Engineering a Rugged Nanoscaffold To Enhance Plug-and-Display Vaccination

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Supporting Information

ABSTRACT: Nanoscale organization is crucial to stimulating an immune response. Using self-assembling proteins as multimerization platforms provides a safe and immunogenic system to vaccinate against otherwise weakly immunogenic antigens. Such multimerization platforms are generally based on icosahedral viruses and have led to vaccines given to millions of people. It is unclear whether synthetic protein nanoassemblies would show similar potency. Here we take the computationally designed porous dodecahedral i301 60-mer and rationally engineer this particle, giving a mutated i301 (mi3) with improved particle uniformity and stability. To simplify the conjugation of this nanoparticle, we employ a SpyCatcher fusion of mi3, such that an antigen of interest linked to the SpyTag peptide can spontaneously couple through isopeptide bond formation (Plug-and-Display). SpyCatcher-mi3 expressed solubly to high yields in Escherichia coli, giving more than 10-fold greater yield than a comparable phage-derived icosahedral nanoparticle, SpyCatcher-AP205. SpyCatcher-mi3 nanoparticles showed high stability to temperature, freeze−thaw, lyophilization, and storage over time. We demonstrate approximately 95% efficiency coupling to different transmission-blocking and blood-stage malaria antigens. Plasmodium falciparum CyRPA was conjugated to SpyCatcher-mi3 nanoparticles and elicited a high avidity antibody response, comparable to phage-derived virus-like particles despite their higher valency and RNA cargo. The simple production, precise derivatization, and exceptional ruggedness of this nanoscaffold should facilitate broad application for nanobiotechnology and vaccine development.

KEYWORDS: bionanotechnology, nanomedicine, vaccination, protein engineering, bioconjugation, virus-like particle, self-assembly

Nanoassembly creates great opportunities for studying and modulating biological systems.1−3 Decorating functional units onto nanoparticles can enhance function in areas including catalysis,4,5 imaging,6 and therapy.7 Protein-based nanoparticles, compared to abiotic synthetic polymers, may have the advantage of atomically precise assembly, but at the cost of low stability and difficulty in scaling up production.8−10 Exploiting the natural immunogenicity of proteinaceous cages as well as the multimerization of antigens is crucially important to the development of the next generation of vaccines.10,11 Developing a modular and robust nanoscaffold with scalable, low-cost production could contribute to major challenges in human and animal health,12 including vaccines to rapidly evolving pathogens (e.g., HIV, malaria) or zoonotic outbreaks (e.g., Ebola virus, Rift Valley fever). Such a platform would also be important for vaccine stockpiling against pandemics and for vaccine delivery to low-resource areas.13,14

Traditional vaccines based on whole-killed or live-attenuated viruses are good at stimulating immune responses but pose a greater risk of rare adverse events.15,16 Recombinant subunit vaccines, while safer, are often insufficiently immunogenic to progress through clinical development.17−20 By using virus-like particles (VLPs) as multimerization platforms, antigens can be delivered to the immune system on a highly immunogenic scaffold in a safe fashion.20 The immunogenicity of VLPs is a product of efficient draining to lymph nodes, efficient uptake by dendritic cells, and a highly repetitive surface that promotes B-cell cross-linking and activation.

Conjugation of antigens onto a VLP or nanoparticle can be achieved through a variety of techniques including chemical conjugation,21−24 genetic fusion,25,26,27 or bio-orthogonal chemistry with unnatural amino acids.28 Genetic fusion frequently runs into difficulty from inefficient folding of complex antigens.29−31 Chemical coupling faces challenges from the heterogeneity in coupling site and particle coverage.32 We recently established another method for VLP decoration, exploiting the spontaneous isopeptide bond that forms between the protein domain SpyCatcher and its peptide partner SpyTag.33 This ability to separate antigen and scaffold production through modular assembly, termed “Plug-and-Display” (Figure 1a), allows...
optimization of production conditions for both components and may shorten time from development to manufacture of a new vaccine.33 These Plug-and-Display platforms were based on SpyTag or SpyCatcher linked to the coat protein cp3 from the bacteriophage AP205. Spy-AP205 VLPs have been investigated as vaccine candidates against a number of targets, including malaria and cancer.18,34,35 However, despite the fact that Spy-AP205 VLPs are good at stimulating the immune system, a number of concerns hinder their clinical development, including limited solubility, stability, and yield (Figure 1a).

