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► To cite this version:

Laura Laneque, Annie Lambert, Delphine Cayet, Caroline Gilleron, Emmanuelle Courtois, et al.. Mutagenesis to Orient conjugation and preserve self-adjutant properties of flagellin in conjugates. ChemBioChem, 2025, pp.e20240100. 10.1002/cbic.202401002 . hal-04940464

HAL Id: hal-04940464

<https://hal.science/hal-04940464v1>

Submitted on 11 Feb 2025

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Mutagenesis to Orient Conjugation and Preserve Self-Adjuvant Properties of Flagellin in Conjugates

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Abstract: Bacterial flagellins are unique for their capacity to activate both the innate and the adaptive immune response through a Toll-like receptor 5 (TLR5) signaling cascade. Used as a carrier protein in conjugate vaccines it is crucial to preserve their self-adjuvant properties during the conjugation step. Considering the absence of cysteine in the *Salmonella enterica* flagellin FliC sequence, we have investigated the impact of five mutations (A2C, K180C, T240C, D251C and S306C) alone or in combination on TLR5 activation. The FliC mutated at the four positions K180C, T240C, D251C and S306C displayed much the same activity as native flagellin whether the cysteine residues were free or conjugated. These results pave the way for the preparation of self-adjuvanting conjugate vaccines based on cysteine-mutated FliC as a carrier protein.

Flagellin is a subunit protein of a whip-like flagellar filament responsible for locomotion of many bacteria. Monomeric form of flagellin of both β - and γ -proteobacteria, noticeably that from *Salmonella enterica* Serovar *Typhimurium* (thereafter referred to as FliC), is unique for its capacity to engage Toll-like receptor 5 (TLR5).^{[1],[2]} TLR5 is widely expressed by cells of both epithelium and of the innate and adaptive immune system. TLR5 signaling augments immune responses by enhancing antigen transport into the host, white blood cell recruitment to the infection, and B cell activation/differentiation in response to bacterial antigens. Taking advantage of TLR5-stimulating activities, flagellin has been actively developed as vaccine adjuvant, either co-administered or fused with antigens or even engineered in the form of chimera.^{[3],[4]} Flagellin has also been used as carrier protein to give rise to built-in adjuvanted conjugate vaccines.^{[5],[6],[7],[8],[9],[10],[11],[12],[13],[14]} However, every reported conjugate preparation relies on random

Introduction

conjugation chemistry involving either free amine or carboxylic acid groups of flagellin side-chain residues, a strategy that obviously does not warrant preserving adjuvant properties of the flagellin carrier protein.^[8] Flagellin such as FliC is composed of four interpenetrated domains D0-D3. D2 and D3 domains consist in hypervariable globular regions while D0 and D1 domains are highly conserved among flagellin sequences from different bacteria (Figure 1). These two domains are essential to TLR5 activation but only the D1 domain makes substantial contributions to binding and TLR5 signaling.^{[1],[15],[16],[17]} TLR5 and FliC interact thanks to two adjacent but spatially distinct interfaces, one of them involving residues from Glu83 to Arg119 accounting for 60% of the interface. In the filament, flagellin monomers stack together exposing the D2 and D3 domains to the environment while D0 and D1 domains are buried in the center of the filament where they interact extensively with neighboring monomers.^[18] Very recently, Peng *et al.* have exploited this self-protecting flagellin topology to selectively orient modifications at the sole D2 and D3 of *Pseudomonas aeruginosa* flagellin. These authors demonstrated that, opposite to randomly modified flagellins used as controls, the site-selectively modified flagellins retained much of the parental flagellin ability to activate the TLR5 signaling pathway.^[19] We report herein an alternative strategy based on mutagenesis for the precise control of the conjugate connectivity. Using FliC as a model flagellin, we took advantage of the absence of cysteine residue in the sequence, to introduce up to 4 mutations and evaluate their impact both before and after conjugation to a hapten using the well-established thiol-maleimide chemistry^{[20],[21],[22]} on TLR5-stimulating activity.

Results and Discussion

Selection of Mutations

Molecular biology techniques such as mutagenesis or the incorporation of non-canonical amino acids carrying a biorthogonal group for conjugation^[23] allow precise engineering of proteins by overcoming the constraints inherent in the nature of the amino acids that make up the sequence. Considering FliC, cysteine mutagenesis appears as a straightforward strategy given the absence of cysteine residue in the native sequence associated with the efficiency and specificity of the thiol-based conjugation chemistry. Knowing that the hapten:carrier protein ratio is one of the parameters that impacts the actual immune response, we decided to mutate arbitrarily four distinct positions within the FliC sequence. Ideally, mutations should involve residues exposed on the surface and located far from each other to avoid steric hindrance and facilitate grafting. Taking into account the immunological characteristics of the carrier protein appears as a second criterion. On the one hand the carrier protein contains major histocompatibility complex (MHC) class II epitopes, responsible for the CD4⁺ T-lymphocytes recruitment which, in turn, provides the necessary help to induce an anti-hapten humoral response.^[24] It might be advantageous to graft the hapten within or in close vicinity of T-helper epitopes to optimizing the immune response.^{[25],[26]} On the other hand the carrier protein also displays B-epitopes potentially interfering with the desired response

directed against the conjugated hapten.^[27] Targeting the conjugation to these domains can contribute to deimmunization by destroying or simply masking the B-cell epitopes of the carrier protein to the immune cells.^{[28],[29]}

Previous mapping of FliC T-helper epitopes led to the identification of four functional epitopes, three out of them located in the D0 and D1 domains (FliC⁸⁰⁻⁹⁴, FliC⁴²⁸⁻⁴⁴² and FliC⁴⁵⁵⁻⁴⁶⁹) and the last one in the D2 domain (FliC³³⁹⁻³⁵⁰).^{[30],[31],[32]} Functionality of FliC T-helper peptides was independently confirmed using a slightly different albeit related flagellin sequence.^[33] Interrogation of the Immune Epitope DataBase (IEDB) (<https://www.iedb.org/>) using C57BL/6 and BALB/c mice alleles retrieved above mentioned epitopes and predicted seven other epitopes in the D2 and D3 domains: FliC³⁷⁹⁻³⁸⁷, FliC¹⁷⁹⁻¹⁸⁷, FliC²⁶⁷⁻²⁷⁵, FliC²⁵⁷⁻²⁶⁵, FliC²⁶¹⁻²⁶⁹ (H2-IAb), FliC²⁸⁹⁻²⁹⁷ (H2-IAd) and FliC²²⁹⁻²³⁶ (H2-IAe). Considering the B-epitopes, it is apparent from the literature that the humoral host immune response is mainly directed against the D3 domain.^{[34],[35]} An elegant approach to deimmunizing FliC consists in depleting FliC from D3 domain^[36] or both D2 and D3 domains.^[37] However, the implementation of this strategy forces the hapten to be grafted onto the D0 or D1 domains at the risk of causing the loss of the FliC adjuvant properties and was therefore not considered.

