SpySwitch enables pH- or heat-responsive capture and release for plug-and-display nanoassembly

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Proteins can be empowered via SpyTag for anchoring and nanoassembly, through covalent bonding to SpyCatcher partners. Here we generate a switchable version of SpyCatcher, allowing gentle purification of SpyTagged proteins. We introduce numerous histidines adjacent to SpyTag’s binding site, giving moderate pH-dependent release. After phage-based selection, our final SpySwitch allows purification of SpyTag- and SpyTag003-fusions from bacterial or mammalian culture by capture at neutral pH and release at pH 5, with purity far beyond His-tag methods. SpySwitch is also thermosensitive, capturing at 4 °C and releasing at 37 °C. With flexible choice of eluent, SpySwitch-purified proteins can directly assemble onto multimeric scaffolds. 60-mer multimerization enhances immunogenicity and we use SpySwitch to purify receptor-binding domains from SARS-CoV-2 and 11 other sarbecoviruses. For these receptor-binding domains we determine thermal resilience (for mosaic vaccine development) and cross-recognition by antibodies. Antibody EY6A reacts across all tested sarbecoviruses, towards potential application against new coronavirus pandemic threats.
Results

Rational engineering of a pH-switchable SpyTag-binder. We aimed to establish a pH-switchable SpyCatcher003-based purification system that allows capture of SpyTagged proteins at neutral pH, before eluting under weakly acidic conditions (Fig. 1a). Therefore, we took a rational pH engineering approach through the introduction of histidine residues at the binding interface of SpyTag/SpyCatcher (Fig. 1b). SpySwitch is based on SpyCatcher003 with S49C mutation for site-specific anchoring to the resin and E77A to prevent isopeptide bond formation.

Based on the crystal structure, we identified 16 residues on SpyCatcher adjacent to the SpyTag binding site. We individually mutated all 16 of these residues to histidine (Fig. 1b). These single histidine mutants of SpyCatcher003 S49C E77A were coupled to resin and assayed for binding to SpyTag003-superfolder green fluorescent protein (sfGFP) at pH 8.0, with sequential elution at pH 6.0, pH 5.0 and pH 4.0. Our original SpyDock11, which shows little pH-sensitivity, was used for comparison. Elution fractions were neutralized, and the fluorescence of sfGFP was measured. S30H, Y84H, G83H, E43H or E85H showed the most promising effect on pH elutability (Fig. 1c) and were chosen to make ten double histidine mutants. D33H gave the best elution at pH 5.0, but more than twice the amount of SpyTag003-sfGFP was lost in the flow-through (Fig. 1c), so we did not pursue this variant. Double mutants S30H + E85H, S30H + G83H or S30H + Y84H showed the best promise, with increased elutability at pH 5.0, but without the compromised binding at pH 8.0 seen for S30H + E43H (Fig. 1d). These encouraging double mutants were combined into three triple mutants and S30H + Y84H + E85H showed best pH elutability at pH 5.0 and 6.0 (Fig. 1e). Therefore, the three mutations S30H, Y84H and E85H were added into SpyCatcher003 S49C E77A, to generate a variant which we termed SpyDock2.0.

Enhancing pH-responsiveness by phage display selection. Individually mutating and combining residues has the limitation that cooperativity between different residues is difficult to assess, without expressing and testing an extensive library of variants. To optimize a SpySwitch that shows strong binding at pH 8.0 but high elutability at pH 5.0 and 6.0, we created a phage display library of SpyDock2.0 fused to the pIII C-terminal domain. We included C49S back-mutation to avoid disulfide-mediated dimer formation on phage (Fig. 2a). We optimized display of SpyDock2.0 on phage after inducing with different concentrations of arabinose. Display was validated by Western blot using an antibody against the HA tag on the N-terminus of SpyDock2.0 C49S, as well as with polyclonal anti-SpyCatcher serum (Fig. 2b).

