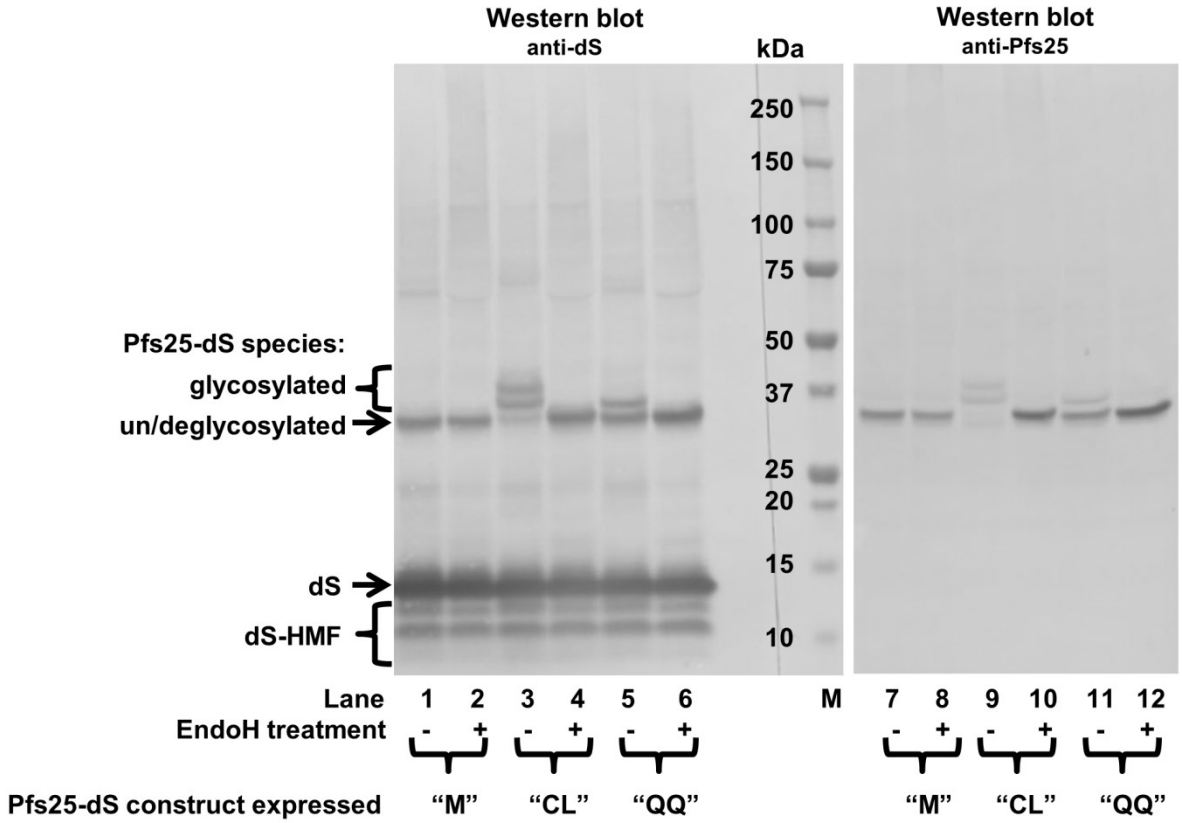
393 *N*-glycosylation of the Pfs25-dS fusion protein was analyzed in crude cell lysates by
394 treatment with EndoH (Fig 2). The Pfs25-dS fusion protein construct “M” was described in
395 Table 2 and was chosen for chimeric VLP production because its recombinant expression in
396 *H. polymorpha* resulted in a homogeneous product (Fig 2, lanes 1 and 7). Despite its three
397 potential *N*-glycosylation sites within the Pfs25 aa sequence, the “M” construct was not
398 sensitive to EndoH treatment. The fusion protein was detected at ~33 kDa MW without and
399 after EndoH treatment indicating it was not *N*-glycosylated. However, in the initial
400 experiments two additional Pfs25-dS constructs were included (“CL” and “QQ”, Fig 2). The
401 “CL” construct analyzed in lanes 3/4 and 9/10 contained the leader sequence of the chicken
402 lysozyme at its *N*-terminus instead of the artificial start-methionine of the “M” construct. The
403 third construct (“QQ”) analyzed in lanes 5/6 and 11/12 was like the “CL” construct but
404 contained two single amino acid exchanges (N112Q, N187Q) eliminating two of the three
405 potential *N*-glycosylation sites. The design of the Pfs25-dS fusion protein affected its degree
406 of *N*-glycosylation. The expression of “QQ” construct led to two Pfs25-dS species detected at
407 ~33 or ~35 kDa MW, respectively (lanes 5 and 11). Expression of the “CL” variant resulted in
408 the detection of four fusion protein species characterized by molecular weights of ~33 kDa
409 (faint band), ~35 kDa, ~38 kDa and ~42 kDa (faint band, lanes 3 and 9). In both cases the
410 signals unified in the ~33 kDa signal after deglycosylation by treatment with EndoH (lanes 4,
411 6, 10 and 12). Thus the ~33 kDa signal refers to the non-glycosylated form of the Pfs25-dS
412 fusion protein.
413



414

415 **Figure 1 Analyses of purified Pfs25-dS/dS VLP derived from strain RK#097.** (A):
416 Reducing SDS-PAGE (left, 10 µg protein loaded) and Western blot (right, 1 µg protein
417 loaded) comparing Pfs25-dS/dS VLP to plain dS VLP which were purified from strain A#299
418 and do not contain any fusion protein. Lanes 1 and 2: Coomassie stained PAA gel. Lanes 3
419 and 4: Western blot probed with anti-dS 7C12 mAb. Lanes 5 and 6: Western blot probed with
420 anti-Pfs25 mAb 32F81 and analyzed on the same membrane. M: molecular weight marker.
421 (B): TEM imaging. (C): Analysis by ELISA in comparison to plain dS VLP purified from strain

422 A#299. The wells of the ELISA plate were coated with 1 µg mL^{-1} (50 µL per well) chimeric
423 Pfs25-dS/dS VLP or same amounts of plain dS VLP, respectively. Error bars indicate
424 standard deviation based on triplicates. (D): Size distribution determined by DLS.
425
426