Protein cages from both nonviral and viral origin have gained increased interest expanding on possible applications in the fields of vaccine technology and biotechnology.36−38 Recently, protein nanocages that mimic the structure of VLPs have been computationally designed, although their immunogenicity remains untested.39 The i301 nanocage is based on the 2-keto-3-deoxy-phosphogluconate (KDPG) aldolase from the Entner−Doudoroff pathway of the hyperthermophilic bacterium Thermotoga maritima. i301 has five mutations that alter the interface between the wild-type protein trimer, promoting assembly into a higher order dodecahedral 60-mer (Figure 1b). In this paper we use rational design to improve i301 stability and establish modular antigen coupling by fusing SpyCatcher to the N-terminus of the protein. We then characterize the conformation, ruggedness, and immunogenicity of the SpyCatcher-mi3 scaffold to evaluate its potential to accelerate vaccine development.

RESULTS AND DISCUSSION
To create a Plug-and-Display nanoscaffold, ΔN1-SpyCatcher was genetically fused to the N-terminus of the previously
described i301 sequence.39 A C-tag was installed on the C-terminus to allow more efficient downstream processing for vaccine applications.40,41 This construct expressed solubly in *E. coli*, and particles were purified from cell lysate by C-tag resin affinity chromatography followed by size-exclusion chromatography (Figure 1c). We found difficulties during purification arising from aggregation of the cell lysate. We also saw a double-banded SpyCatcher-i301 product by SDS-PAGE, which converted to a single band upon reduction, indicative of intrachain disulfide bond formation (Figure 1c). Therefore, we designed mutations of two surface-exposed cysteines C76A and C100A (Figure 1b, Supporting Information, Figure S1a) to avoid potential disulfide bond-mediated heterogeneity. Evidence from sequence conservation of KDPG aldolases indicates that alanine is the preferred amino acid at position 76 (Supporting Information, Figure S1b). Removing complicating disulfide bond formation or the need for the presence of reducing agent38 would also assist the conjugation of many antigens that require intact disulfide bonds. We termed this mutated i301 sequence mi3. SpyCatcher-mi3 was efficiently purified under the same conditions, with no double-banded species visible by SDS-PAGE (Figure 1c).

After purification, SpyCatcher-mi3 gave 2-fold higher yield than SpyCatcher-i301 (Figure 1d). This yield is more than 10-fold higher than we could achieve for SpyCatcher-AP205, despite extensive optimization of DNA constructs, bacterial strains, and conditions for growth and induction (Figure 1d).18 25 mg/L is close to the maximum we achieve for any soluble protein in shake-flask *E. coli* culture.20,42,43 Higher yields should be accessible through fermentation.44

The mutations in SpyCatcher-mi3 also eliminated the aggregation of SpyCatcher-i301 seen by dynamic light scattering (DLS) after 5 weeks of storage (Figure 1e). Therefore, all further studies were performed with SpyCatcher-mi3.
cells (CyRPA-SpyTag, 42 kDa, 5 disulfide bonds; Pfs25-SpyTag, 21 kDa, 11 disulfide bonds). Conjugation efficiency ranged from 91 to 96% (Figure 3e). Proper assembly of conjugated SpyCatcher-mi3 was validated by DLS. SpyCatcher-mi3 had a hydrodynamic radius of 17.6 ± 4.4 nm before conjugation and 20.9 ± 5.8 nm after conjugation to CyRPA-SpyTag (Figure 3f). Thus, SpyCatcher-mi3 was able to react with high efficiency to a range of antigens, bearing either N- or C-terminally fused SpyTag and produced in eukaryotic or bacterial cells.

