SpyStapler-mediated assembly of nanoparticle vaccines

Songzi Kou1,2, Weitao Chen1, Chenbo Sun3, and Fei Sun1,2,3

1 Biomedical Research Institute, Shenzhen Peking University-The Hong Kong University of Science and Technology Medical Center, Shenzhen 518036, China
2 Greater Bay Biomedical Innocenter, Shenzhen Bay Laboratory, Shenzhen 518132, China
3 Department of Chemical and Biological Engineering, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

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ABSTRACT
The COVID-19 pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has wreaked havoc around the globe, with no end in sight. The rapid emergence of viral mutants, marked by rapid transmission and effective immune evasion, has also posed unprecedented challenges for vaccine development, not least in its speed, mass production, and distribution. Here we report a versatile “plug-and-display” strategy for creating protein vaccines, including those against malaria parasites and SARS-CoV-2, through the combined use of the intrinsically disordered protein ligase SpyStapler and computationally designed viral-like particles. The resulting protein nanoparticles harboring multiple antigens induce potent neutralizing antibody responses in mice, substantially stronger than those induced by the corresponding free antigens. This modular vaccine design enabled by SpyStapler furnishes us with a new weapon for combatting infectious diseases.

KEYWORDS
subunit vaccine, SpyTag, SARS-CoV-2, malaria

1 Introduction
Vaccines are crucial in our battles against infectious diseases. As much of the globe is still mired in the unprecedented public health crisis caused by COVID-19, the need of vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the culprit of COVID-19, has never been so urgent and desperate. The past two years have witnessed the quick development and deployment of several COVID vaccines, including those based on traditional inactivated pathogens, recombinant subunit vaccines, and the cutting-edge mRNA technology, a truly remarkable feat in the history of modern medicine. However, it has become evident that SARS-CoV-2, because of its unceasing evolution and agile immune evasion, will remain as a huge threat to public health in the foreseeable future. The recent emergence of mutant strains, Omicron in particular, would have made all the existing vaccines obsolete, if it was not for booster shots. To keep up with the pace of this quickly evolving virus, an ideal vaccine needs to be amenable to speedy redesign and readjustment, without affecting its mass production.

Typical host–pathogen interactions draw on multivalency to convert a relatively weak and monovalent molecular interaction into a strong one [1]. To stimulate a potent host immune response, an effective vaccine must be able to recapitulate the multivalent feature of the host–pathogen interactions. Virus-like particles (VLPs) are gaining attraction in recent years, for their abilities to display antigens in a multivalent and highly symmetric manner, which provides a modular and versatile platform for designing subunit vaccines. The VLPs are made of self-assembling proteins of viral or non-viral origin, with size (~ 10–150 nm) and geometry well-suited for the symmetric display of multimeric antigens [2–4]. Traditional methods have relied on recombinant DNA technology to genetically fuse self-assembling and immunogenic domains. The production of the large fusion proteins using simple cell factories such as Escherichia coli and Bacillus subtilis can be challenging; while the self-assembling VLP motifs can be produced economically using these bacterial cells, the immunogenetic domains often possess posttranslational modifications such as glycosylation and phosphorylation that elude from these bacteria and thus have to be produced with more costly mammalian cells [5]. To reconcile the need for different expression systems, an alternative strategy involves the two-step synthesis of the VLP-based vaccines; the VLPs are first produced in a bacterial cell factory, followed by a plug-and-display step, i.e., the decoration with antigens that might be obtained from different sources. Rosetta-designed, porous dodecahedral Mi3 60-mer protein nanoparticles, thanks to their easy production in E. coli, have become a popular scaffold for designing VLP vaccines [6], which, alongside genetically encoded click chemistries (GECCs) [7], has opened the venue to a variety of synthetic vaccines. Moreover, this plug-and-display method is highly modular and minimizes the uncertainties over vaccine production by dissecting it into two separate parts, corresponding to the productions of VLP precursors and antigens.