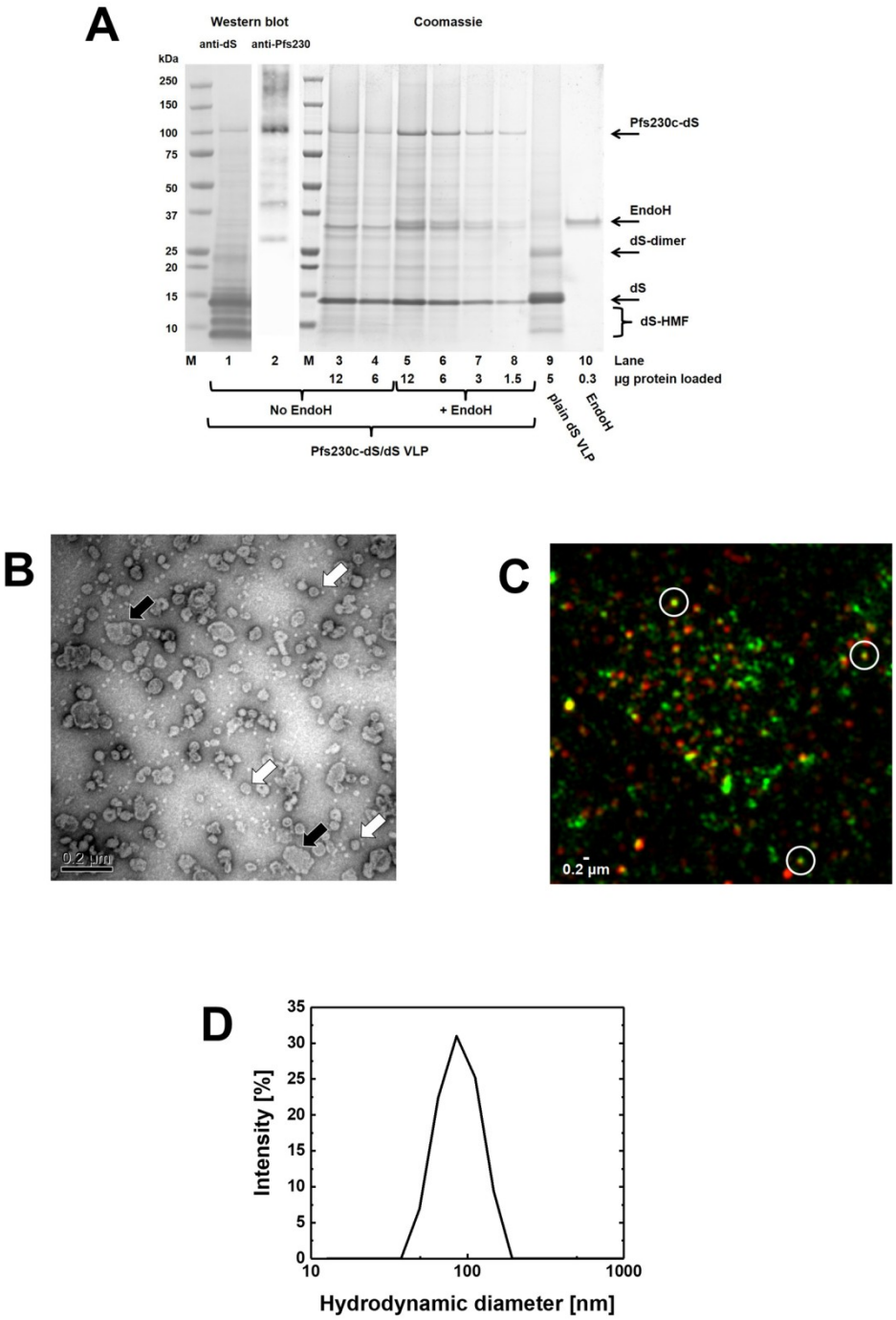
427

428 **Figure 2 Western blot analysis of different Pfs25-dS constructs in crude cell lysates**
429 **before and after treatment with EndoH.** Crude cell lysates of three different recombinant
430 *H. polymorpha* strains were analyzed by anti-dS (mAb 7C12) and anti-Pfs25 (mAb 32F81)
431 Western blots. The strains co-expressed the wildtype dS and one of the three Pfs25-dS
432 fusion protein constructs: "M" (*N*-terminal artificial start-methionine), "CL" (*N*-terminal chicken
433 lysozyme signal sequence) or "QQ" (*N*-terminal chicken lysozyme signal sequence and
434 N112Q, N187Q aa exchanges), respectively Samples were treated with EndoH where
435 indicated. M: molecular weight marker.
436

437 ## 4.4. Production of chimeric Pfs230c-dS/dS VLP

438 From 73.4±8 g DCW of strain RK#114, 14.5±2.6 mg chimeric Pfs230c-dS/dS VLP composed
439 of wild-type dS and the fusion protein Pfs230c-dS were isolated ($Y_{P/X}$ = 0.2±0.06 mg g^{-1}). At
440 different stages during processing of Pfs230c-dS/dS VLP substantial losses of product were
441 observed due to precipitation. Therefore, the purification protocol was adjusted compared to
442 the purification of Pfs25-dS/dS VLP: the PEG and NaCl concentrations were reduced for
443 clarification of the crude cell lysate, the Capto Core 700 matrix was used instead of the
444 Mustang Q membrane adsorber and the dialysis procedure was modified. To reach higher
445 product purity with the modified process, an additional wash step during the Aerosil batch
446 procedure and a second Capto Core 700 run were added to the purification process.
447
448 Purified Pfs230c-dS/dS VLP were analyzed in native and non-native assays (Fig 3). VLP
449 forming proteins were identified by anti-dS Western blot (Fig 3 A, lane 1) or by Pfs230-
450 specific Western blot (lane 2), respectively. No cross-reactivity of the anti-Pfs230 polyclonal
451 antibodies with the dS was observed. The purity on protein level (64 %) was investigated by
452 densitometry for lane 3 of the Coomassie stained PAA gel (Fig 3 A). All bands detected
453 between the fusion protein and the ds were considered as impurities. A subset of these
454 bands was reactive in an anti HCP Western blot (data not shown). The most prominent host
455 cell protein band beside the two product proteins (dS and Pfs230c-dS) was detected at
456 32 kDa apparent MW and represented 12 % of the total band volume of Coomassie stained
457 lane 3. The Pfs230c-dS specific signal appears diffuse in lanes 3 and 4 (Fig 3 A). Upon
458 treatment with EndoH, the diffuse smear disappeared and the main band became intensified
459 by factor 2.6 according to analysis by densitometry. This revealed that the six potential *N*-
460 glycosylation sites within the aa sequence of the Pfs230c antigen were in a subpopulation of
461 the molecules at least partially occupied. Signal intensities of wild-type dS and
462 deglycosylated Pfs230c-dS obtained by densitometry from lanes 5 to 8 were used to
463 calculate the ratio of the two target proteins. For that purpose, the intensities were plotted
464 against the protein amount loaded in the corresponding lane. The slopes obtained from linear

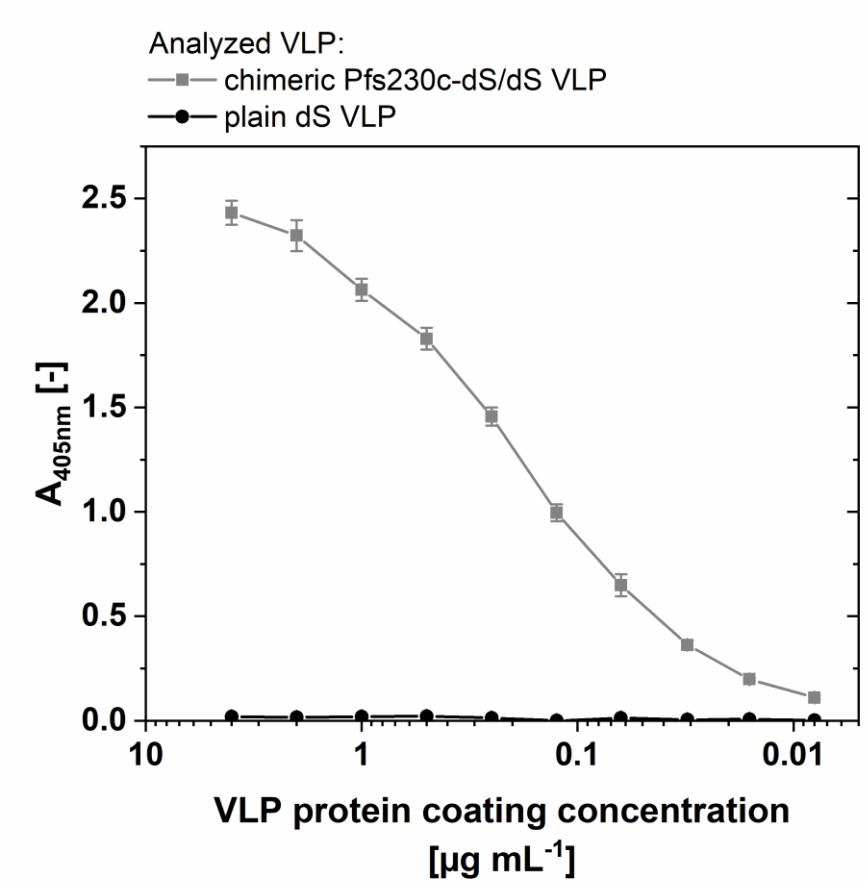
465 regression revealed a ratio of wild-type dS to Pfs230c-dS in the chimeric Pfs230c-dS/dS VLP
466 of approximately 70 % wild-type dS to 30 % fusion protein.
467
468 Formation of VLP was confirmed for both samples by TEM and DLS (Fig 3 B and D). DLS
469 indicated a monomodal and monodisperse (PDI 0.09) particle population characterized by
470 hydrodynamic diameter of 91 nm. However, the appearance of the Pfs230c-dS/dS VLP in
471 TEM imaging was rather heterogeneous (Fig 3 B). The dominating species of detected
472 objects were in the range of 44-60 nm diameter but also larger structures (>120 nm) were
473 observed frequently and this could be due to particle aggregation.
474
475 Analysis by N-SIM was applied as native immunoassay using polyclonal antibodies
476 described to have transmission-blocking activity [53]. This demonstrated co-localization of dS
477 and Pfs230c antigens in nano-scaled particles (circled spots, Fig 3 C) providing supporting
478 evidence of the formation of chimeric VLP by wild-type dS and Pfs230c-dS. Also, Pfs230c-
479 dS/dS VLP were reactive with anti-Pfs230 polyclonal mouse antibodies [53] in ELISA (Fig 4
480 and Fig S 2) but failed to react with monoclonal anti-Pfs230 antibody 1B3 [10] (Fig S 2).
481 Cross reactivity of the anti-Pfs230 polyclonal antibody with the dS VLP scaffold in form of
482 plain dS VLP [42] was not observed in this native assay (Fig 4).
483