For a nanoparticle platform to transition from small-scale laboratory production to clinical and field-settings, it is desirable that the platform is robust and tolerates long-term storage and varying temperature. Inactivation of vaccines through failure of the cold-chain is a major challenge for the cost and efficacy of vaccines in the developing world. In addition, using this modular vaccine assembly strategy, it would be desirable to be able to stockpile the platform, to use in a newly emerging medical or veterinary outbreak challenge. To test nanoparticle heat stability, purified SpyCatcher-mi3 was incubated in neutral buffer at temperatures ranging from 25 to 95 °C for 1 h. Aggregates were removed by centrifugation, and we measured the proportion of protein in the soluble fraction. Up to 75 °C, at least 80% of the protein remained in the soluble fraction (Figure 4a). At elevated temperatures a small increase was observed in the hydrodynamic radius, as measured by DLS (Supporting Information, Figure S2b).

Many protein nanoparticles are disrupted by freezing. After four rounds of freeze–thawing, ~15% of SpyCatcher-mi3 was lost to aggregation. However, addition of the common sugar stabilizer trehalose minimized aggregation, resulting in only 3% loss of protein over four rounds (Figure 4b). Beyond the solubility, the SpyCatcher-mi3 particles also remained well-formed after four rounds of freeze–thawing, based on DLS (Figure 4c).

We also evaluated stability to lyophilization. The SpyCatcher-mi3 nanoparticle could be lyophilized and reconstituted without damage to the particle shape, based on DLS (Figure 4d), or to the solubility (Figure 4e). After lyophilization, reconstituted SpyCatcher-mi3 retained good reactivity, as tested by conjugation to SpyTag-MBP (Figure 4f). For vaccine formulation and to facilitate reaction with antigens which are not soluble themselves at high concentration, it is valuable for the nanoparticles to have high solubility. We found that SpyCatcher-mi3 was highly soluble and could be concentrated to >1 mM (36 mg/mL) (Supporting Information, Figure S2c). This high concentration...
was sustainable following freeze−thaw or storage at 4 °C for 1 week (Supporting Information, Figure S2c).

To streamline modular nanoparticle assembly, it was interesting to explore whether the nanoparticles could conjugate with target proteins without purification of each partner. We added purified SpyTag-mClover3 to E. coli cleared lysate expressing SpyCatcher-mi3. mClover3 remains fluorescent in SDS-PAGE, as long as the sample is not boiled before loading. Using fluorescent imaging and Coomassie staining, we saw substantial depletion of the SpyCatcher-mi3 band and formation of a SpyCatcher-mi3:SpyTag-mClover3 conjugate band (Supporting Information, Figure S2d), indicating that nanoparticle decoration can occur without purification.

In considering the mi3 nanoparticle scaffold for vaccine applications, it was important to check for similarity to human sequences, to minimize the chance of activating an autoimmune response. No ortholog of KDPG aldolase exists in humans.57 Sequence alignment of mi3 against the human protein database using BLAST revealed no substantial similarity, through coincidence, to any human protein. The top hit was to a 32 amino acid fragment of Interleukin-1 receptor-associated kinase 3 (IRAK3), giving a score of 64, sequence identity 40.6%, and E-value: 9.4 (Supporting Information, Figure S3a). E-values of 0.1 or greater are generally not considered to be significant.58 In comparison, a similar alignment was done for ΔN1-SpyCatcher which is an N-terminally truncated version of SpyCatcher for reduced immunogenicity.59 The top hit was a fragment of the Hemicentin-2 human gene that gave a score of 62, sequence identity 29.7%, and E-value: 2.8 (Supporting Information, Figure S3b).