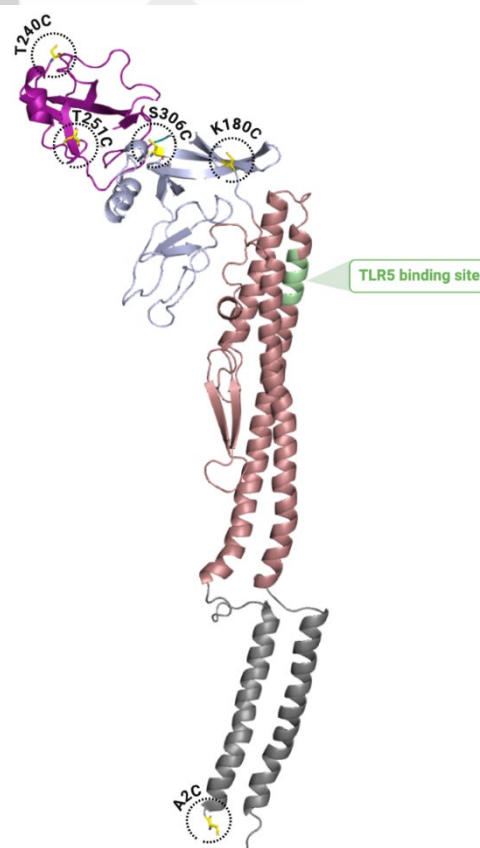


Figure 1. Ribbon diagram of FliC with the four domains D0 (grey), D1 (pink), D2 (light grey) and D3 (magenta) as well as the sequence 86-96 essential for TLR5 recognition (green). Side-chains of the five residues targeted for cysteine mutagenesis are represented in yellow and surrounded by a dashed circle; Structure generated by PHYRE2 Protein Fold Recognition Server using NCBI Reference sequence: WP_000079805.1 and represented with PyMOL.

B-cell epitopes can be continuous or discontinuous, linear or, most commonly, conformational.^{[38],[39]} They can include two to several hundred of residues, with the majority of epitopes being 6-30 amino acids long.^[40] Given this variability, B-cell epitope prediction remains elusive but is the only approach in absence of detailed experimental analysis as for FliC. IEDB server proposes several prediction tools to scrutinize protein sequences based on hydrophilicity, surface accessibility and flexibility (e.g. Emini surface accessibility or Karplus and Schulz flexibility scale) or X-

ray structures (e.g. ElliPro or BepiPred). The FliC sequence was submitted to every implemented prediction tool plus additional tools freely accessible on the web. The different predictions were compiled (Figure S1) and compared in order to find consensus B-cells epitopes (Table 1). Tools such as BepiPred 2.0 predicting that the whole D2 and D3 domains are antigenic and sequence similarity-based method (BepiBlast)^[41] only predicting epitopes within the highly conserved D0 and D1 domains, were not taken into consideration

Table 1. FliC B-cell prediction covering D2/D3 domains (FliC¹⁷⁷⁻⁴⁰¹)

| Method | Peptide 1 ^[a] | Peptide 2 | Peptide 3 | Peptide 4 | Peptide 5 | Peptide 6 | Peptide 7 | Peptide 8 | Peptide 9 | Peptide 10 |
|--------------------------------------|--------------------------|----------------------------------|-------------------------------|-----------|----------------------------------|-------------------------------|---------------------------|----------------|------------------------|--------------------|
| ElliPro | 173-181 ^[b] | 194-282 ^[c] | 194-282 | 194-282 | 194-282 | | 312-319 | | | |
| Chou & Fasman Beta-Turn | | 209-228 | 238-246 ^[d] | 252-254 | 263-271 | | 311-322 | 330-344 | 352-357 | |
| Emini surface accessibility | 175-181 | | | | | | 311-318 | 329-339 | 346-357 | |
| Karplus & Schulz Flexibility | 177-178 | 211-220 225-228 | | | 263-269 277-280 | | 314-322 | 336-341 | 353-358 | |
| Kolaskar and Tongaonkar antigenicity | 167-191 | | | | 279-287 | 292-302 304-311 | | | | 371-377 394-400 |
| Parker Hydrophilicity | 180-186 | 211-217 | 238-244 | 250-255 | 275-280 | | 312-320 | 332-341 | 349-357 | 385-387 |
| BepiPred 3.0 | 188-192 | 207-217 222-225 | 236-242 | 251-255 | 260-290 | | 314-320 | 338-341 | 348-379 ^[e] | 348-379 394-401 |
| SVMtrip | 184-199 | | | | | | | | 342-357 | |
| ABCpred | | 214-229 | 235-250 | | 260-275 269-284 | 305-320 ^[e] | 305-320 317-332 | | 348-363 | 380-395 |
| LBtope | 177-190 | 213-224 | | | 279-281 | 297-301 | 313-314 | | | 380 389-401 |
| Total occurrence ^[e] | 8 | 7 | 5 | 4 | 8 | 3 | 8 | 5 | 7 | 5 |

[a] Predicted antigenic sequence. [b] Start/end peptide sequence, if not filed = not predicted as a B-cell epitope. [c] Long antigenic predicted sequence spreading over several distinct and discontinuous peptides identified by other methods. [d] Start/end peptide sequence in bold = ranked as an immune-dominant B-cell epitope. [e] Cumulative number of times the peptide was predicted to be antigenic by the different methods (maximum 10).