484

485 **Figure 3 Analyses of purified Pfs230c-dS/dS VLP derived from strain RK#114.** (A):
486 Western blot probed with anti-dS 7C12 mAb (lane 1) or probed with polyclonal anti-Pfs230
487 antibody (lane 2) and Coomassie stained PAA gel (lanes 3-10). Samples loaded in lanes 5-8
488 were treated with an EndoH. Lane 9: purified plain dS VLP. Lane 10: EndoH used. M:
489 molecular weight marker. (B): TEM imaging at 100,000-fold magnification. (C): N-SIM
490 analysis of purified VLP containing Pfs230c antigen showing immunolabeling of dS (green)

491 or Pfs230c (red). Three nano-scaled spots that showed co-localization of the fluorescence
492 markers (yellow) were representatively circled. (D): Size distribution determined by DLS.
493



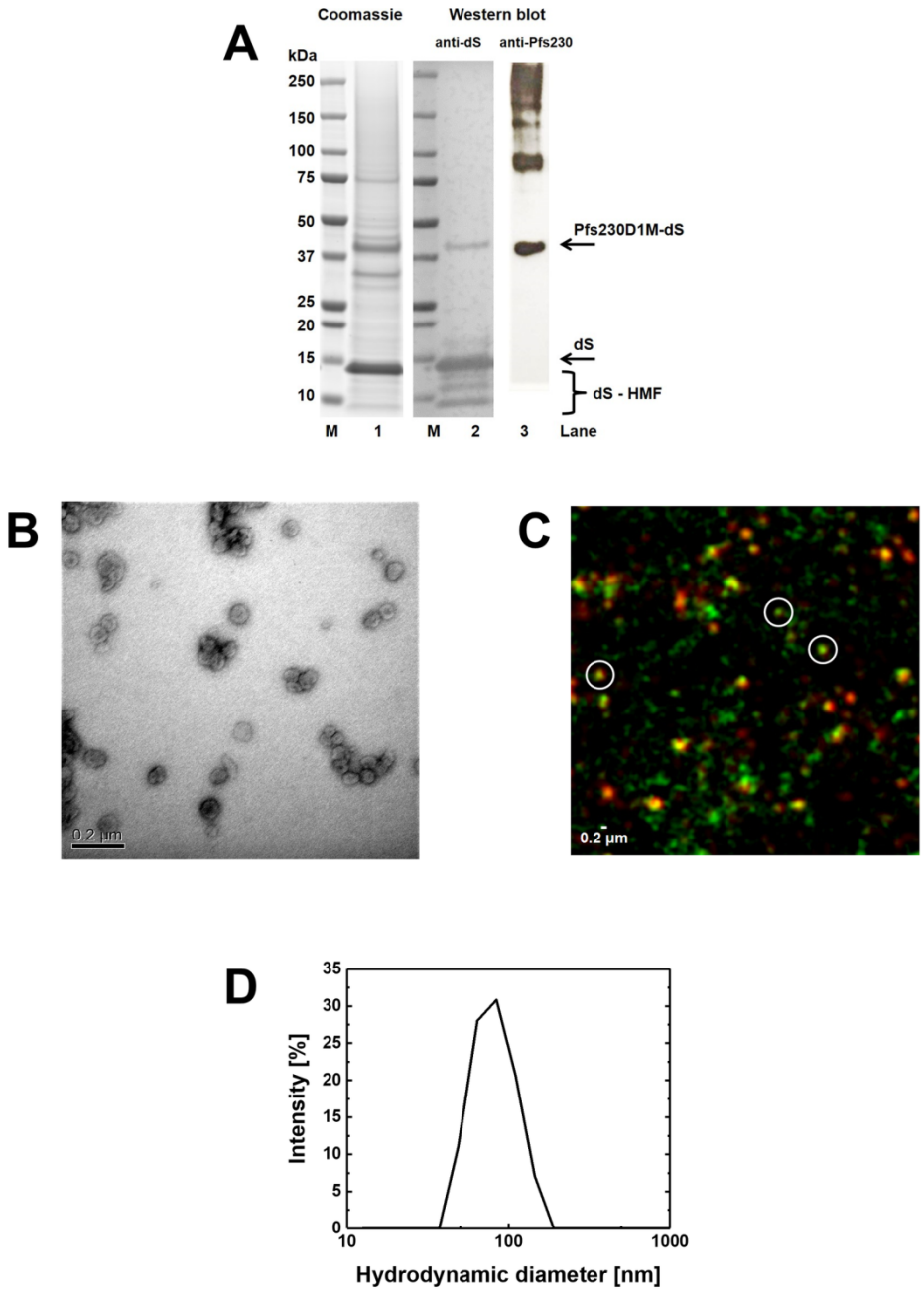
494

495 **Figure 4 ELISA data on purified Pfs230c-dS/dS VLP derived from strain RK#114.** The
496 ELISA plate wells were coated with the indicated chimeric Pfs230c-dS/dS VLP or plain dS
497 VLP, respectively. The mouse polyclonal antibody was applied at 10 µg mL^{-1}.
498

499 ## 4.5. Production of chimeric Pfs230D1M-dS VLP

500 Processing 8.1±0.9 g DCW of strain Ko#119 yielded 5.1±0.4 mg of chimeric
501 Pfs230D1M-dS/dS VLP ($Y_{P/X}$ = 0.64±0.1 mg g^{-1}) that were composed of wild-type dS and the
502 fusion protein Pfs230D1M-dS. Processing of Pfs230D1M-dS/dS VLP was easier than
503 processing of Pfs230c-dS/dS VLP. No unexpected product losses during downstream
504 processing (DSP) were observed and thus a less complex DSP could be chosen. The

505 specific yield $Y_{P/X}$ of Pfs230D1M-dS/dS VLP was about three times as much as for the
506 Pfs230c-dS/dS VLP.
507
508 Both VLP-forming proteins were detected by anti-dS Western blot (Fig 5 A, lane 2). The
509 fusion protein Pfs230D1M-dS was specifically detected by anti-Pfs230 Western blot (lane 3).
510 Judging by their MW, the additional high MW signals in this lane likely correspond to
511 oligomeric forms (dimers, trimers, etc.) of the fusion protein. Most likely, these forms were not
512 detected by the anti-dS mAb in lane 2 because the signals were below the detection limit.
513 Analysis of a Coomassie stained PAA gel (Fig 5 A, lane 1) by densitometry indicated 72 %
514 purity on protein level and a composition of 24 % fusion protein and 76 % wild-type dS.
515 Formation of VLP was confirmed by TEM (Fig 5 B) and indicated 42 – 62 nm diameter for the
516 VLP. Size distribution analyzed by DLS (Fig 5 D) indicated monomodal size distribution and
517 a monodisperse particle population characterized by a hydrodynamic diameter of 84 nm
518 (PDI 0.09). N-SIM was performed as for Pfs230c-dS/dS VLP with a similar result (Fig 5 C).
519 The Pfs230D1M-specific and the dS-specific signals co-localized in nano-scaled particles
520 (circled spots).
521



522

523 **Figure 5 Analyses of purified Pfs230D1M-dS/dS VLP derived from strain Ko#119.** (A):
524 Coomassie stained PAA gel (lane 1, 12 µg protein loaded), Western blot probed with anti-dS
525 7C12 mAb (lane 2) or probed with polyclonal anti-Pfs230 antibody (lane 3). M: molecular
526 weight marker. (B): TEM imaging at 100,000-fold magnification. (C): N-SIM analysis of
527 purified VLP containing Pfs230c antigen showing immunolabeling of dS (green) or

528 Pfs230D1M (red). Three nano-scaled spots that showed co-localization of the fluorescence
529 markers (yellow) were representatively circled. (D): Size distribution determined by DLS.
530
531
532 ## 4.6. Summary and comparison

533 The production processes and the compositions of the three different VLP preparations are
534 summarized in Table 4. The purification of chimeric Pfs25-dS/dS VLP from strain RK#097
535 was the most productive process and yielded 1.0±0.1 mg VLP per g DCW or 39±5 mg VLP
536 per L cell culture with 90 % purity on protein level. However, the fusion protein content in the
537 VLP was lowest (~3 %) in comparison to the other VLP preparations.
538
539 Despite the size of the Pfs230c antigen and difficulties due to product precipitation, isolation
540 of chimeric Pfs230c-dS/dS VLP was successful. However, the VLP yield was considerably
541 lower (0.2±0.06 mg g^{-1} or 8±2 mg L^{-1}) than for the chimeric Pfs25-dS/dS VLP but the fusion
542 protein content of the VLP was approximately 10-times higher than for Pfs25. The purity of
543 Pfs230c-dS/dS VLP on the protein level was substantially lower (64 % on protein level) and
544 could not be improved by SEC (Capto Core 700 matrix), ultrafiltration (MWCO: 100 kDa) or
545 HP-SEC (data not shown).
546
547 VLP yield and purity, were improved by modification of the fusion protein. The truncated
548 Pfs230D1M-dS version led to tripling in VLP yield ($Y_{P/X}$) combined with improvement in purity
549 on the protein level by 8 %. However, the yield per culture volume was lower due to
550 cultivation in shake flasks compared to fermentation in 2.5 L scale. The Pfs230D1M-dS/dS
551 and the Pfs230c-dS/dS VLP preparations had comparable fusion protein contents.
552
553 The hydrodynamic diameters determined by DLS were consistently slightly larger than the
554 respective diameters specified by manual evaluation of the TEM images. Nevertheless, all
555 data collected are within dimensions that could be expected for this kind of VLP [42,58]. The