Having established good assembly, reactivity, and robustness of the SpyCatcher-mi3 platform, we investigated its immunogenicity. Because of the urgent need to improve malaria vaccination,60,61 we focused on immunizing against CyRPA. We validated conjugation of CyRPA-SpyTag to SpyCatcher-mi3 by SDS-PAGE, and free antigen was removed using a high molecular weight cutoff (MWCO) membrane (Figure 5a,b). Mice were injected intramuscularly with 1 μg total CyRPA for each group. CyRPA-SpyTag alone was compared to the same antigen multimerized using SpyCatcher-mi3 or the previous leading nanoassembly platform for Plug-and-Display Spy-
All immunizations were performed with AddaVax, a potent squalene-oil-in-water emulsion adjuvant based on the MF59 adjuvant that is licensed and used in influenza vaccines. Mice were boosted with the same dosage at 14 days, and antibody titer against CyRPA was measured after prime or postboost via enzyme-linked immunosorbent assay (ELISA) (Figure 5c). After priming, 6/6 mice in each group responded to the nanoparticle platforms, while 4/6 responded to the monomeric antigen (Figure 5d). This indicates that there is variability in the immune response to free antigen. However, this difference in titer did not reach significance (Figure 5d).

After boosting, 6/6 mice in each group had an anti-CyRPA antibody response at day 27. There was not a significantly different response comparing SpyCatcher-AP205 multimerization against monomeric antigen (Figure 5e). There was a significantly higher antibody response with SpyCatcher-mi3 assembly compared to monomeric antigen (p = 0.011, n = 6, Kruskal–Wallis test followed by Dunn’s multiple comparison post-test) (Figure 5e).

The avidity of CyRPA-specific antibodies was analyzed at day 28 (14 days postboost) using a sodium thiocyanate (NaSCN) antibody displacement ELISA. The molar concentration of NaSCN required to reduce the A405 to 50% (IC50) compared to untreated samples was determined. Both SpyCatcher-mi3 and SpyCatcher-AP205 induced CyRPA-specific antibodies with significantly higher avidity compared to CyRPA-SpyTag (p = 0.028 for SpyCatcher-mi3; p = 0.011 for SpyCatcher-AP205, n = 6, Kruskal–Wallis test followed by Dunn’s multiple comparison post-test) (Figure 5f). There was no significant difference in avidity between SpyCatcher-mi3 and SpyCatcher-AP205 (Figure 5f).
CONCLUSION
Nanoparticles must fulfill a stringent series of criteria to be suitable for medical application.75,8 This work establishes a simple and robust nanoscaffold, suitable for modular multimerization of complex proteins just by mixing. The experiments here also provide insight into nanoparticle features important for high stability and for potent stimulation of an immune response.

Rational modification of a protein computationally designed to assemble into a dodecahedron (i301) enhanced the uniformity of the isolated protein, while increasing both the yield and stability of the resultant nanoparticle. The cysteines mutated in i301 are not accessible for intersubunit disulfide bond formation in the predicted folded structure.79 Therefore, consideration of rare misfolded structures may be important to obtain robust nanoscaffolds.

SpyCatcher-mi3 shows various positive features toward application in vaccine assembly. The platform is robust to freezing, heating, and lyophilization. The high thermoresilience is consistent with the hyperthermophilic origin of the aldolase, suggesting that the mutations to generate i301 and then mi3 did not greatly impair the protein stability. SpyCatcher itself is from a mesophilic organism, Streptococcus pyogenes.66 However, SpyCatcher has shown the ability to enhance protein thermal resilience in the context of cyclizing enzymes.67,68

For the malaria antigen CyRPA, multimerizing on the SpyCatcher-mi3 scaffold increased the antibody response compared to immunization with monomeric antigen. Analysis of the avidity of the anti-CyRPA specific antibodies also indicated a qualitatively superior response from multimerized antigen. The importance of high avidity antibodies has been emphasized for resisting HIV immune escape69,70 and for increasing antibody antiviral effector function.71 It might be predicted that AP205 would be a more immunogenic nanoscaffold than mi3, having a higher valency (maximum 180 antigens on AP205 versus 60 on mi3)72 and efficiently encapsulating RNA, which can stimulate Toll-like receptor (TLR) 7 and 8 signaling.73 Also, AP205 cp3 forms a conventional tightly packed capsid surface, whereas i301 has large pores. In fact, we found comparable antibody titer and avidity from immunization using SpyCatcher-AP205 or SpyCatcher-mi3. Therefore, the more efficient production by E. coli may make SpyCatcher-mi3 a more favorable nanoscaffold for future development. A limitation of the SpyCatcher-mi3 platform is that it is important that the protein to be conjugated does not self-associate and thereby promote aggregation. Also, we have not tested the induction of cytotoxic T cell responses using SpyCatcher-mi3, where one would expect viral vectored vaccines to be superior.74