Almost all of the domains are predicted to be antigenic by the different considered methods. It is nevertheless possible to identify 16 more particularly targeted segments, of which the 10 most important are reported in Table 1. Peptides 1, 5 and 7, on the one hand, as well as peptides 2 and 9, on the other hand, are predicted to be antigenic by, respectively, 8 and 7 of the 10 methods tested. Combining these B-cell epitope predictions with

that of T-helper epitopes and apparent amino acid surface accessibility, the following mutations have been targeted: Lys180 located within both Peptide 1 and a H2-IAb T-helper epitope; Thr240 within Peptide 3 and close to H2-IAe FliC²²⁹⁻²³⁶ epitope; Asp251 within Peptide 4 and close to Peptide 5 as well as to 3 H2-IAb T-helper epitopes; Ser306 in between Peptides 6 and 7 which are close from each other and also at the proximity of H2-

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IAd FliC²⁸⁹⁻²⁹⁷ epitope. A final mutation could have been chosen near Peptides 8 and 9 roughly covering sequence from amino acid 329 to amino acid 363 and comprising functional T-helper FliC³³⁹⁻³⁵⁰.^[32] We, however, privileged a mutation at Ala2 position considering that protein immunogens fused with flagellin at its *N*-terminus, benefiting of its adjuvant properties, have already been successfully proposed as vaccine candidates.^{[42],[43]} It should be noted that the number of cysteine mutations at this stage was limited to four because uncertainty remains regarding the impact of excess cysteines on the expression and purification of recombinant FliC.

Production of Recombinant Native and Mutant FliC Proteins

E. coli BL21(DE3) expression strain was transformed with a pET22b(+) plasmid coding either wild type FliC or FliC₈₉₋₉₆. In the latter, residues 89 to 96 (QRVRELAV) essential to TLR5 detection^{[44],[45]} are replaced by the corresponding sequence of a non-signaling flagellin (DTVKVKAT).^{[46],[47]} Both proteins were produced with an additional sequence (GAAEPEA) at their C-terminus: the tripeptide GAA is a spacer aiming at facilitating the binding of the immunosilent tetrapeptide C-tag (EPEA) to the resin-grafted camelid antibody fragment during affinity chromatography purification step.^[48] Mutants were prepared according to a step-by-step approach using the QuikChangeTM method. First, single mutation A2C, K180C, T240C and D251C were introduced independently in both FliC and FliC₈₉₋₉₆ sequences to ascertain the feasibility of mutation incorporation at the selected positions. Next, all combinations of double mutations were obtained from the plasmids containing *fliC* or *fliC*₈₉₋₉₆ mutated at the A2, the K180 or the T240 positions (12 mutants). Iteration of the process gave rise to further 8 plasmids each harboring 3 mutations. The fourth A2C mutation was introduced in pET22b(+)_{fliC}-K180C-T240C-D251C and pET22b(+)_{fliC}₈₉₋₉₆-K180C-T240C-D251C plasmids. Later the S306C mutation was also introduced in pET22b(+)_{fliC}-K180C-T240C-D251C as suggested by TLR5 activation assessment (*vide infra*).

At every step, effective incorporation of the mutation was checked by sequencing after DNA plasmid extraction of the corresponding clones. Then *E. coli* BL21(DE3) were transformed with 15 among the 31 pET22b(+) vectors coding FliC or FliC₈₉₋₉₆ mutants to give rise to a series of 15 proteins after affinity chromatography purification in a yield ranging from 2.50 to 5.70 mg/400mL of culture. Noticeably, the introduction of cysteine does not appear to have an impact on production yield, with the highest isolated yield being observed for the tetracysteine mutant (FliC-A2C-K180C-T240C-D251C) (Table S4 and Figure S2). All mutants and the two parent proteins were analyzed by SDS-PAGE and mass spectroscopy as exemplified for FliC-K180C-T240C-D251C-S306C (thereafter named FliC_{4C}), in Figure 2. Samples were sometimes contaminated by a protein of slightly lower apparent molecular weight related to FliC (as determined by Western-blot analysis using an anti-C-tag antibody, data not shown). Mass spectrometry analyses indicate that the contaminant corresponds to a truncated form of FliC partially digested at its *N*-terminus. Primary cleavage site is between Ser11 and Leu12 residues and is characterized by a loss of 1,029 Da. The instability of flagellin

towards proteases has long been recognized and ascribed to the marginal stability of the alpha-helical structure of FliC as a monomer in solution.^{[49],[50]} Content of contaminant, when present, could be determined to be up to 8% and 15% for the FliC-related and FliC₈₉₋₉₆-related proteins (Figure S3), respectively, suggesting that replacement of the TLR5 binding sequence although distant from both the *N*- and *C*-terms of FliC contributes to the disordered state of the protein ends, facilitating their degradation.

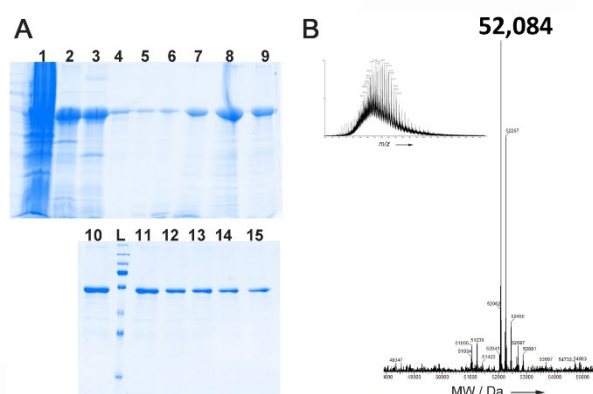


Figure 2. A) SDS-PAGE analysis (12% acrylamide gel with coomassie blue) of FliC_{4C} purified by chromatography using CaptureSelectTM affinity resin. L = Ladder; 1 = cell lysate; 2 = supernatant; 3 = flowthrough fraction; 4-5 = wash buffer; 6-7 = elution fractions ([MgCl₂] = 0.4 M in 20mM Tris.HCl, pH 7.3); 8-15 = elution fractions ([MgCl₂] = 2 M in 20mM Tris.HCl, pH 7.3) (see Figure S2 for affinity chromatography profile); B) ESI mass spectra of FliC_{4C} before and after deconvolution obtained by UPLC-HRMS (measured molecular mass observed with an adduct with mass of 98u^[51]).

FliC Conjugates Preparation and Characterization

FliC_{4C} and FliC wt (the native FliC modified by the C-tag at its C-term), were further used as platforms to investigate the impact of conjugation on TLR-5 activation.