Having optimized display, we performed error-prone PCR using Mutazyme II to generate phage library 1 from SpyDock2.0 C49S, with induction in the presence of 0.2% arabinose. We carried out four rounds of selection based on library 1, gradually using more challenging conditions, including changing the elution pH from 5.0 to 6.0. We also introduced free SpyTag003 as a competitor before elution, to select for variants with a low off-rate for the SpyTag003-fused bait. From this screen, we saw repeatedly the three mutations R32H, S59T and A111P (Fig. 2c). We then constructed a second phage display library based on SpyDock2.0 R32H S59T A111P with C49S back-mutation. Library 2 used either the original codons or a template with different codons (104 out of 113 codons changed), to allow for a greater number of amino acids to be accessed through mutation of a single base in the error-prone PCR. Through the rounds of selection, we continued to increase the stringency, including moving the elution buffer from pH 6.5 to 7.0. We also mixed the bait with Escherichia coli (E. coli) lysate, to favor SpyDock variants with low non-specific binding to the various competing protein targets. Several mutations from the second library were screened, but in the end we only included V94I for increased binding stability. Of the mutations added from phage display selections, R32H and A111P...
Fig. 1 Rational engineering of SpySwitch. a Overview of SpySwitch concept. SpyTag fused to a protein of interest interacts with SpySwitch at neutral pH and is eluted at weakly acidic pH through charge-charge repulsion (represented by red stars). b Residues at the interface of the SpyTag:SpyCatcher interaction chosen for histidine mutagenesis are marked in orange. SpyCatcher is in dark blue, based on the CnaB2 domain of PDB ID: 2X5P. SpyTag is in cyan, based on PDB ID: 4MLI. Reactive residues K31 and D117 that form an isopeptide bond in SpyTag:SpyCatcher are colored gray. H112 and K120 side-chains on SpyTag (K123 is not resolved in PDB ID: 4MLI) are marked as potential sites of electrostatic repulsion. Certain residues that were selected for mutation in the SpyCatcher003 parent are different to the SpyCatcher grandparent. c Single histidine variants of non-reactive SpyCatcher003 were assayed for SpyTag003-sfGFP binding and stepwise pH elution from pH 6.0 to pH 4.0. Each fraction was neutralized before fluorescence detection of SpyTag003-sfGFP. FT, flow-through; W, wash (pH 8.0). d Double histidine variants of non-reactive SpyCatcher003 were assayed as in (c). e Triple histidine mutants were assayed as in (c), compared to the best single and double mutant. Individual data points are shown as crosses. The connecting line goes through the mean, with the error bars ± 1 s.d. (n = 3). Fluorescence is given in arbitrary units. Source data are provided as a Source data file.
Mutations from phage selection

i. Phage-SpyDock2.0 library binds to biotinylated SpyTag003-MBP at pH 8.0

ii. Streptavidin-bead capture of biotinylated bait

iii. Phage is eluted at pH 5.0-7.0

iv. Phage amplification

3-4 cycles

Library 1 Mutations relative to SpyDock2.0

Round 4, clone 1: G12R R32H H84Y A111P
Round 4, clone 4: R32H S55C S59T
Round 4, clone 6: M17L T18A R32H S55R S59T
Round 4, clone 9: S23R R32H S59T H85N A111P
Round 4, clone 10: R32H T43A E96G A111P

Selected mutations: R32H S59T A111P

Library 2 Mutations relative to SpyDock2.0 R32H S59T A111P

Round 2, original codons: V94I T101A
Round 2, alternative codons: S50R V94I D97V
Round 3, original codons: V94I