The simple production of SpyCatcher-mi3, accessible to any molecular biology laboratory, should facilitate its widespread use by the research community as well as favoring potential clinical development. The lack of sequence homology between SpyCatcher-mi3 and the human genome reduces potential concerns about the platform inducing an autoimmune response. Beyond mi3, there are a number of computationally derived protein nanocages that vary in composition, structure, and cargo packaging.75,76 Thus, future work in synthetic nanobiology may establish the factors maximizing nanoparticle immunogenicity, such as the ideal antigen spacing, number, and orientation.32,77,78

METHODS

Cloning. Constructs were cloned using standard PCR methods and Gibson assembly. Inserts were verified by Sanger sequencing. In all cases, the version of SpyCatcher used was ΔN1-SpyCatcher.79 The i301 insert79 was synthesized by Genscript. pET28-SpyCatcher-i301 has the organization: SpyCatcher, (GGS), spacer, i301, GSG spacer, C-tag. pET28a-SpyCatcher-mi3 (GenBank accession no. MH425515 and Addgene plasmid ID 112255) has the same organization, except for mutations C76A and C100A (numbering based on i301 from Hsia et al., Supporting Information Figure S1A)79 introduced by Gibson cloning. pENTR4-LPTOS-CyRPA-SpyTag has the organization: IgG leader of V-kappa sequence, P. falciparum CyRPA (3D7), (GSG) spacer, SpyTag, GSG spacer, C-tag (GenBank accession no. MH425516) in the pENTR4-LPTOS backbone.80 pENTR4-LPTOS-CyRPA has the organization: IgG leader of V-kappa sequence, CyRPA, GGGs spacer, C-tag, pENTR4-LPTOS-P625-SpyTag for expression in mammalian cells has been previously described (GenBank accession no. KU302811.1).81 pET28a-SpyTag-MBP80 (Addgene plasmid ID 35050), pET28-SpyTag-mClover3,81 pGEM-SpyCatcher-AP205 cp3,81 and pET15b-SpyTag-CIDR-IT4variant79 have been previously described.

Expression of SpyCatcher-mi3 Particles. The pET28a expression plasmid of either SpyCatcher-i301 or SpyCatcher-mi3 was transformed into E. coli BL21 (DE3)-RIPL (Agilent), and cells were grown for 16 h at 37 °C on LB-Agar plates containing 50 μg/mL kanamycin. A single colony was picked into a 10 mL starter culture of LB medium containing 50 μg/mL kanamycin and incubated for 16 h at 37 °C with shaking at 200 rpm. The entire 10 mL culture was then diluted into 1 L LB containing 50 μg/mL kanamycin and incubated at 37 °C with shaking at 200 rpm. At A600 0.8, cultures were induced with 0.5 mM IPTG and grown for 16–20 h with shaking at 200 rpm at 32 °C.