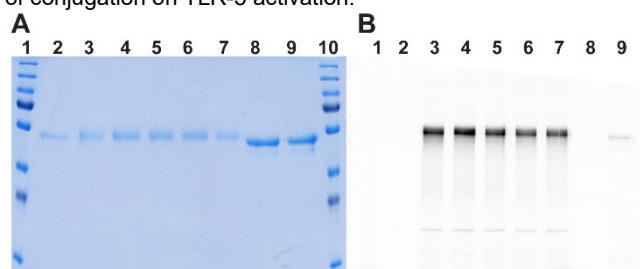


Figure 3. SDS-PAGE analysis (12% acrylamide gel) of ~ 3 µg of protein / well stained with coomassie blue (left) or ~ 0.05 µg of protein / well with fluorescence detection (excitation: 498 nm; emission: 517 nm) on GelDoc Go Imaging System (Bio-Rad) (right); A) 1 = Ladder; 2 = FliC_{4C}; 3 = crude reaction mixture of FliC_{4C} conjugated to 5-maleimido-fluorescein, 8 equivalents; 4 = 16 equivalents; 5 = 24 equivalents; 6 = 32 equivalents; 7 = 40 equivalents; 8 = FliC wt; 9 = crude reaction mixture of FliC wt reacted with 40 equivalents of 5-maleimido-fluorescein; 10 = Ladder; B) 1 = Ladder; 2 = FliC_{4C}; 3 = crude reaction mixture of FliC_{4C} conjugated to 5-maleimido-fluorescein, 8 equivalents; 4 = 16 equivalents; 5 = 24 equivalents; 6 = 32 equivalents; 7 = 40 equivalents; 8 = FliC wt; 9 = crude reaction mixture of FliC wt reacted with 40 equivalents of 5-maleimido-fluorescein.

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First, FliC_4C was reacted with increasing amount (8, 16, 24, 32 or 40 mol/mol equivalents) of 5-maleimido-fluorescein to ascertain accessibility and reactivity of cysteines. FliC wt was reacted in parallel with 40 mol/mol equivalent of 5-maleimido-fluorescein as a negative control.

For this purpose, FliC_4C was first treated overnight with an excess of DTT (200 mol/mol equivalent per Cys) in degassed PBS 0.2M pH8 at room temperature to reduce any intra- or intermolecular disulfide bonds and make each cysteine available for conjugation. Removal of excess DTT and buffer exchange to thoroughly degassed PBS 0.2M pH7 was next carried out simultaneously by gravity flow using a desalting column and the recovered protein immediately reacted with the maleimide derivative under an argon atmosphere.^{[52],[26]} Crude reaction mixtures were analyzed by SDS-PAGE using both coomassie blue staining and fluorescence detection.

As observed in Figure 3B, fluorescein-labeling is observed for every tested condition. All spots appear at both similar molecular weight and intensity, suggesting that conjugation is effective from 2 equivalents of maleimide reagent per cysteine residue.

Additionally, when FliC wt, which does not have a cysteine residue, reacts with the larger amount of maleimide, only a small band is observed at a lower molecular weight (Figure 3B, Lane 8). This result indicates that either covalent or non-covalent aspecific labeling remains highly marginal.

On the basis of these results, FliC_4C and FliC wt were next conjugated to a known tetrasaccharide, β -D-Gal-(1 \rightarrow 4)- β -D-Glc(1 \rightarrow 6)-[β -D-Gal-(1 \rightarrow 4)]- β -D-GlcNAc referred to as Pn14TS, corresponding to the repeat unit of *Streptococcus pneumoniae* serotype 14 capsular polysaccharide, included in every commercial pneumococcal conjugate vaccine. Pn14TS is the smallest structure capable of inducing opsonophagocytic antibodies in mice when conjugated to a protein carrier.^[53] Equipped with a maleimido-functionalized spacer arm, Pn14TS was successfully used in the past to prepare pneumococcal conjugates.^{[54],[22]} This tetrasaccharide was herein coupled to either FliC_4C (at 10 molar equivalent Pn14TS/cysteine residue) or FliC wt (at 15 molar equivalent Pn14TS/FliC), after random derivatization of the free amine groups of its surface-exposed lysine side-chains with *S*-acetylthioacetate groups). FliC_4C was treated with DTT prior to conjugation to ensure that thiol groups are freely available. Both conjugates were purified by size-exclusion chromatography (SEC) and analyzed by gel electrophoresis (Figure 4).

As observed in Figure 4B the band corresponding to the conjugate appears at higher molecular weight than parent FliC_4C protein (Lane 3 vs Lanes 4 & 5) suggesting that the reaction took place at least partially. Indeed, apparent molecular weight of Pn14TS-FliC_4C is around 55 kDa when compared to the protein markers (Lane 1) *i.e.* slightly below the expected 56 kDa MW for a complete reaction. Random conjugate appears at higher molecular weight suggesting that a higher Pn14TS/protein ratio was reached (Lane 7). As expected, the band corresponding to the latter is less defined and spreads over a broad range of molecular weights, representative of a mixture of glycoconjugates differing by their Pn14TS payload.

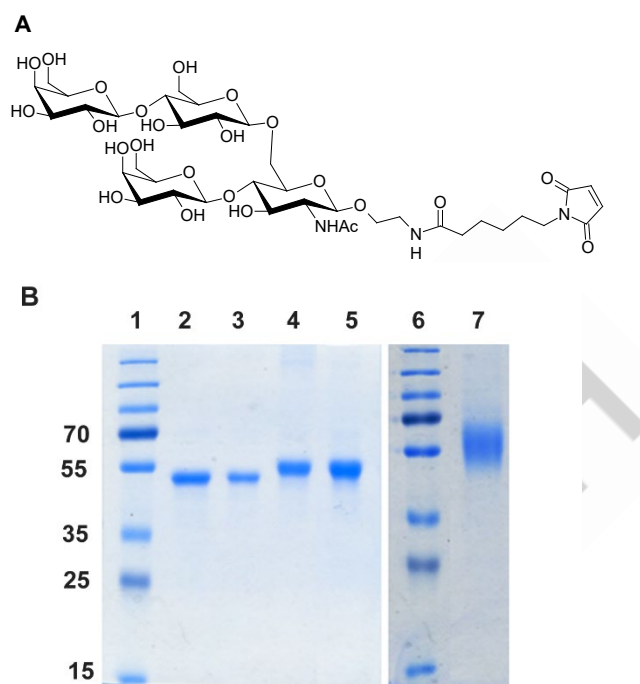


Figure 4. A) Structure of Pn14TS derivative used for conjugation; B) SDS-PAGE analysis (12% acrylamide gel stained with coomassie blue). **1** = Ladder; **2** = FliC_4C treated with excess DTT; **3** = FliC_4C after Zeba spin desalting prior to conjugation; **4** = crude Pn14TS-FliC_4C conjugation mixture; **5** = homogeneous Pn14TS-FliC_4C conjugate after purification by SEC on a Superdex 200 column; **6** = Ladder; **7** = random Pn14TS-FliC wt conjugate after purification by SEC on a Superdex 200 column.