556 determined buoyant densities (1.13 - 1.16 g cm^{-3}) and lipid contents (30-40 %) were also
557 consistent throughout the VLP preparations and plausible for lipoproteins or VLP [59].
558
559 **Table 4 Summary of production processes leading to the three VLP preparations containing Pfs25-dS,**
560 **Pfs230c-dS or Pfs230D1M-dS, respectively.**

	VLP forming proteins		
	dS and Pfs25-dS	**dS and Pfs230c-dS**	**dS and Pfs230D1M-dS**
***H. polymorpha* strain**	RK#097	RK#114	Ko#119
Cell mass generation	Fermentation 2.5 L scale	Fermentation 2.5 L scale	Shake flask
DCW used for VLP purification [g]	97±3	73.4±8	8.1±0.9
VLP purity on protein level[(a)] [%]	90	64	72
Isolated VLP [mg]	97.6±10.3	14.5±2.6	5.1±0.4
VLP yield per biomass, $Y_{P/X}$ [mg g^{-1}]	1.0±0.1	0.2±0.06	0.64±0.1
Product yield per culture volume [mg L^{-1}]	39±5	8±2	3.2±0.3
Fusion protein per total VLP-forming protein[(a)] [%]	~3	~30	~24
VLP diameter by TEM [nm]	20 - 40	44 – 60	42 - 62
Hydrodynamic VLP diameter by DLS [nm]	64 (PDI: 0.11)	91 (PDI: 0.09)	84 (PDI: 0.09)
Buoyant density [g cm^{-3}]	1.14	1.13 - 1.16	1.14 – 1.15
Lipid content of purified VLP [%]	30±4	NE	38±4

561 [(a)] Based on analysis of Coomassie-stained PAA gels by densitometry.
562 NE.: not evaluated
563

564 # 5. Discussion

565 This work introduces a novel VLP platform for the display of malaria transmission-blocking
566 antigens derived from *P. falciparum* on the surface of a nano-scaled VLP scaffold. Chimeric
567 VLP containing the leading malaria vaccine candidates Pfs25 and Pfs230 were engineered,
568 purified and characterized.
569
570 A DSP approved for hepatitis B vaccine production from yeast [49] was used for isolation of
571 highly pure chimeric Pfs25-dS/dS VLP. Initially, three different Pfs25-dS fusion protein
572 constructs were constructed and it was shown that depending on the design of the construct,
573 the degree of *N*-glycosylation varied (Fig 2). Due to product homogeneity and therewith
574 potential regulatory advantages, the non-glycosylated variant was chosen for chimeric Pfs25-
575 dS/dS VLP production. The purified Pfs25 containing VLP were reactive with transmission-
576 blocking monoclonal antibodies 32F81 and 4B7 in ELISA [56,57] and could be identified as
577 particulate structures in TEM imaging (Fig 1)These can be considered as a promising

578 findings for upcoming immunization studies. However, cross reactivity of the dS with the
579 antibodies 32D81 and 4B7 was observed in Western blot (Fig 1A) and ELISA (Fig 1C) which
580 complicates interpretation of the results. In case of the antibody 4B7, the difference in
581 reactivity with the chimeric Pfs25-dS/dS VLP or with plain dS VLP not containing the Pfs25
582 antigen was only about factor 3. This difference in reactivity could be expected to be greater
583 and may be caused by misfolded Pfs25 antigen e.g. due to incomplete formation of disulfide
584 bonds or because of the low Pfs25 fusion protein content in the isolated chimeric VLP.
585 Depletion of Pfs25-dS relative to dS in the course of the DSP was not observed and thus this
586 low inclusion rate could have two possible reasons.

587 (1) The ratio of Pfs25-dS to wild-type dS produced by RK#097 was too low to
588 facilitate isolation of chimeric VLP with higher fusion protein content.
589 (2) The chosen fusion protein construct Pfs25-dS is suboptimal for high fusion protein
590 content in this kind of VLP.

591 To overcome possibility 1, recombinant *H. polymorpha* strains producing improved fusion
592 protein to dS ratios were generated by applying alternative strain generation approaches
593 described in [42]. Solubility of the VLP forming proteins in homogenates of these new strains
594 was however reduced compared to strain RK#097 (data not shown). As a next step,
595 solubilization of the VLP proteins would need to be optimized and purified chimeric VLP
596 should then be compared head-to-head with purified particles from strain RK#097. To
597 address possibility 2, the fusion protein construct may be modified e.g. by truncation of the
598 Pfs25 antigen. The EGF2-domain of the protein was described to retain the transmission-
599 blocking activity [60] and might be used instead of the more complex, full-length Pfs25.
600

601 For production of VLP expressing a Pfs230 construct, the 630 aa Pfs230c fragment [22] of
602 the *Plasmodium* antigen Pfs230 was fused to the dS and chimeric Pfs230c-dS/dS VLP were
603 purified. The incorporation of the 80 kDa Pfs230c fragment into chimeric VLP demonstrated
604 that the integration of foreign antigens in dS-based VLP is not necessarily limited by their
605 size; this is a substantial advantage over other VLP platforms [13]. The observed

606 precipitation of the Pfs230c-dS containing material during DSP may be due to misfolded
607 protein. This may also be an explanation for missing response in ELISA applying the 1B3
608 monoclonal antibody (Fig S 2). The Pfs230c-dS aa sequence contains 16 cysteine residues
609 which could be linked incorrect via disulfide bonds. In our experiments, overexpression of a
610 recombinant protein disulfide isomerase did not result in detectable reduction of product loss
611 during DSP (data not shown) which does not support the hypothesis of incorrectly formed
612 disulfide bonds. Additionally, our observations regarding the solubility of the Pfs230c
613 construct correlate with reports on improving the solubility of Pfs230 fragments by choosing
614 particular fusion partners [22,61]. Although this issue did not hinder the isolation of chimeric
615 VLP, it resulted in changes in the DSP and led to reduced VLP yields. Nontheless, the
616 obtained yields and fusion protein contents of the VLP (~30 %) are good in the context of
617 chimeric VLP vaccines [62] and the reactivity with the polyclonal anti-Pfs230 antibody in
618 native ELISA (Fig 4) is a promising result.
619
620 A remaining challenge regarding the chimeric Pfs230c-dS/dS VLP purification is the relatively
621 low purity of 64 % of the final preparation. It can be speculated that a contributor to the sub-
622 optimal purity is that the residual HCP impurities were tightly associated with the particles or
623 the Pfs230c antigen. Separation of product from contaminative proteins was not possible by
624 CsCl density gradient separation, ultrafiltration (MWCO 100 kDa), multimodal
625 chromatography (Capto Core 700 matrix, GE Healthcare) or HP-SEC (data not shown). A
626 revised purification protocol may need to be developed addressing reduction of the most
627 prominent, persisting protein contaminants already present in earlier purification steps.
628
629 Compared to the Pfs230c-dS/dS VLP, the Pfs230D1M [36] variant was easier to process; no
630 loss of product due to precipitation was observed and higher VLP yield per biomass was
631 achieved with comparable fusion protein content. This finding of improved solubility agrees
632 with successful heterologous production of soluble Pfs230D1M fragment in *P. pastoris* and

633 secretion of the product into the culture supernatant [36]. This was also feasible with
634 *H. polymorpha* with promising yields (data not shown).
635
636 For the two chimeric VLP preparations containing Pfs230 fragments, the co-localization of dS
637 and Pfs230 in nano-scaled particles was observed by N-SIM analysis. However, the
638 resolution of the test set-up may not be sufficient for the detection of single chimeric VLP.
639 Due to physicochemical homogeneity of the analyzed samples, it can be concluded that co-
640 localization of both proteins in clusters of few VLP support the occurrence of both proteins in
641 single VLP. Together, N-SIM analysis (Figs 3 C and 4 C) and ELISA (not shown) proved the
642 accessibility of both Pfs230 constructs under native conditions for immunolabeling which
643 substantiates the display of the respective malaria antigens on the VLP surface. Additionally,
644 these native immunoassays demonstrated reactivity with antibodies that are known to have
645 transmission-blocking activity. This is again promising regarding applying these VLP in
646 vaccine immunogenicity studies.
647
648 The expression system *H. polymorpha* was shown to be a reliable and productive host for
649 production of chimeric VLP displaying the difficult-to-express transmission-blocking antigens
650 Pfs25 and Pfs230 on their surface. Yeasts combine the ease of genetic manipulation and the
651 option for simple fermentation strategies of bacterial expression systems with the ability to
652 modify proteins according to a general eukaryotic scheme [63]. Mammalian and insect cell
653 expression systems might be the favorable systems in case of production and assembly of
654 highly complex multi-layer VLP. However, the advantages of yeast-based VLP production is
655 especially valued in the domain of simpler, single-layered VLP production [43,64–66].
656 Particularly, the methylotrophic yeast *H. polymorpha* should be considered for production of
657 chimeric VLP vaccine candidates since it is already established as a safe and reliable
658 microbial cell factory for the production of biopharmaceuticals like hepatitis B VLP vaccines
659 [49,67,68] or recombinant products that have been granted “generally recognized as safe”
660 (GRAS) status.