Purification of SpyCatcher-mi3 Particles. One 500 mL culture-derived pellet was resuspended in 10 mL lysis buffer [25 mM Tris-HCl pH 8.5, 150 mM NaCl, 0.1 mg/mL lysisomize, 1 mg/mL Complete mini EDTA-free protease inhibitor (Sigma-Aldrich), 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich) at 4 °C and rotated at 25 °C for 1 h. The lysate was then sonicated on ice for 5 min with rounds of 10 s on and 10 s off, before centrifugation at 16,900 × g for 30 min at 4 °C. Capture Select C-tag Affinity Matrix (Thermo Fisher Scientific) (7.5 mL), equilibrated to 25 mM Tris-HCl pH 8.5 with 150 mM NaCl at 4 °C, was added to cleared lysate and incubated on a tube roller at 4 °C for 1 h. The mixture was then added to a polypropylene column by gravity filtration and washed with 10 column volumes of 25 mM Tris-HCl pH 8.5 with 150 mM NaCl at 4 °C. Elution buffer [20 mM Tris-HCl pH 7.4 with 2 M MgCl2, at 4 °C] was added to the column and incubated for 5 min before each elution. Protein in elution was monitored by A280. Total eluate was then diluted with a 10X stock of 250 mM Tris-HCl pH 8.5 with 1.5 M NaCl at 4 °C, before concentrating in a 20 mL 100 kDa MWCO Vivaspin ultrafiltration unit (Sartorius) and applying to a previously equilibrated HiPrep 16/60 Sepharyl S-400 or S-500 HR (GE Healthcare) on a fast protein liquid chromatography (FPLC) system AKTA Purifier 10 (GE Healthcare). The mobile-phase column buffer was 25 mM Tris-HCl pH 8.5 with 150 mM NaCl, and the applied flow-rate was 1.0 mL/min, all at 4 °C. A high molecular weight gel filtration standard (Bio-Rad, Cat. No. 151–1901) was run on the same column with the same buffer and flow-rate specification. Elution of proteins and standard was monitored at A280. The SpyCatcher-mi3 elution in the 65–90 mL range was collected. Fractions were concentrated using a 100 kDa spin column, and the concentration was determined using the Pierce bichromonic acid (BCA) Assay Kit (Thermo Fisher Scientific).

Expression of SpyCatcher-AP205 VLPs. C41 E. coli cells, a kind gift of Anthony Watts (University of Oxford), were transformed with pGEM-SpyCatcher-AP20518 (GenBank accession number KU302810) and grown for 16 h at 37 °C on an LB-agar plate containing 100 μg/mL ampicillin. A single colony was picked into a 10 mL starter culture of 2×TY medium containing 100 μg/mL...
ampicillin and incubated at 37 °C with shaking at 200 rpm for 16 h. The entire 10 mL culture was then diluted into 1 L 2×TY containing 100 µg/mL ampicillin and incubated at 37 °C with shaking at 200 rpm. At A
to 0.5, cultures were induced with 0.42 mM IPTG and grown for 4–6 h with shaking at 200 rpm at 30 °C. Following the conditions for expression of SpyCatcher-mi3 above decreased the yield of SpyCatcher-AP205.

**Purification of SpyCatcher-AP205 VLPs.** One 1 L culture-derived pellet was resuspended at 25 °C for 1 h in 10 mL lysis buffer [20 mM Tris-HCl pH 7.8, 150 mM NaCl, 0.1% (v/v) Tween-20, 75 mM imidazole, 0.2 mg/mL lysozyme, 1 mg/mL cOmplete mini EDTA-free protease inhibitor cocktail, 1 M PMSF, 25 U/mL benzamidine]. The lysis was sonicated 4 times for 30 s with a minimum of 1 min between each pulse. The lystate was centrifuged twice at 16,900 g for 45 min at 4 °C. The supernatant was then filtered through a Minisart NML Glass Fiber Filter (1.2 µm pore size) (Sartorius) and then through a Syringe Filter (0.45 µm pore size) (StarLab, cat. no. E4780-1453). The filtrate was incubated with 250 U benzamidine (Sigma-Aldrich) for 5 min at 25 °C. One mL of packed Ni-NTA agarose (Qiagen) equilibrated to 75 mM imidazole in buffer [20 mM Tris-HCl pH 7.8, 150 mM NaCl, 0.1% (v/v) Tween-20] was added to the cleared lysate and incubated on a tube roller for 30 min at 4 °C. The resin was then added to a polyprep column by gravity filtration and washed 5 times with 10 column volumes of wash buffer at 4 °C [50 mM Tris-HCl, 150 mM NaCl, 100 mM imidazole, 0.1% (v/v) Tween-20, pH 7.8]. For elution, the polyprep column was capped, 1 mL of elution buffer added [2 M imidazole, 50 mM glycine, 25 mM sodium citrate, 0.1% (v/v) Tween-20, pH 8.5 at 4 °C], the resin incubated for 5 min, and the process repeated until all protein was eluted. Eluate was centrifuged for 30 min at 16,900 g at 4 °C, before transfer to a 300 kDa molecular weight cutoff cellulose ester dialysis tubing (SpectrumLabs) and dialyzed overnight at 4 °C against 1000-fold excess of 50 mM glycine, 25 mM sodium citrate, 0.1% (v/v) Tween-20, pH 8.0, for buffer exchange and depletion of VLP monomer. Dialysis was repeated an additional two times for 3 h. After dialysis, the sample was centrifuged once more at 16,900 g at 4 °C for 30 min to remove any aggregates.