To ascertain the conjugation, both conjugates were tentatively analyzed by mass spectrometry. Neither electrospray ionization (ESI) nor MALDI-TOF mass spectrometry were successful in analyzing the Pn14TS-FliC wt conjugate. Carbohydrate content was therefore measured by a colorimetric method based on the anthrone reaction.^[55] Extent of derivatization was determined to be 8.2:1 (mol/mol) Pn14TS/FliC wt. ESI-MS was also inconclusive for Pn14TS-FliC_4C. However, peak corresponding to the conjugate could be detected and compared to that observed for FliC_4C using MALDI-TOF MS (Figure 5).

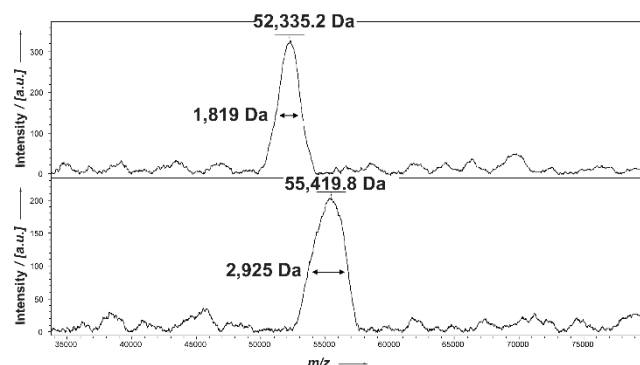


Figure 5. MALDI-TOF mass spectra (+) of FliC-4C-Ctag before (top) and after (bottom) conjugation with the tetrasaccharide 34 – 80 kDa *m/z* range.

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The molecular weight of parent FliC_{4C} was found equal to 52,335 Da (Figure 5, top chromatogram). The peak corresponding to the conjugate was shifted to higher mass (54,419 Da) and slightly broaden (full width at half maximum 2,925 vs 1,819 Da), indicating that the conjugation took place and gave rise to a mixture of conjugates having different, possibly 2, 3 or 4 tetrasaccharide/protein ratios (Figure 5, bottom chromatogram). The mass of the conjugate was incremented by 3,184 mass unit which corresponds to the grafting of 3.4 tetrasaccharide on average (based on calculated +944 Da per Pn14TS derivative).

Tryptic in-gel digestion of both FliC_{4C} and Pn14TS-FliC_{4C} conjugate was further carried out to confirm that the conjugation is selective for the cysteine residues and determine whether some cysteine residues react preferentially to the others. The proteolytic mass analysis confirmed the identity of the protein and the correct insertion of the cysteine residues (97% protein sequence coverage). Identification of peptides carrying the Pn14TS modification proved tedious. It was only possible when omitting the classic denaturing treatment with DTT at 56°C for 1 hour of the sample which precedes the digestion and reducing the peptide detection threshold during the analysis. It seems that the conjugation preferentially took place at position 180 then at position 306 on the basis of recorded events by mass analysis. Consequently, heterogeneity of the Pn14TS-FliC_{4C} conjugate likely results from incomplete substitution at positions 240 and 251.

TLR5 Activation

Next, the ability of the FliC mutants as well as the two conjugates to activate TLR5 was evaluated by a secreted Interleukin-8 (IL-8) reporter assay by using the HEK-hTLR5 cell line. This cell line derives from HEK293 cells and co-expresses the TLR5 receptor and the Lucia luciferase, placed under the control of the endogenous IL-8 promoter, in replacement of the IL-8 gene. Since IL-8 is a chemokine produced in response to TLR5 activation, FliC proteins' adjuvant properties can be simply assessed by monitoring the luciferase activity.

Hence, reporter cells were treated with serial dilutions of the recombinant FliCs, including their TLR5-inactive FliC₈₉₋₉₆ counterparts in comparison with native flagellin, (FliC not modified by the C-tag, referred to as FliC native) and PBS as positive and negative controls, respectively (Figure S4).^[56] We calculated the effective flagellin concentration required for 50%-maximal stimulation (EC₅₀) for each mutant. TLR5-activation by any of FliC₈₉₋₉₆ recombinant flagellins was significantly reduced compared to FliC native (Figure S5). In contrast, FliC proteins non-mutated in their TLR-5 binding region were all potent cell activators, with EC₅₀ falling in the nanogram per milliliter range as previously reported (Figure 6).^[1]

Noticeably, neither introduction of a C-tag at the C-term of the D0 domain or combination of up to 4 cysteine mutations in FliC (EC₅₀ ~ 3.50 ng/mL) impact adjuvant properties. The apparent slightly lower activity of FliC-A2C among the single cysteine-mutant flagellins incited us to consider its replacement by the S306C mutation and further use this mutant to investigate the impact of Pn14TS conjugation on the activity.

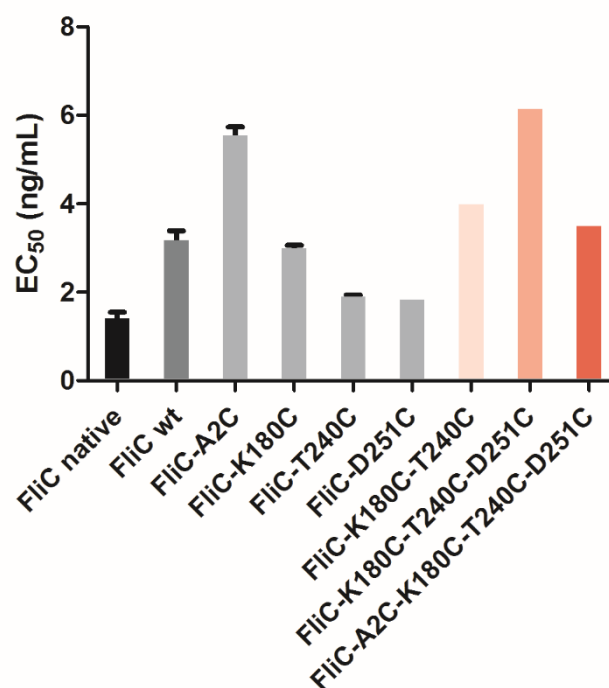


Figure 6. EC₅₀ for each FliC mutant in comparison with FliC native. Results are representative of one or two independent experiments. FliC native (positive control) (black), FliC wt (dark grey), Mono-mutants (grey), di- mutant (light pink), tri-mutant (pink) and tetra-mutant (red).