661

662 The generation of recombinant *H. polymorpha* strains is more laborious than for other yeast
663 species, including *Saccharomyces cerevisiae* [48]. However, these additional difficulties are
664 compensated by a number of positive characteristics which are advantageous in
665 biotechnological applications (for review see e.g. [69–71]). These include mitotic stability of
666 recombinant strains even under non-selective conditions due to stable integration of
667 plasmids in high copy numbers into the host`s genome [48], the availability of strong and
668 regulated promoters derived from the methanol utilization pathway [72], the applicability of
669 different carbon sources, especially glycerol [73,74] and the ease to grow *H. polymorpha* to
670 high cell densities reaching dimensions of 100 g DCW per L culture broth [75]. Additional
671 advantages of the methylotrophic yeast for the production of recombinant proteins are its
672 relatively high optimal growth temperature of 37 °C which allows a better temperature
673 management in large-scale fermentations and the tendency to reduced *N*-linked
674 hyperglycosylation of recombinant proteins compared to *Saccharomyces cerevisiae* [72,76]
675 combined with the lack of the terminal, hyperallergenic α-1,3-linked mannose [77].

676

677 # 6. Conclusion

678 This study introduces a novel platform for the presentation of leading malaria transmission-
679 blocking antigens of up to 80 kDa on the surface of chimeric VLP. Each of the generated
680 chimeric VLP preparations was reactive under native conditions with antibodies described to
681 have transmission-blocking activity. Regarding VLP yield, their purity and fusion protein
682 content, the chimeric Pfs230D1M-dS/dS VLP appears to be the most promising candidate
683 that emerged from this study. The obtained product yields in combination with the versatility
684 and reliability of the described VLP production platform makes it a competitive system and
685 should be considered for future malaria vaccine development. However, the potential of the
686 three developed, chimeric VLP as effective vaccine candidates cannot be disclosed unless
687 studies to assess their immunogenicity and transmission-blocking performance are

688 completed. This represents together with improving the product purity especially for the
689 chimeric Pfs230c-dS/dS VLP the future key tasks.
690

691 # References

692 1. WHO. World Malaria Report 2017. World Health Organization. 2017.
693 doi:10.1071/EC12504

694 2. Agnandji ST, Lell B, Fernandes JF, Abossolo BP, Methogo BGNO, Kabwende AL, et
695 al. A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants. N Engl J Med.
696 2012;367: 2284–2295. doi:10.1056/NEJMoa1208394

697 3. Malaria Vaccine Funders Group. Malaria Vaccine Technology Roadmap. 2013; 1–9.

698 4. Halbroth BR, Draper SJ. Recent Developments in Malaria Vaccinology. Adv Parasitol.
699 2015;88: 1–49. doi:10.1016/bs.apar.2015.03.001

700 5. Huang DB, Wu JJ, Tyring SK. A review of licensed viral vaccines, some of their safety
701 concerns, and the advances in the development of investigational viral vaccines. J
702 Infect. 2004;49: 179–209. doi:10.1016/j.jinf.2004.05.018

703 6. Birkett AJ, Moorthy VS, Loucq C, Chitnis CE, Kaslow DC. Malaria vaccine R&D in the
704 Decade of Vaccines: Breakthroughs, challenges and opportunities. Vaccine. 2013;31:
705 B233–B243. doi:10.1016/j.vaccine.2013.02.040

706 7. Yoshida N, Nussenzweig RS, Potocnjak P, Nussenzweig V, Aikawa M. Hybridoma
707 produces protective antibodies directed against the sporozoite stage of malaria
708 parasite. Science. 1980;207: 71–73. doi:10.1126/science.6985745

709 8. Beeson JG, Drew DR, Boyle MJ, Feng G, Fowkes FJI, Richards JS. Merozoite surface
710 proteins in red blood cell invasion, immunity and vaccines against malaria. FEMS
711 Microbiol Rev. 2016;40: 343–372. doi:10.1093/femsre/fuw001

712 9. Vermeulen AN, Ponnudurai T, Beckers PJ, Verhave JP, Smits MA, Meuwissen JH.
713 Sequential expression of antigens on sexual stages of Plasmodium falciparum
714 accessible to transmission-blocking antibodies in the mosquito. J Exp Med. 1985;162:
715 1460–1476. doi:10.1084/jem.162.5.1460

716 10. Quakyi IA, Carter R, Rener J, Kumar N, Good MF, Miller LH. The 230-kDa gamete
717 surface protein of Plasmodium falciparum is also a target for transmission-blocking
718 antibodies. J Immunol. 1987;139: 4213–4217.

719 11. Delves M, Angrisano F, Blagborough A. Antimalarial Transmission-Blocking
720 Interventions: Past, Present, and Future. Trends Parasitol. 2018;1776: 1–12.
721 doi:10.1016/j.pt.2018.07.001

722 12. Bobbala S, Hook S. Is There an Optimal Formulation and Delivery Strategy for Subunit
723 Vaccines? Pharm Res. 2016;33: 2078–2097. doi:10.1007/s11095-016-1979-0

724 13. Frietze KM, Peabody DS, Chackerian B. Engineering virus-like particles as vaccine
725 platforms. Curr Opin Virol. 2016;18: 44–49. doi:10.1016/j.coviro.2016.03.001

726 14. Grgacic EVL, Anderson DA. Virus-like particles: Passport to immune recognition.
727 Methods. 2006;40: 60–65. doi:10.1016/j.ymeth.2006.07.018

728 15. McAleer WJ, Buynak EB, Maigetter RZ, Wampler DE, Miller WJ, Hilleman MR. Human
729 hepatitis B vaccine from recombinant yeast. Nature. 1984;307: 178–180.
730 doi:10.1038/307178a0

731 16. Tan M, Jiang X. Subviral particle as vaccine and vaccine platform. Curr Opin Virol.
732 2014;6: 24–33. doi:10.1016/j.coviro.2014.02.009

733 17. “RTS/S Clinical Trials Partnership.” Efficacy and safety of RTS,S/AS01 malaria

734 vaccine with or without a booster dose in infants and children in Africa: Final results of
735 a phase 3, individually randomised, controlled trial. Lancet. 2015;386: 31–45.
736 doi:10.1016/S0140-6736(15)60721-8

737 18. Wu Y, Sinden RE, Churcher TS, Tsuboi T, Yusibov V. Development of Malaria
738 Transmission-Blocking Vaccines: From Concept to Product. Adv Parasitol. 2015;
739 doi:10.1016/bs.apar.2015.04.001

740 19. Rener J, Graves PM, Carter R, Williams JL, Burkot TR. Target antigens of
741 transmission-blocking immunity on gametes of Plasmodium falciparum. J Exp Med.
742 1983;158/: 976–981. doi:10.1084/jem.20142182

743 20. Stone WJR, Dantzler KW, Nilsson SK, Drakeley CJ, Marti M, Bousema T, et al.
744 Naturally acquired immunity to sexual stage P. Falciparum parasites. Parasitology.
745 2016;143: 187–198. doi:10.1017/S0031182015001341

746 21. Kaslow DC, Quakyi IA, Syin C, Raum MG, Keister DB, Coligan JE, et al. A vaccine
747 candidate from the sexual stage of human malaria that contains EGF-like domains.
748 Nature. 1988;333: 74–76. doi:10.1038/333074a0

749 22. Williamson KC, Keister DB, Muratova O, Kaslow DC. Recombinant Pfs230, a
750 Plasmodium falciparum gametocyte protein, induces antisera that reduce the
751 infectivity of Plasmodium falciparum to mosquitoes. Mol Biochem Parasitol. 1995;75:
752 33–42. doi:10.1016/0166-6851(95)02507-3