Various VLP-based anti-malaria vaccines have been created using this plug-and-display strategy [3,8–12], some of which were even designed to display several types of malaria antigens simultaneously, leading to enhanced antibody response in mice [8,
The abrupt development of COVID-19 prompted the researchers to quickly adopt this plug-and-display strategy to create COVID vaccines; the de novo-designed Mi3 VLPs were modified via Spy chemistry, or its optimized version Spy003, to display the receptor binding domain (RBD) of SARS-CoV-2 spike protein, which showed the abilities to induce potent neutralizing antibody responses in vivo [13–18]. This plug-and-display approach has expanded not only the existing arsenal for combating SARS-CoV-2, but also perhaps some other coronaviruses that have yet to jump from animals to humans [13, 18, 19]. To date, most of the plug-and-display designs involve the creation of SpyCatcher-fusion Mi3 proteins, which, upon self-assembly into VLPs, are amenable to covariant decoration by SpyTagged antigens. Modular and flexible as it is, this approach still suffers from at least two limitations. Since SpyCatcher is originated from Streptococcus pyogenes, a human pathogen that infects 700 million people worldwide every year, a large portion of the population have preexisting antibodies against SpyCatcher, which confounds the efforts of developing any vaccine based on Spy chemistry [20]. In addition, SpyCatcher (M_w ∼ 12 kDa) is not a small protein, which, when unwittingly fused with some VLP precursors, may interfere their self-assembly due to steric hindrance. An improved protein ligation strategy, less-trace and involving only short and flexible reactive motifs, may help circumvent the issues, such as preexisting immunity and steric hindrance, facing the VLP vaccine design.

The folded SpyCatcher has recently been dissected into two smaller segments—SpyStapler (62 aa) and BDTag (ATHIKFSKREDGKELAGATMELRD). SpyStapler, though intrinsically disordered, can catalyze the ligation between BDTag and SpyTag (AHIVMVDAYKPTK), via the formation of a Lys-Asp isopeptide bond [20]. In this study, the unstructured ligase, SpyStapler, furnished us with a new plug-and-display strategy, less-trace and less-constrained sterically, for creating VLP vaccines. By varying the antigens plugged, we demonstrated the creation of the vaccines against human pathogens such as malaria parasites and SARS-CoV-2, both of which were efficacious in triggering the corresponding antibody response in mice. The SpyStapler-mediated plug-and-display method has strengthened our abilities to create vaccines for combating infectious diseases.

2 Results and discussion

2.1 Protein construct design

Despite being intrinsically disordered, SpyStapler has proved to be an efficient ligase that catalyzes the isopeptide bond formation between SpyTag and BDTag [20]. This SpyStapler-mediated ligation could provide us with an alternative plug-and-display approach to nanoparticle vaccines. Compared with other commonly used ligases such as sortase [21], the major advantages of SpyStapler lie in (1) its unstructured nature, which renders it more resilient under various conditions and poses less steric constraints than the folded globular protein like sortase, and (2) the flexibility of introducing its cognate sequences, BDTag and SpyTag, to any site, whether N-, C-terminal, or the middle, of a given protein, in contrast to sortase, of which the recognition sequences, LPXTG, and (Gly)_n must be placed at the C- and N-terminals of a protein, respectively. We chose as the VLP scaffold the computationally designed protein, Mi3, which can be heterologously produced in E. coli and spontaneously assemble into a 36-nm dodecahedral cage with 60 subunits. We created two Mi3 constructs by genetically fusing SpyTag and BDTag to its N-terminus and C-terminus, respectively. An unstructured peptide sequence, GGGSGGS, was introduced as a spacer to minimize the steric effect between Mi3 and the appended tag (SpyTag or BDTag) (Fig.1 and Fig.S1 in the Electronic Supplementary Material (ESM)). Five types of His6-tagged VLP precursors, including Mi3, Mi3-SpyTag, SpyTag-Mi3, Mi3-BDTag, and BDTag-Mi3, were produced in E. coli BL21(DE3) and purified using Ni-NTA chromatography. Size-exclusion chromatography (SEC) analyses of these products with a Superose-6 10/300 GL column revealed two major peaks at low retention volumes (Peak 1 and Peak 2 at 8.4–8.7 and 10.8–11.5 mL, respectively) (Fig.S2 in the ESM), corresponding to the agglomerated and dispersed VLPs, respectively [6, 22, 23]. Static light scattering experiments showed that the measured M_w of the products at Peak 2 were in rough agreement with the theoretical M_w of the corresponding 60-mer nanoassemblies, further confirming the assembled structures at Peak 2 (Fig.S2 in the ESM). The SEC fractions corresponding to the dispersed nanoparticles were collected for further use.

To examine the versatility of the SpyStapler-mediated plug-and-display strategy, we chose two types of antigens, Plasmodium falciparum cysteine-rich protective antigen (CyRPA) and SARS-CoV2-RBD (or RBD, residue 333-526 of SARS-CoV2 spike protein), which were originated from the human pathogens, Plasmodium parasites and SARS-CoV2, respectively. These antigens were produced heterologously using an HEK293 expression system. As another proof of the versatility, the two antigens were prepared in different ways prior to the plug-and-display; CyRPA was fused at its C-terminus with the sorting signal, LPETGG, enabling the ensuing sortase-mediated conjugation with synthetic peptides such as GGG-SpyTag or GGG-BDTag, while SARS-CoV-2-RBD was genetically fused with SpyTag at its C-terminus.