The resulting FliC_{4C} was found to stimulate HEK-hTLR5 cells similarly to FliC (Figure 7).

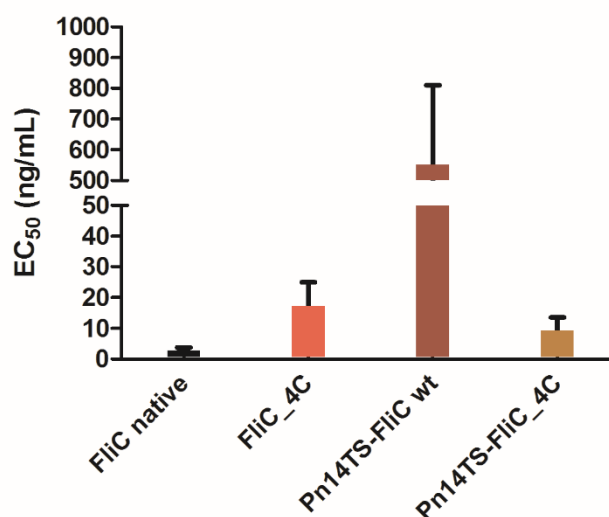


Figure 7. EC₅₀ for FliC_{4C} and conjugates in comparison with FliC native as positive control. FliC native (black), FliC_{4C} (red), Pn14TS-FliC wt (brown) and Pn14TS-FliC_{4C} (light brown),

Strikingly, the random conjugate, Pn14TS-FliC wt, shows a considerably diminished activity compared to FliC native, being almost 200 times less active (EC₅₀ = 550 ng/mL). Thio/maleimide chemistry has largely been applied for the preparation of glycoconjugate vaccines and is not associated with an alteration

of the tertiary structure, and consequently of the immunogenicity, of the carrier protein when used in a dual role^{[57],[22]} including FliC.^[12] As indicated in the literature for *S. enterica* serovar *enteridis* or *Pseudomonas aeruginosa* flagellins, the loss of TLR5 stimulation is more likely linked to the modification of lysine residues involved in the recognition of flagellins by the TLR5 receptor.^{[8],[19],[14]} Among the 28 lysines of FliC, 8 are located in the highly conserved D0 and D1 domains. It has been shown that the preservation of the lysines of these domains from excessive conjugation thanks to steric self-protection is correlated with the maintenance of adjuvant properties comparable to that of unmodified flagellin.^{[19],[14]} Consistently with these observations, TLR5 activation is also attenuated as hapten density increases, likely due to a higher rate of modification of lysine residues, noticeably those in the D0 and D1 domains which are all surface-exposed when FliC is in monomeric form.^[8] Considering the 8.2:1 carbohydrate antigen/protein molar ratio determined for the Pn14TS-FliC wt conjugate, the decrease in TLR5 activity compared to unmodified FliC appears to be greater than that reported in the literature for conjugates having 15.6:1 or 17:1 hapten/flagellin ratios for which differences in stimulation was significant from 10 ng/mL or 1 ng/ml, respectively.^{[8],[14]} It is thus possible that FliC wt derivatization carried out with a large excess of SATA reagent contributed in part to the loss of adjuvant properties. In sharp contrast with the herein presented results and reported data for randomly prepared conjugates, Pn14TS-FliC_{4C} which has been obtained by site-selective modification appears as a strong TLR5 receptor activating agent. Peng *et al.* relied on flagellin's reversible self-association properties to guide conjugation to the D2 and the D3 domains.^[19] This original strategy is straightforward and does not require flagellin sequence engineering. However, according to this strategy, partial changes at lysine K42 and K448 of the D0 and the D1 domains, respectively, cannot be completely avoided (even if it seems that these modifications did not impact the binding to TLR5 in the context of their studies). Alternatively, site-selective mutagenesis offers a full control of the conjugation step. Along this line, Lockner *et al.* have suggested to replace specific lysine residues of the flagellin (particularly in the D0 and D1 domains) with arginine residues to preclude hapten modification.^[8] The replacement of residues exposed on the surface by cysteine residues as depicted herein obviously appears to be a complementary strategy.

Conclusion

The ability of bacterial flagellins to stimulate both innate and adaptive immune response by binding to TLR5 receptor has long motivated their use as adjuvant molecules within vaccine formulations. Along this line it has been proposed to use flagellin as carrier protein to give rise to self-adjuncting conjugate vaccines. Masking or alteration of the sequences necessary for flagellin/TLR5 recognition leading to the abrogation of all or part of the adjuvant properties is a major risk during the derivatization and conjugation steps of flagellin to the hapten. In this study we demonstrated that the use of cysteine mutagenesis was a viable strategy for site-selective conjugation of haptens to *S. enterica* flagellin FliC. This approach allowed us to direct the introduction

of four potentially five haptens on the D2 and D3 domains or area of the D0 domain not involved in TLR5 binding. Expression in *E. coli* and purification of FliC harboring four cysteines on its surface has been successful suggesting that further cysteine mutations could be introduced to increase the hapten/FliC ratio upon requirement. More importantly, it is *a priori* possible to select key positions within the D3 and D2 domains to deimmunize FliC and avoid any immune interference on the part of the carrier protein. Conversely, mutations can be selected so as to maintain the immunogenicity of flagellin if the vaccine candidate is directed against the pathogen from which it originates in a dual role. Next step consists in the *in vivo* immune evaluation of model conjugates. This will be achieved in comparison with conjugates prepared with their TLR5-inactive (FliC₈₉₋₉₆) counterparts. Ultimately, FliC could be used as a carrier protein, of which only five are currently authorized for use in humans, with the advantage of having self-adjuvant properties.

Supporting Information

Additional figures, tables and experimental procedures can be found in the supplementary information material.