753 23. Barr PJ, Green KM, Gibson HL, Bathurst IC, Quakyi IA, Kaslow DC. Recombinant
754 Pfs25 protein of Plasmodium falciparum elicits malaria transmission-blocking immunity
755 in experimental animals. J Exp Med. 1991;174: 1203–1208.
756 doi:10.1084/jem.174.5.1203

757 24. Kaslow DC, Shiloach J. Production, purification and immunogenicity of a malaria
758 transmission-blocking vaccine candidate: TBV25H expressed in yeast and purified
759 using nickel-NTA agarose. Bio/Technology. 1994;12: 494–499. doi:10.1038/nbt0594-
760 494

761 25. Zou L, Miles AP, Wang J, Stowers AW. Expression of malaria transmission-blocking
762 vaccine antigen Pfs25 in Pichia pastoris for use in human clinical trials. Vaccine.
763 2003;21: 1650–1657. doi:10.1016/S0264-410X(02)00701-6

764 26. Coban C, Ishii KJ, Stowers AW, Keister DB, Klinman DM, Kumar N. Effect of CpG
765 Oligodeoxynucleotides on the Immunogenicity of Pfs25, a Plasmodium falciparum
766 Transmission-Blocking Vaccine Antigen. Infect Immun. 2004;72: 584–588.
767 doi:10.1128/IAI.72.1.584-588.2004

768 27. Qian F, Wu Y, Muratova O, Zhou H, Dobrescu G, Duggan P, et al. Conjugating
769 recombinant proteins to Pseudomonas aeruginosa ExoProtein A: A strategy for
770 enhancing immunogenicity of malaria vaccine candidates. Vaccine. 2007;25: 3923–
771 3933. doi:10.1016/j.vaccine.2007.02.073

772 28. Kubler-Kielb J, Majadly F, Wu Y, Narum DL, Guo C, Miller LH, et al. Long-lasting and
773 transmission-blocking activity of antibodies to Plasmodium falciparum elicited in mice
774 by protein conjugates of Pfs25. Proc Natl Acad Sci U S A. 2007;104: 293–298.
775 doi:10.1073/pnas.0609885104

776 29. Wu Y, Przysiecki C, Flanagan E, Bello-Irizarry SN, Ionescu R, Muratova O, et al.
777 Sustained high-titer antibody responses induced by conjugating a malarial vaccine
778 candidate to outer-membrane protein complex. Proc Natl Acad Sci U S A. 2006;103:
779 18243–18248. doi:10.1073/pnas.0608545103

780 30. Jones RM, Chichester JA, Mett V, Jaje J, Tottey S, Manceva S, et al. A plant-

781 produced Pfs25 VLP malaria vaccine candidate induces persistent transmission
782 blocking antibodies against plasmodium falciparum in immunized mice. PLoS One.
783 2013;8: e79538. doi:10.1371/journal.pone.0079538

784 31. Li Y, Leneghan DB, Miura K, Nikolaeva D, Brian IJ, Dicks MDJ, et al. Enhancing
785 immunogenicity and transmission-blocking activity of malaria vaccines by fusing Pfs25
786 to IMX313 multimerization technology. Sci Rep. 2016;6: 18848.
787 doi:10.1038/srep18848

788 32. Brune KD, Leneghan DB, Brian IJ, Ishizuka AS, Bachmann MF, Draper SJ, et al. Plug-
789 and-Display: decoration of Virus-Like Particles via isopeptide bonds for modular
790 immunization. Sci Rep. 2016;6: 19234. doi:10.1038/srep19234

791 33. Leneghan DB, Miura K, Taylor IJ, Li Y, Jin J, Brune KD, et al. Nanoassembly routes
792 stimulate conflicting antibody quantity and quality for transmission-blocking malaria
793 vaccines. Sci Rep. 2017;7: 3811. doi:10.1038/s41598-017-03798-3

794 34. Carter R, Coulson A, Bhatti S, Taylor BJ, Elliott JF. Predicted disulphide-bonded
795 structures for three uniquely related proteins of Plasmodium falciparum, Pfs230,
796 Pfs48/45 and pf12. Mol Biochem Parasitol. 1995;71: 203–210.

797 35. Gerloff DL, Creasey A, Maslau S, Carter R. Structural models for the protein family
798 characterized by gamete surface protein Pfs230 of Plasmodium falciparum. Proc Natl
799 Acad Sci U S A. 2005;102: 13598–13603. doi:0502378102
800 [pii]\r10.1073/pnas.0502378102

801 36. MacDonald NJ, Nguyen V, Shimp R, Reiter K, Herrera R, Burkhardt M, et al. Structural
802 and immunological characterization of recombinant 6-cysteine domains of the
803 plasmodium falciparum sexual stage protein Pfs230. J Biol Chem. 2016;291: 19913–
804 19922. doi:10.1074/jbc.M116.732305

805 37. Tachibana M, Wu Y, Iriko H, Muratova O, MacDonald NJ, Sattabongkot J, et al. N-
806 terminal prodomain of Pfs230 synthesized using a cell-free system is sufficient to
807 induce complement-dependent malaria transmission-blocking activity. Clin Vaccine
808 Immunol. 2011;18: 1343–4350. doi:10.1128/CVI.05104-11

809 38. Nikolaeva D, Draper SJ, Biswas S. Toward the development of effective transmission-
810 blocking vaccines for malaria. Expert Rev Vaccines. 2015;14: 653–680.
811 doi:10.1586/14760584.2015.993383

812 39. Anderson DA, Grgacic EVL. Viral vectors expressing fusion of viral large envelope
813 protein and protein of interest. Patent WO2004092387A1. 2004. doi:10.1016/j.(73)

814 40. Grgacic EVL, Anderson DA, Loke P, Anders R. Recombinant proteins and virus-like
815 particles comprising L and S polypeptides of avian hepadnaviridae and methods,
816 nucleic acid constructs, vectors and host cells for producing same. Patent
817 WO2008025067A1. 2006.

818 41. Suh SO, Zhou JJ. Methylotrophic yeasts near Ogataea (Hansenula) polymorpha: A
819 proposal of Ogataea angusta comb. nov. and Candida parapolymorpha sp. nov.
820 FEMS Yeast Res. 2010;10: 631–638. doi:10.1111/j.1567-1364.2010.00634.x

821 42. Wetzel D, Rolf T, Suckow M, Kranz A, Barbian A, Chan JA, et al. Establishment of a
822 yeast-based VLP platform for antigen presentation. Microb Cell Fact. 2018;17: 1–17.
823 doi:10.1186/s12934-018-0868-0

824 43. Roldão A, Mellado MCM, Castilho LR, Carrondo MJT, Alves PM. Virus-like particles in
825 vaccine development. Expert Rev Vaccines. 2010;9: 1149–1176.
826 doi:10.1586/erv.10.115

827 44. Degelmann A, Müller F, Sieber H, Jenzelewski V, Suckow M, Strasser AWM, et al.

Strain and process development for the production of human cytokines in Hansenula polymorpha. FEMS Yeast Res. 2002;2: 349–361. doi:10.1016/S1567-1356(02)00096-X

45. Bertani G. Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli. J Bacteriol. 1951;62: 293–300. doi:citeulike-article-id:149214

46. Lahtchev KL, Semenova VD, Tolstorukov II, Van der Klei I, Veenhuis M. Isolation and properties of genetically defined strains of the methylotrophic yeast Hansenula polymorpha CBS4732. Arch Microbiol. 2002;177: 150–158. doi:10.1007/s00203-001-0370-6

47. Faber KN, Haima P, Harder W, Veenhuis M, Ab G. Highly-efficient electrotransformation of the yeast Hansenula polymorpha. Curr Genet. 1994;25: 305–310. doi:10.1007/BF00351482

48. Guengerich L, Kang HA, Behle B, Gellissen G, Suckow M. A platform for heterologous gene expression based on the methylotrophic yeast Hansenula polymorpha. In: Kueck U, editor. Genetics and Biotechnology. Berlin: Springer; 2004. pp. 273–287.

49. Schaefer S, Piontek M, Ahn SJ, Papendieck A, Janowicz ZA, Timmermans I, et al. Recombinant hepatitis B vaccines—disease characterization and vaccine production. In: Gellissen G, editor. Hansenula polymorpha—biology and applications. Weinheim: Wiley-VCH; 2002. pp. 175–210.

50. Lünsdorf H, Gurramkonda C, Adnan A, Khanna N, Rinas U. Virus-like particle production with yeast: Ultrastructural and immunocytochemical insights into Pichia pastoris producing high levels of the Hepatitis B surface antigen. Microb Cell Fact. 2011;10: 1–16. doi:10.1186/1475-2859-10-48

51. Frings CS, Fendley TW, Dunn RT, Queen CA. Improved determination of total serum lipids by the sulfo-phospho-vanillin reaction. Clin Chem. 1972;18: 673–674.