Protein concentration was measured using the BCA assay kit. Protein concentration for nanoparticles refers to the concentration of monomer. The comparison of nanoparticle yield was based upon BCA assays of preps grown and purified on independent days (n = 3).
100% soluble. All samples were run in triplicate (plotted as mean ± s.d.). Samples were adjusted to 0.125 mg/mL before analyzing by DLS as above.

**Freeze–Thaw Stability Assay.** 180 μL SpyCatcher-mi3 at 20 μM in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4 at 4 °C) was mixed by SDS-PAGE and DLS, either at the initial time point or after 1 or 4 cycles of freeze–thaw. Each cycle of freeze–thaw consisted of placing the sample in a thin-wall PCR tube (StarLab) for 20 min into a −80 °C freezer, followed by 15 min in an Eppendorf ThermoMixer C at 25 °C. Each sample was spun at 16,900 g for 30 min at 4 °C to remove aggregates, before the supernatant was assayed by SDS-PAGE with Coomassie staining or DLS. The sample without freeze–thaw was defined as 100% soluble.

**Lyophilization Stability Assay.** A 100 μL aliquot of SpyCatcher-mi3 particles at 10 μM in 25 mM Tris-HCl, 150 mM NaCl, pH 8.5 at 4 °C was prepared in a 100 μL thin-wall PCR tube. Samples were snap-frozen in a dry ice-ethanol bath for 30 min. A BenchTop 2K freeze-dryer (VirTis) was used for 24 h at 0.14 mbar and lyophilization, 5 μM SpyCatcher-mi3 was reacted with 15 μM SpyTag-MBP for 16 h at 25 °C, before analysis by SDS-PAGE with Coomassie staining.

**Concentration Stability Assay.** SpyCatcher-mi3 in 25 mM Tris-HCl, 150 mM NaCl, pH 8.5 at 4 °C was concentrated in a 500 μL 300 kDa MWCO Vivaspin ultrafiltration unit (Sartorius) to a final concentration of 1090 μM. Concentrated protein sample was then either stored at 4 °C or underwent one round of freeze–thaw (following the procedure in the Freeze–Thaw Stability Assay section). The samples were then centrifuged for 30 min at 16,900 g at 4 °C, before the protein concentration in the supernatant was measured by BCA assay on triplicate samples.