Acknowledgements

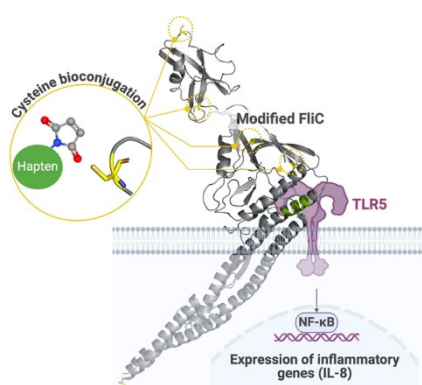
This work was carried out with financial support from the National Agency for Research (ANR project SiSho ANR-21-CE07-0053) and from Région Pays de la Loire – Trajectoire Nationale. L.L. and C.G. are grateful for the ANR for a PhD thesis funding and an internship fellowship, respectively.

Keywords: conjugate vaccines • flagellin • TLR5 • connectivity • epitope

- [1] K. D. Smith, E. Andersen-Nissen, F. Hayashi, K. Strobe, M. A. Bergman, S. L. R. Barrett, B. T. Cookson, A. Aderem, *Nat Immunol* **2003**, *4*, 1247–1253.
- [2] E. A. Miao, E. Andersen-Nissen, S. E. Warren, A. Aderem, *Semin Immunopathol* **2007**, *29*, 275–288.
- [3] S. B. Mizel, J. T. Bates, *J Immunol* **2010**, *185*, 5677–5682.
- [4] I. A. Hajam, P. A. Dar, I. Shahnawaz, J. C. Jaume, J. H. Lee, *Exp Mol Med* **2017**, *49*, e373.
- [5] P. J. Brett, D. E. Woods, *Infect Immun* **1996**, *64*, 2824–2828.
- [6] V. L. Campodónico, N. J. Llosa, L. V. Bentancor, T. Maira-Litran, G. B. Pier, *Infect Immun* **2011**, *79*, 3455–3464.
- [7] R. Simon, J. Y. Wang, M. A. Boyd, M. E. Tulapurkar, G. Ramachandran, S. M. Tennant, M. Pasetti, J. E. Galen, M. M. Levine, *PLoS One* **2013**, *8*, e64680.
- [8] J. W. Lockner, L. M. Eubanks, J. L. Choi, J. M. Lively, J. E. Schlosburg, K. C. Collins, D. Globisch, R. J. Rosenfeld-Gunn, I. A. Wilson, K. D. Janda, *Mol Pharm* **2015**, *12*, 653–662.
- [9] S. M. Baliban, M. Yang, G. Ramachandran, B. Curtis, S. Shridhar, R. S. Laufer, J. Y. Wang, J. Van Druff, E. E. Higginson, N. Hegerle, K. M. Varney, J. E. Galen, S. M.