52. Pink M, Verma N, Rettenmeier AW, Schmitz-Spanke S. CBB staining protocol with higher sensitivity and mass spectrometric compatibility. Electrophoresis. 2010;31: 593–598. doi:10.1002/elps.200900481

53. Miura K, Takashima E, Deng B, Tullo G, Diouf A, Moretz SE, et al. Functional comparison of plasmodium falciparum transmission-blocking vaccine candidates by the standard membrane-feeding assay. Infect Immun. 2013;81: 4377–4382. doi:10.1128/IAI.01056-13

54. Grgacic EVL, Anderson DA. St, a truncated envelope protein derived from the S protein of duck hepatitis B virus, acts as a chaperone for the folding of the large envelope protein. J Virol. 2005;79: 5346–5352. doi:10.1128/JVI.79.9.5346-5352.2005

55. Pugh JC, Di Q, Mason WS, Simmons H. Susceptibility to duck hepatitis B virus infection is associated with the presence of cell surface receptor sites that efficiently bind viral particles. J Virol. 1995;69: 4814–4822.

56. Mulder B, Roeffen W, Sauerwein R, Tchuinkam T, Boudin C, Verhave JP. Anti-Pfs25 monoclonal antibody 32F81 blocks transmission from Plasmodium falciparum gametocyte carriers in Cameroon. Trans R Soc Trop Med Hyg. 1996;90: 195. doi:10.1016/S0035-9203(96)90139-X

57. Stura EA, Kang AS, Stefanko RS, Calvo JC, Kaslow DC, Satterthwait AC. Crystallization, sequence and preliminary crystallographic data for transmission-blocking anti-malaria Fab 4B7 with cyclic peptides from the Pfs25 protein of P. falciparum. Acta Crystallogr Sect D Biol Crystallogr. 1994;50: 535–542. doi:10.1107/S0907444994001356

875 58. Cova L, Abdul F, Buronfosse T. Avihepadnavirus. In: Tidona C, Darai G, editors. The
876 Springer Index of Viruses. Berlin: Springer; 2011. pp. 615–624.

877 59. Mason WS, Gerlich WH, Taylor JM, Kann M, Mizokami T, Loeb D, et al.
878 Hepadnaviridae. In: King AMQ, Adams AJ, Carstens EB, Lekowitz EJ, editors. Virus
879 taxonomy. Amsterdam: Elsevier; 2013. pp. 445–455.

880 60. Stowers AW, Keister DB, Muratova O, Kaslow DC. A region of Plasmodium falciparum
881 antigen Pfs25 that is the target of highly potent transmission-blocking antibodies.
882 Infect Immun. 2000;68: 5530–5538. doi:10.1128/IAI.68.10.5530-5538.2000

883 61. Farrance CE, Rhee A, Jones RM, Musiychuk K, Shamloul M, Sharma S, et al. A plant-
884 produced Pfs230 vaccine candidate blocks transmission of Plasmodium falciparum.
885 Clin Vaccine Immunol. 2011;18: 1351–1357. doi:10.1128/CVI.05105-11

886 62. Gordon DM, Mc Govern TW, Krzych U, Cohen JC, Schneider I, La Chance R, et al.
887 Safety, immunogenicity, and efficacy of a recombinantly produced plasmodium
888 falciparum circumsporozoite protein-hepatitis b surface antigen subunit vaccine. J
889 Infect Dis. 1995;171: 1576–1585. doi:10.1093/infdis/171.6.1576

890 63. Gellissen G, Kunze G, Gaillardin C, Cregg JM, Berardi E, Veenhuis M, et al. New
891 yeast expression platforms based on methylotrophic Hansenula polymorpha and
892 Pichia pastoris and on dimorphic Arxula adeninivorans and Yarrowia lipolytica - A
893 comparison. FEMS Yeast Res. 2005;5: 1079–1096. doi:10.1016/j.femsyr.2005.06.004

894 64. Fuenmayor J, Gòdia F, Cervera L. Production of virus-like particles for vaccines. N
895 Biotechnol. 2017;39: 174–180. doi:10.1016/j.nbt.2017.07.010

896 65. Ahmad M, Hirz M, Pichler H, Schwab H. Protein expression in Pichia pastoris: Recent
897 achievements and perspectives for heterologous protein production. Appl Microbiol
898 Biotechnol. 2014;98: 5301–5317. doi:10.1007/s00253-014-5732-5

899 66. Liu F, Wu X, Li L, Liu Z, Wang Z. Use of baculovirus expression system for generation
900 of virus-like particles: Successes and challenges. Protein Expr Purif. 2013;90: 104–
901 116. doi:10.1016/j.pep.2013.05.009

902 67. Heijtink RA, Bergen P Van, Melber K, Janowicz ZA, Osterhaus ADME. Hepatitis B
903 surface antigen (HBsAg) derived from yeast cells (Hansenula polymorpha) used to
904 establish an influence of antigenic subtype (adw2, adr, ayw3) in measuring the
905 immune response after vaccination. Vaccine. 2002;20: 2191–2196.
906 doi:10.1016/S0264-410X(02)00145-7

907 68. Janowicz ZA, Melber K, Merckelbach A, Jacobs E, Harford N, Comberbach M, et al.
908 Simultaneous expression of the S and L surface antigens of hepatitis B, and formation
909 of mixed particles in the methylotrophic yeast, Hansenula polymorpha. Yeast. 1991;7:
910 431–443. doi:10.1002/yea.320070502

911 69. Romanos MA, Scorer CA, Clare JJ. Foreign gene expression in yeast: a review.
912 Yeast. 1992;8: 423–488. doi:10.1002/yea.320080602

913 70. Faber KN, Harder W, Ab G, Veenhuis M. Methylotrophic yeasts as factories for the
914 production of foreign proteins. Yeast. 1995;11: 1331–1344.
915 doi:10.1002/yea.320111402

916 71. Gellissen G, Hollenberg CP. Application of yeasts in gene expression studies: A
917 comparison of Saccharomyces cerevisiae, Hansenula polymorpha and Kluyveromyces
918 lactis - A review. Gene. 1997;190: 87–97. doi:10.1016/S0378-1119(97)00020-6

919 72. Gellissen G, Hollenberg C, Janowicz Z. Gene expression in methylotrophic yeasts. In:
920 Smith A, editor. Gene expression in recombinant microorganisms. 1st ed. New York:
921 Marcel Dekker; 1995. pp. 195–239.

922 73. Swinnen S, Klein M, Carrillo M, McInnes J, Nguyen HTT, Nevoigt E. Re-evaluation of
923 glycerol utilization in Saccharomyces cerevisiae: Characterization of an isolate that
924 grows on glycerol without supporting supplements. Biotechnol Biofuels. 2013;157: 1–
925 12. doi:10.1186/1754-6834-6-157

926 74. Jenzelewski V. Fermentation and primary product recovery. In: Gellissen G, editor.
927 Hansenula polymorpha—biology and applications. Weinheim: Wiley-VCH; 2002. pp.
928 156–174.

929 75. Mayer AF, Hellmuth K, Schlieker H, Lopez-Ulibarri R, Oertel S, Dahlems U, et al. An
930 expression system matures: A highly efficient and cost-effective process for phytase
931 production by recombinant strains of Hansenula polymorpha. Biotechnol Bioeng.
932 1999;63: 373–381. doi:10.1002/(SICI)1097-0290(19990505)63:3<373::AID-
933 BIT14>3.0.CO;2-T

934 76. Kang HA, Sohn JH, Choi ES, Chung BH, Yu MH, Rhee SK. Glycosylation of human
935 α1-antitrypsin in Saccharomyces cerevisiae and methylotrophic yeasts. Yeast.
936 1998;14: 371–381. doi:10.1002/(SICI)1097-0061(19980315)14:4<371::AID-
937 YEA231>3.0.CO;2-1

938 77. Kim MW, Rhee SK, Kim JY, Shimma YI, Chiba Y, Jigami Y, et al. Characterization of
939 N-linked oligosaccharides assembled on secretory recombinant glucose oxidase and
940 cell wall mannoproteins from the methylotrophic yeast Hansenula polymorpha.
941 Glycobiology. 2004;14: 243–251. doi:10.1093/glycob/cwh030

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944 **Author contributions statement**

945 Conceptualization, DW, JAC, JB, MP and DA; Methodology, DW, JAC, AB, EH, BK, CP, VJ,
946 MW, MS, DA and MP; Investigation, DW, JAC, LR and AB; Administration, JB, MP, VJ, JR
947 and DA; Writing – Original Draft, DW; Writing – Review and Editing, DW, JAC, MP, JB, MS,
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958 manuscript as a supervisor as articulated in the 'author contributions' section. Most of the
959 research presented in this work was performed in the company`s facilities.