2.2 SpyStapler-mediated decoration of VLPs

To examine the feasibility of covalently decorating Mi3-VLPs with functional proteins via SpyStapler-mediated ligation, we first used the fluorescent proteins, BDTag-CFP and YFP-SpyTag, as model substrates, both of which were produced using the E. Coli expression. The reactions were initiated by mixing the designated Mi3 (SpyTagged Mi3 (M_w ∼ 24.1 kDa) or BDTagged Mi3 (M_w ∼ 26.8 kDa)), the fluorescent protein (BDTag-CFP (32.4 kDa) or YFP-SpyTag (30.9 kDa)), and SpyStapler (7.8 kDa) at a molar ratio of 1:1:2 in tris-buffered saline (TBS, pH 8.5) or phosphate buffered saline (PBS, pH 7.4) at 4 °C. After 12 h, the products were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The reaction of SpyTagged Mi3 (SpyTag-Mi3 or Mi3-SpyTag) and BDTag-CFP catalyzed by SpyStapler yielded a product (M_w ∼ 57 kDa), with a conjugation efficiency (∼ 70%) barely affected by the position of SpyTag, at the N- or C-terminal of Mi3, and the buffer condition, in TBS or PBS (Fig.2(a)). In addition, swapping the reactive motifs between Mi3 and the fluorescing proteins exerted negligible influence on the conjugation of BDTagged Mi3 (BDTag-Mi3 or Mi3-BDTag) and YFP-SpyTag, with a comparable efficiency (∼ 75%), showing considerable flexibility in designing the building blocks of VLP vaccines (Fig.2(b)). Time-course experiments showed that these ligation reactions mediated by SpyStapler all reached maximum within ~ 6-8 h (Figs. S3 and S4 in the ESM), where a suitable stoichiometry (i.e., 5:4:10 for SpyTagged Mi3 + BDTag-CFP + SpyTag or BDTagged Mi3 + YFP-SpyTag + SpyStapler) was also needed for maximal conjugation (Figs. S5–S8 in the ESM).

Prompted by the success with the fluorescent proteins, we further tested SpyStapler-mediated plug-and-display of the antigens, CyRPA-BDTag (43.8 kDa) and RBD-SpyTag (27.8 kDa), on the Mi3-VLPs. In the presence of SpyStapler (2 equiv.), both Mi3-SpyTag and SpyTag-Mi3 exhibited marked reactivities to
CyRPA-BDTag (0.7 equiv.) in PBS (pH 8.5), yielding 36.7% and 24.0% conjugation, respectively, according to the SDS-PAGE analysis (Fig. 2(c) and Fig. S9 in the ESM). As to RBD-SpyTag, its conjugation to the VLP turned out to be sensitive to the location of BDTag in Mi3, as considerable conjugation (39.5%) was observed only when BDTag resided in the C-terminal of Mi3 (i.e., RBD-SpyTag + BDTag-Mi3 + SpyStapler) at a molar ratio of 2:1:2 in PBS (pH 7.4) (Fig. 2(d) and Fig. S10 in the ESM). The resulting conjugate exhibited an apparent $M_w$ of ~68 kDa, larger than the theoretical (~54 kDa), presumably due to the glycosylation of RBD during the mammalian cell expression. A dialysis process against PBS was performed, which was able to remove over 95% of the non-reacted antigen molecules and the catalyst, SpyStapler (Fig. S11 in the ESM). As these antigens were prone to aggregation, the overall efficiency of conjugating them onto the VLPs was lower than that with BDTag-CFP or YFP-SpyTag, showing the contingency of this SpyStapler-mediated plug-and-display strategy on the physicochemical properties of the functional domains to be appended, including their structure, solubility, and oligomeric state. It is also noteworthy that the excess use of SpyStapler (e.g., 10 equiv. or more) can be problematic, of which the unstructured nature resulted in undesirable precipitation.

2.3 Characterization of decorated VLPs
The Mi3 protein is noted for its ability to self-assemble into monodisperse 60-mer nanoparticles, with a porous dodecahedral structure [6]. Transmission electron microscopy (TEM) was used to verify the structures of these nanoparticles. The undecorated VLPs, including Mi3-SpyTag, SpyTag-Mi3, Mi3-BDTag, and BDTag-Mi3, were shown to be uniformly sized particles, with diameters of 22.97 ± 1.84, 24.08 ± 2.29, 20.33 ±1.82, and 23.72 ± 1.31 nm, respectively (Figs. 3(a) and 3(b)).