- Tennant, A. Lees, A. D. MacKerell, M. M. Levine, R. Simon, *PLoS Negl Trop Dis* **2017**, *11*, e0005493.
- [10] A. E. Gregory, B. M. Judy, O. Qazi, C. A. Blumentritt, K. A. Brown, A. M. Shaw, A. G. Torres, R. W. Titball, *Nanomedicine* **2015**, *11*, 447–456.
- [11] H. Rostami, M. Ebtekar, M. S. Ardestani, M. H. Yazdi, M. Mahdavi, *Immunol Lett* **2017**, *187*, 19–26.
- [12] S. M. Baliban, J. C. Allen, B. Curtis, M. N. Amin, A. Lees, R. N. Rao, G. Naidu, R. Venkatesan, D. Y. Rao, V. K. Mohan, K. M. Ella, M. M. Levine, R. Simon, *Molecules* **2018**, *23*, 1749.
- [13] O. Schuster, K. T. Sears, G. Ramachandran, F. J. Fuche, B. Curtis, S. M. Tennant, R. Simon, *Hum Vaccin Immunother* **2019**, *15*, 1436–1444.
- [14] T.-W. Chiu, C.-J. Peng, M.-C. Chen, M.-H. Hsu, Y.-H. Liang, C.-H. Chiu, J.-M. Fang, Y. C. Lee, *J Biomed Sci* **2020**, *27*, 89.
- [15] T. D. Eaves-Pyles, H. R. Wong, K. Odoms, R. B. Pyles, *J Immunol* **2001**, *167*, 7009–7016.
- [16] K. G. K. Murthy, A. Deb, S. Goonesekera, C. Szabó, A. L. Salzman, *J Biol Chem* **2004**, *279*, 5667–5675.
- [17] S. Yoon, O. Kurnasov, V. Natarajan, M. Hong, A. V. Gudkov, A. L. Osterman, I. A. Wilson, *Science* **2012**, *335*, 859–864.
- [18] K. Yonekura, S. Maki-Yonekura, K. Namba, *Nature* **2003**, *424*, 643–650.
- [19] C.-J. Peng, H.-L. Chen, C.-H. Chiu, J.-M. Fang, *ChemBiochem* **2018**, *19*, 805–814.
- [20] G. T. Hermanson in *Bioconjugate Techniques*, 3rd Edition, Academic Press, **2013**.
- [21] V. Verez-Bencomo, V. Fernández-Santana, E. Hardy, M. E. Toledo, M. C. Rodríguez, L. Heynngnezz, A. Rodríguez, A. Baly, L. Herrera, M. Izquierdo, A. Villar, Y. Valdés, K. Cosme, M. L. Deler, M. Montane, E. Garcia, A. Ramos, A. Aguilar, E. Medina, G. Toraño, I. Sosa, I. Hernandez, R. Martinez, A. Muzachio, A. Carmenates, L. Costa, F. Cardoso, C. Campa, M. Diaz, R. Roy, *Science* **2004**, *305*, 522–525.
- [22] M. Prasanna, D. Soulard, E. Camberlein, N. Ruffier, A. Lambert, F. Trottein, N. Csaba, C. Grandjean, *Eur J Pharm Sci* **2019**, *129*, 31–41.
- [23] T. Violo, A. Lambert, A. Pillot, M. Fanuel, J. Mac-Béar, C. Broussard, C. Grandjean, E. Camberlein, *Chemistry* **2023**, *29*, e202203497.
- [24] A. J. Pollard, K. P. Perrett, P. C. Beverley, *Nat Rev Immunol* **2009**, *9*, 213–220.
- [25] Z. Ma, H. Zhang, P. G. Wang, X.-W. Liu, M. Chen, *Oncotarget* **2018**, *9*, 75–82.
- [26] A. Pillot, A. Defontaine, A. Fateh, A. Lambert, M. Prasanna, M. Fanuel, M. Pipelier, N. Csaba, T. Violo, E. Camberlein, C. Grandjean, *Front Chem* **2019**, *7*, 726.
- [27] R. Dagan, J. Poolman, C.-A. Siegrist, *Vaccine* **2010**, *28*, 5513–5523.
- [28] Q. Zhou, H. Qiu, *J Pharm Sci* **2019**, *108*, 1366–1377.
- [29] P. L. Turecek, M. J. Bossard, F. Schoetens, I. A. Ivens, *J Pharm Sci* **2016**, *105*, 460–475.
- [30] M. A. Bergman, L. A. Cummings, R. C. Alaniz, L. Mayeda, I. Fellnerova, B. T. Cookson, *Infect Immun* **2005**, *73*, 7226–7235.
- [31] B. T. Cookson, M. J. Bevan, *J Immunol* **1997**, *158*, 4310–4319.
- [32] S. J. McSorley, B. T. Cookson, M. K. Jenkins, *J Immunol* **2000**, *164*, 986–993.
- [33] S. Vij, R. Thakur, L. Kumari, C. R. Suri, P. Rishi, *Microb Pathog* **2023**, *174*, 105936.
- [34] K. Yoshioka, S. Aizawa, S. Yamaguchi, *J Bacteriol* **1995**, *177*, 1090–1093.
- [35] A. H. López-Yglesias, C.-C. Lu, X. Zhao, T. Chou, T. VandenBos, R. K. Strong, K. D. Smith, *Immunohorizons* **2019**, *3*, 422–432.
- [36] C. Nempont, D. Cayet, M. Rumbo, C. Bompard, V. Villeret, J.-C. Sirard, *J Immunol* **2008**, *181*, 2036–2043.
- [37] J. H. Rhee, K. Khim, S. Puth, Y. Choi, S. E. Lee, *Curr Opin Virol* **2023**, *60*, 101330.
- [38] D. J. Barlow, M. S. Edwards, J. M. Thornton, *Nature* **1986**, *322*, 747–748.
- [39] J. V. Kringelum, M. Nielsen, S. B. Padkjær, O. Lund, *Mol Immunol* **2013**, *53*, 24–34.
- [40] H.-W. Wang, T.-W. Pai, *Methods Mol Biol* **2014**, *1184*, 217–236.
- [41] A. Ras-Carmona, A. A. Lehmann, P. V. Lehmann, P. A. Reche, *Sci Rep* **2022**, *12*, 13739.
- [42] N. S. Savar, A. Jahanian-Najafabadi, M. Mahdavi, M. A. Shokrgozar, A. Jafari, S. Bouzari, *J Biotechnol* **2014**, *175*, 31–37.
- [43] K. N. Delaney, J. P. Phipps, J. B. Johnson, S. B. Mizel, *Viral Immunol* **2010**, *23*, 201–210.
- [44] L. Zhang, Z. Pan, X. Kang, Y. Yang, H. Kang, N. Zhang, J. M. Rosati, X. Jiao, *Cell Mol Immunol* **2015**, *12*, 625–632.
- [45] X. Kang, Z. Pan, X. Jiao, *Cell Mol Immunol* **2017**, *14*, 1023–1025.
- [46] E. Andersen-Nissen, K. D. Smith, K. L. Strobe, S. L. R. Barrett, B. T. Cookson, S. M. Logan, A. Aderem, *Proc Natl Acad Sci U S A* **2005**, *102*, 9247–9252.
- [47] R. Porte, D. Fougeron, N. Muñoz-Wolf, J. Tabareau, A.-F. Georgel, F. Wallet, C. Paget, F. Trottein, J. A. Chabalgoity, C. Carnoy, J.-C. Sirard, *Antimicrob Agents Chemother* **2015**, *59*, 6064–6072.
- [48] J. Jin, K. A. Hjerrild, S. E. Silk, R. E. Brown, G. M. Labbé, J. M. Marshall, K. E. Wright, S. Bezemer, S. B. Clemmensen, S. Biswas, Y. Li, A. El-Turabi, A. D. Douglas, P. Hermans, F. J. Detmers, W. A. de Jongh, M. K. Higgins, R. Ashfield, S. J. Draper, *Int J Parasitol* **2017**, *47*, 435–446.
- [49] F. Vonderviszt, S. Kanto, S. Aizawa, K. Namba, *J Mol Biol* **1989**, *209*, 127–133.
- [50] F. Vonderviszt, S. Aizawa, K. Namba, *J Mol Biol* **1991**, *221*, 1461–1474.
- [51] S. K. Chowdhury, V. Katta, R. C. Beavis, B. T. Chait, *J Am Soc Mass Spectrom* **1990**, *1*, 382–388.
- [52] J. M. Chalker, L. Lercher, N. R. Rose, C. J. Schofield, B. G. Davis, *Angew Chem Int Ed Engl* **2012**, *51*, 1835–1839.
- [53] D. Safari, H. A. T. Dekker, J. A. F. Joosten, D. Michalik, A. C. de Souza, R. Adamo, M. Lahmann, A. Sundgren, S. Oscarson, J. P. Kamerling, H. Snippe, *Infect Immun* **2008**, *76*, 4615–4623.
- [54] M. Prasanna, R. Varela Calvino, A. Lambert, M. Arista Romero, S. Pujals, F. Trottein, E. Camberlein, C. Grandjean, N. Csaba, *Bioconjug Chem* **2023**, *34*, 1563–1575.
- [55] D. Herbert, P. J. Phipps, R. E. Strange, in *Methods in Microbiology* (Eds.: J.R. Norris, D.W. Ribbons), Academic Press, **1971**, pp. 209–344.
- [56] M. E. Biedma, D. Cayet, J. Tabareau, A. H. Rossi, K. Ivičak-Kocjan, G. Moreno, A. Errea, D. Soulard, G. Parisi, R. Jerala, P. Berguer, M. Rumbo, J. C. Sirard, *Vaccine* **2019**, *37*, 652–663.
- [57] M. Muse, C. Grandjean, T. K. Wade, W. F. Wade, *FEMS Immunol Med Microbiol* **2012**, *66*, 98–115.

Entry for the Table of Contents



Guide to control: Rational replacement of surface-exposed residues of flagellin with cysteines directs conjugation to specific residues while preserving the critical TLR5 binding domains D0 and D1 and potentiates the use of flagellin as self-adjuvanting carrier in conjugate vaccines.

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