**Preparation of Immunogens.** SpyCatcher-mi3 nanoparticles (10 μM) with 3X molar excess of CyRPA-SpyTag or SpyCatcher-AP205 VLPs with 1.5X molar excess of CyRPA-SpyTag were incubated for 16–18 h at 25 °C in PBS. The reaction was then dialyzed 4 times in a 300 kDa MWCO cellulose ester dialysis tubing against a 4000-fold excess of PBS with 0.1% (v/v) Tween-20 to remove unreacted CyRPA-SpyTag. Conjugation of CyRPA-SpyTag to SpyCatcher-mi3 or SpyCatcher-AP205 was validated by SDS-PAGE with Coomassie staining. Reducing conditions were employed for SpyCatcher-mi3 or SpyCatcher-AP205 was validated by SDS-PAGE and DLS, either at the initial time point or after 1 or 4 cycles of freeze–thaw. Following a wash step with PBS/T, goat antinouse total IgG conjugated to alkaline phosphatase (Sigma-Aldrich) (1:3000 dilution in PBS/T) was added to the plates, and the plates were incubated for 1 h at 25 °C. After a final wash step with PBS/T, p-nitrophenylphosphate (Sigma-Aldrich) (1 mg/mL) diluted in 1 M diethanolamine, pH 9.8 (Thermo Scientific) was used as a developing substrate. A405 was obtained using a SpectraMAX M3 plate reader (Molecular 322 Devices). The end point titer is defined as the x-intercept of the dilution curve at an absorbance value greater than the mean A405 ± three standard deviations for a serum sample from a naive mouse at a serum dilution of 1:100.

**Antibody Avidity ELISA.** Antibody avidity was assessed at day 28 using a sodium thiocyanate (NaSCN) displacement ELISA. MaxiSorp plates (Thermo Fisher Scientific) were coated with CyRPA overnight at 4 °C and blocked and washed with PBS/T as described above. Serum samples were individually diluted to reach an A405 of approximately 1.5 and plated in duplicate. Three of the samples from the CyRPA-SpyTag immunized group were analyzed at their maximum reachable A405 of 0.4, 0.5, and 0.9. Following 2 h incubation at 25 °C and washing with PBS/T, the indicated concentration of NaSCN (0–7 M) was added to the wells. The plates were incubated for 15 min at 25 °C, followed by washing with PBS/T, and then incubation with secondary antibody and substrate as above. The intercept at which the molar concentration of NaSCN had reduced the A405 to 50% for individual samples was used to give IC50.

**Statistical Analysis of Immunizations.** Statistical analysis was performed using GraphPad Prism 7.0d. Comparisons were made using a Kruskal–Wallis test. Dunn’s multiple comparison post-test was performed for significant values. The p values above 0.05 were reported as nonsignificant (n.s.).

Further information and request for resources and reagents should be directed to and will be fulfilled by the lead contact, Mark Howarth (mark.howarth@bioch.ox.ac.uk).

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.8b02805.

**Sequence alignment of Thermotoga maritima KDPG aldolase, i301, and mi3 and conservation of aldolase sequence; time-course of SpyCatcher-mi3 reactivity, heat stability determined by DLS, stability at high concentration and reactivity in cleared lysate; sequence similarity of mi3 and SpyCatcher to the human genome (PDF)**

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**ASSOCIATED CONTENT**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.8b02805.
Author Contributions

These authors contributed equally. T.U.J.B. and A.C.A. performed all experiments. T.U.J.B., A.C.A., S.J.D., and M.H. designed the experiments. T.U.J.B., A.C.A., and M.H. wrote the paper. All authors analyzed the data.

Notes

The authors declare the following competing financial interest(s): M.H. is an inventor on a patent regarding peptide targeting via spontaneous amide bond formation (EP2534484). M.H. and S.J.D. are SpyBiotech shareholders and consultants.

ACKNOWLEDGMENTS

Funding for T.U.J.B. was provided by the Clarendon Scholarship and St. Edmund Hall, Oxford. Funding for A.C.A., S.J.D., and M.H. was from the Medical Research Council (MR/P001351/1). S.J.D is also a Jenner Investigator, a Lister Institute Research Prize Fellow, and a Wellcome Trust Senior Fellow (106917/Z/15/Z). We gratefully acknowledge Errin Johnson (Bioimaging Facility, Sir William Dunn School of Pathology, University of Oxford) for assistance with TEM, David Pattinson for assistance with malaria immunology (Jenner Institute, University of Oxford), and Matteo Ferla (Department of Biochemistry, University of Oxford) for assistance with bioinformatics.

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