The decorated VLPs, including Mi3-SpyTag + BDTag-CFP (24.57 ± 2.17 nm in diameter), Mi3-SpyTag + CyRPA-BDTag (24.14 ± 2.03 nm), SpyTag-Mi3 + BDTag-CFP (25.69 ± 2.59 nm), SpyTag-Mi3 + CyRPA-BDTag (14.33 ± 1.97 nm), Mi3-BDTag + YFP-SpyTag (25.42 ± 1.55 nm), Mi3-BDTag + RBD-SpyTag (24.03 ± 1.48 nm), BDTag-Mi3 + YFP-SpyTag (26.52 ± 2.25 nm), and BDTag-Mi3 + RBD-SpyTag (23.72 ± 1.37 nm), exhibited increased diameters in comparison to the undecorated, while maintaining the uniform spherical geometry (Fig. 3).
observed increase in particle size further confirmed the successful decoration of these VLPs with various protein molecules, exemplifying the versatility of this SpyStapler-mediated plug-and-display strategy.

2.4 SpyStapler-mediated display of CyRPA on VLPs enhances antibody response in mice

To evaluate the possible benefits arising from the multimeric display of the *Plasmodium* antigen CyRPA on the VLPs, we compared these antigens in different forms, including free CyRPA, VLP-CyRPA, and CyRPA-VLP, in their abilities to trigger antibody response in mice. The animals were immunized intramuscularly, and then boosted with the same dose of antigens after 14 days (Fig. 4(a)). Sera were collected at days 14 and 28 via tail vein and cardiac puncture, respectively (Fig. 4(a)). Fourteen days after the primary immunization, only a low level of antibody response was detected in the mice treated with VLP-CyRPA and with CyRPA-VLP, while no detectable antibody response arose from those with free CyRPA-BDTag or with blank VLP (i.e., Mi3-SpyTag or SpyTag-Mi3) (Fig. 4(b), and Figs. S12 and S13 in the ESM). The level of antibody response after the primary immunization was also contingent on the purity of the VLPs; detectable antibody response was observed from the dialyzed VLP-CyRPA and CyRPA-VLP products (Fig. 4(b), and Figs. S12 and S13 in the ESM).

Booster shots enhanced the antibody response, as the three groups of mice, boosted with free CyRPA, crude VLP-CyRPA, and dialyzed VLP-CyRPA, respectively, all exhibited robust antibody response; according to the enzyme-linked immunosorbent assay (ELISA), alongside the analyses of serum reciprocal endpoint titers (ET) and absorbance summation (AS), the level of antibody response triggered by the VLP-CyRPA product, whether it be crude or dialyzed, was dramatically higher than that by the free antigen, clearly showing the benefits of displaying the antigens on the VLPs for eliciting immune response (Fig. 4(b), and Figs. S12 and S13 in the ESM). The avidity of the anti-CyRPA antibody in the sera harvested after boost (at day 28) was determined using sodium thiocyanate (NaSCN)-displacement ELISA [6]. The anti-CyRPA antibody elicited by dialyzed VLP-CyRPA exhibited an avidity significantly higher than that by free CyRPA (p < 0.01, n = 4), (Fig. 4(c)). As to CyRPA-VLP, in which the antigen, CyRPA, was conjugated with Mi3 at its N-terminal, this alternative vaccine design exhibited an efficacy comparable to VLP-CyRPA. The mice which were dosed twice with CyRPA-VLP exhibited pronounced antibody responses compared with those with free CyRPA (p < 0.0001 for the crude, n = 5; p < 0.0001 for the dialyzed, n = 5) (Fig. 4(b)). One subtle difference between the two designs, CyRPA-VLP and VLP-CyRPA, lies in the finding that the avidity of anti-CyRPA-specific antibody induced by the dialyzed VLP-CyRPA was significantly higher than that by the VLP-CyRPA (p < 0.05, n = 5) according to the NaSCN-displacement ELISA (Fig. 4(c)). This nuance points to the intricate nature of vaccine design, which must take into account multiple seemingly nuanced factors, such as the relative location of the antigens on the VLPs, as well as their preparation processes in this case.