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Competing interests

The authors VJ, MP, MS, DW and MW are associated with ARTES Biotechnology GmbH which owns the license for the VLP technology (patents cited as references [39] and [40]): Viral vectors expressing fusion of viral large envelope protein and protein of interest (No. WO2004092387A1). Recombinant proteins and virus-like particles comprising L and S polypeptides of avian hepadnaviridae and methods, nucleic acid constructs, vectors and host cells for producing same (No. WO2008025067A1).

Author JM is affiliated with Evonik Technology & Infrastructure GmbH.

There are no further patents, products in development or marketed products to declare. This does not alter our adherence to all the PLOS ONE policies on sharing data and materials.

Data Availability

All relevant data are within the paper.

Supplementary material

of the manuscript entitled

Display of malaria transmission-blocking antigens on chimeric DHBV-derived virus-like particles produced in *Hansenula polymorpha*

Authors:

Wetzel, David; Chan, Jo-Anne; Suckow, Manfred; Barbian, Andreas; Weniger, Michael; Jenzelewski, Volker; Reiling, Linda; Richards, Jack S; Anderson, David A; Kouskousis, Betty; Palmer, Catherine; Hanssen, Eric; Schembecker, Gerhard; Merz, Juliane; Beeson, James G; Piontek, Michael

1008 # Section S1

1009 ## Additional data on the chimeric Pfs25-dS/dS VLP

1010 Fig 1 A of the main manuscript shows Western blot analyses on the chimeric Pfs25-dS/dS
1011 VLP (lanes 3 to 6). Cross reactivity of the anti-Pfs25 antibody with the dS scaffold protein
1012 was observed (Fig 1A, lanes 5 and 6). Therefore, Western blot analysis was repeated
1013 (shown in Fig S 1) with two different chimeric VLP preparations originating from two different
1014 *H. polymorpha* cell lines producing different amounts of two target proteins dS and Pfs25-dS.
1015 The blocking of the membrane was modified compared to the methodology described in the
1016 main manuscript. Instead of the Roti Block reagent (Carl Roth GmbH, Karlsruhe, Germany),
1017 3 % (w/v) dry milk (in PBS-T) were applied as for the Pfs230-related Western blots. With this
1018 modified procedure we did not observe cross-reactivity of the anti-Pfs25 antibody with the dS
1019 in Western blot.

1020 Lanes 2 and 4 in Fig S1 represent the actual Pfs25-dS/dS VLP preparation discussed in the
1021 main manuscript whereas lanes 1 and 3 represent a substantially lower concentrated
1022 Pfs25-dS/dS VLP preparation originating from a different *H. polymorpha* cell line. In both VLP
1023 preparation the dS was detected by the anti-dS antibody (lanes 1 and 2). However, only for
1024 the preparation applied to lanes 2 and 4 the presence of the Pfs25-dS fusion protein was
1025 substantiated by the anti-Pfs25 antibody. Potentially, the Pfs25-dS fusion protein content in
1026 the sample applied to lanes 1 and 3 is below the detection limit. However, cross reactivity
1027 with the VLP scaffold protein dS was not observed for neither of the samples most likely due
1028 to the altered Western blot procedure compared to the analysis shown in the main
1029 manuscript (Fig 1 A).

1030

1031

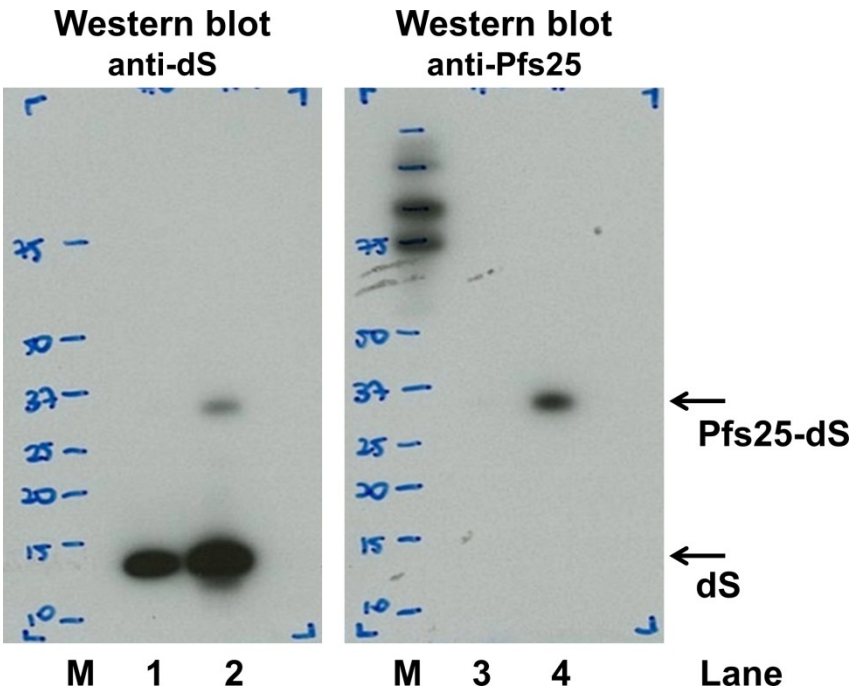
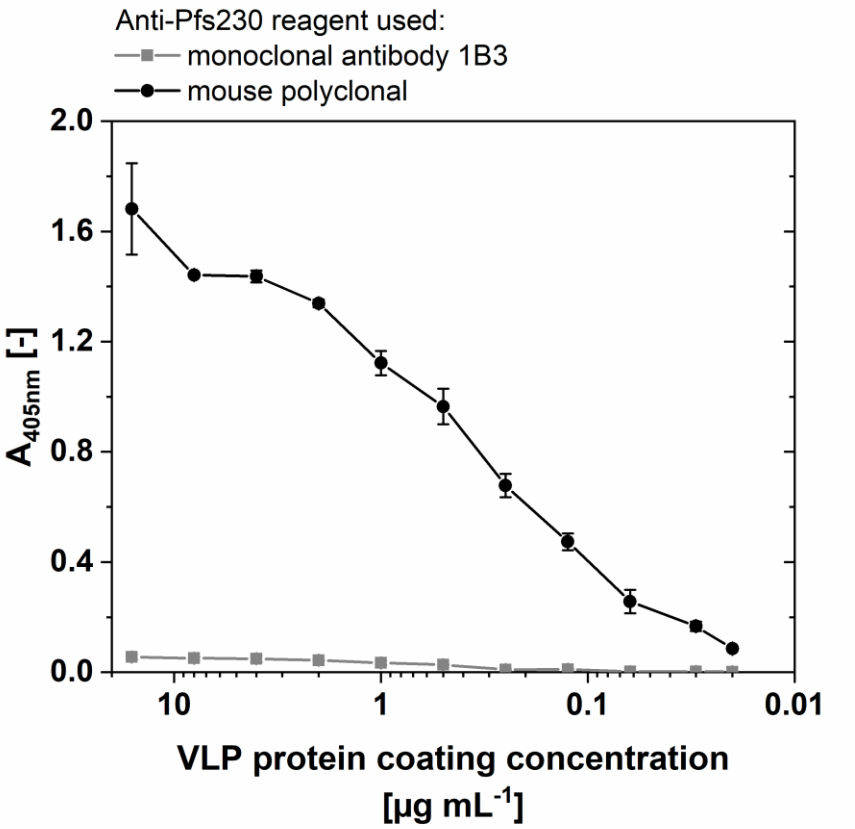


Figure S 1 Repetition of Western blot analyses on chimeric Pfs25-dS/dS VLP. Chimeric Pfs25-dS/dS VLP preparations obtained from two different *H. polymorpha* cell lines were applied. Lanes 2 and 4: the Pfs25-dS/dS VLP preparation discussed in the main manuscript. Lanes 1 and 3: A lower concentrated VLP preparation derived from a different cell line expressing lower levels of dS and Pfs25-dS. Left: Membrane probed with anti-dS 7C12 mAb. Right: Membrane probed with anti-Pfs25 mAb 32F81 and analyzed on the same membrane. M: molecular weight marker

1042 # Section S2

1043 ## Additional data on the chimeric Pfs230c-dS/dS VLP

1044 During the development of methodologies to analyze the Pfs230c-dS/dS VLP, different
1045 primary immunoreagents were tested. The mouse polyclonal antibody was found to be
1046 substantially more reactive than the 1B3 monoclonal antibody (Fig S2).



1047

1048 **Figure S 2 ELISA data on purified Pfs230c-dS/dS VLP derived from strain RK#114**. Titration of
1049 VLP coating concentration. Primary antibodies were applied as 10µg/mL for both the mouse polyclonal
1050 and monoclonal 1B3). Error bars indicate standard deviation based on triplicates.

1051