2.5 SpyStapler-mediated display of SARS-CoV-2 RBD on VLP enhances antibody response in mice

To assess the efficacy of SARS-CoV-2 RBD/VLP conjugates in
vivo, we immunized mice twice in a prime-boost regimen (days 0 and 28) with the RBD antigens in various forms, including free RBD, RBD-VLP, and VLP-RBD. Sera were harvested via tail vein at days 14, 28, and 42 and via cardiac puncture at day 56 (Fig. 5(a)). The levels of antibody response were quantified using ELISA. After the prime immunization (at days 14 and 28), both free RBD and conjugated RBD (i.e., RBD-VLP or VLP-RBD) induced only a low-level antibody response (Fig. 5(b)).

On the other hand, the boost immunization enhanced the anti-RBD-specific antibody response to various degrees, depending on the formulation and preparation of the antigens (Fig. 5(b)). The free RBD antigens induced detectable post-boost antibody response, while the crude RBD/VLP conjugates, RBD-VLP, or VLP-RBD gave rise to significantly enhanced antibody responses (VLP-RBD, $p < 0.0001$ at day 42 and $p < 0.001$ at day 56, $n = 5$; RBD-VLP, $p < 0.005$ at days 42 and 56, $n = 5$), according to the area under the curve (AUC) (Fig. 5(b), ET and AS analysis in Fig. S14 in the ESM). Preparation processes might affect the efficacy of these VLP vaccines. Unlike the crude VLP-RBD, which was efficacious in inducing the antibody response, its dialyzed product failed to so at days 42 and 56. This discrepancy might be attributed to the poor conjugation efficiency between Mi3-BDTag and RBD-SpyTag (Fig. 2(d)), resulting in the loss of the antigens throughout dialysis. By contrast, the other construct, dialyzed RBD-VLP, in which BDTag-Mi3 and RBD-SpyTag conjugated more efficiently (Fig. 2(d)), retained the ability to sustain a robust antibody response over a prolonged period of time, consistently stronger than those by the crude product and by the free antigen ($p < 0.0001$, $n = 5$ at days 42 and 56) (Fig. 5(b) and Fig. S15 in the ESM).

The neutralizing ability of the antibody induced is another critical parameter for gauging a vaccine. We therefore tested the capabilities of the sera collected from the immunized mice to block the binding of RBD to ACE2, a host surface receptor crucial for the viral attachment. It turned out that the sera from the mice treated with VLP-RBD were able to block the RBD/ACE2 interaction, but in an efficiency comparable to those treated with the free antigen (Fig. 5(c)). This result could again be attributed to the inadequate display of the antigens on the VLP surface due to the poor conjugation between Mi3-BDTag and RBD-SpyTag (Fig. 2(d)). Indeed, efficient conjugation between the antigen and VLP was crucial for the neutralizing activity, as the sera from the mice dosed twice with the dialyzed RBD-VLP—a product resulting from the efficient conjugation between BDTag-Mi3 and RBD-SpyTag—turned out to be more effective in blocking the RBD/ACE2 interaction than those treated with the free RBD ($p < 0.01$, $n = 5$) (Fig. 5(c)). Together, these results demonstrated the feasibility of using this SpyStapler-mediated plug-and-display
strategy to create an entirely protein-based vaccine, with the potential to combat COVID-19.

3 Conclusions

In this study, we demonstrated the combined use of SpyStapler and a de novo designed VLP as a versatile platform for designing subunit vaccines, including those against malaria parasites and SARS-CoV-2. The designated antigens can be placed at either N- or C-terminal of the VLP precursor, showing considerable leeway enabled by SpyStapler, an intrinsically disordered ligase. Clustering multiple antigens on the VLP surface has proved to be advantageous in strengthening the neutralizing antibody response in mice, in comparison to the corresponding free antigens. Thanks to its versatility and modularity, this SpyStapler-mediated plug-and-display vaccine design strategy has enriched our arsenal against infectious diseases.

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Figure 5  SpyStapler-mediated display of the SARS-CoV-2 RBD antigen (RBD) on VLPs enhanced antibody titer and neutralization against RBD. (a) Immunization schedule. (b) Antibody production against RBD from mice immunized and boosted with unconjugated VLP, free RBD, and RBD displayed on VLP. “Antigen”, free RBD-SpyTag "VLP/antigen conj.", the crude VLP-BDTag/RBD-SpyTag conjugate or BDTag-VLP/RBD-SpyTag conjugate. "VLP/antigen conj. pure", the dialyzed VLP-BDTag/SpyTag conjugate or BDTag-VLP/RBD-SpyTag conjugate, with unreacted RBD-SpyTag removed. (c) Influence of neutralizing antibody response on the RBD/ACE2 interaction. One-way ANOVA analysis was used. ns, not significant. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Four or five mice were used in each group.

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