Selected mutations: V94I

Mutations in SpySwitch compared to SpyCatcher003

Fig. 2 Library selection of SpySwitch. a SpySwitch selection strategy. Biotinylated AviTag-SpyTag003-MBP was incubated with a library of M13 phage displaying SpyDock2.0 variants, before pull-down and pH elution. b Validation of phage display. Reducing Western blot of phage, induced with increasing arabinose, displaying SpyDock2.0 C49S-pIII, bearing an N-terminal HA tag. The membrane was blotted with anti-HA tag (left gel) or anti-SpyCatcher (right gel). Molecular weight markers represent kDa. c Amino acid sequences of phage display libraries. Library 1 is based on SpyDock2.0 C49S. Library 2 is based on successful mutations from library 1. d Mutations in SpySwitch. His scanning mutations in orange and phage display mutations in magenta are marked on the structure with side-chains in stick format, based on PDB ID: 2X5P for SpyCatcher (in dark blue) and PDB ID: 4MLI for SpyTag (in cyan). H112 and K120 side-chains on SpyTag (K123 is not resolved in PDB ID: 4MLI) are marked as potential sites of electrostatic repulsion. Source data are provided as a Source data file.
are in direct proximity to SpyTag, while S59T and V94I are distant from the SpyTag binding site (Fig. 2d).

The effect of a histidine mutation is likely to depend on what residues surround it. Therefore, several mutations from the initial rational histidine mutagenesis screen (Fig. 1) were tested in the context of the best phage-derived sequence (SpyDock2.0 R32H S59T V94I A111P). K28H improved pH elutability, especially for SpyTag003, and was added into the final SpySwitch construct (Fig. 2d). The N-terminal region of SpyDock2.0 was further modified by removing the tobacco etch virus (TEV) protease cleavage site and preceding amino acids present in the SpyCatcher003 parent, leaving a His$_6$-tag and GSG$_2$ linker before the SpySwitch sequence (final sequence in Supplementary Fig. 1).

**Purification of model proteins by pH switch.** Having decided on a final SpySwitch construct, we carefully characterized its performance in purification. We initially tested pH-dependent purification from *E. coli* lysate. Affinity purification of highly expressed proteins is much easier than for low abundance proteins. Therefore, we set up a challenging situation by doping 0.09 mg of N-terminally tagged SpyTag-sfGFP or SpyTag003-C-terminal SpyTag003 on the heavy chain, as well as a His$_6$-tag. antibody fragment in the human embryonic kidney-based eukaryotic cells, we transiently expressed an anti-HER2 Fab cell lysate from 250 mg of 0.09 mg of N-terminally tagged SpyTag-sfGFP or SpyTag003-sfGFP into cell lysate from 250 mg of *E. coli* cell weight. This approach also allowed direct comparison between the two tags, since SpyTag-sfGFP and SpyTag003-sfGFP might not show identical levels of expression. Both proteins also contained a His$_6$-tag at the C-terminus to allow comparison with the most widely used purification method of nickel-nitrilotriacetic acid (Ni-NTA). For SpySwitch, we eluted with pH 5.0 buffer at 4°C. For Ni-NTA, we followed a standard procedure of eluting with 200 mM imidazole. We saw that both SpyTag-sfGFP and SpyTag003-sfGFP might not show identical levels of expression. Both proteins also contained a His$_6$-tag at the C-terminus to allow comparison with the most widely used purification method of nickel-nitrilotriacetic acid (Ni-NTA). For SpySwitch, we eluted with pH 5.0 buffer at 4°C. For Ni-NTA, we followed a standard procedure of eluting with 200 mM imidazole. We saw that both SpyTag-sfGFP and SpyTag003-sfGFP might not show identical levels of expression. Both proteins also contained a His$_6$-tag at the C-terminus to allow comparison with the most widely used purification method of nickel-nitrilotriacetic acid (Ni-NTA). For SpySwitch, we eluted with pH 5.0 buffer at 4°C. For Ni-NTA, we followed a standard procedure of eluting with 200 mM imidazole. We saw that both SpyTag-sfGFP and SpyTag003-sfGFP might not show identical levels of expression. Both proteins also contained a His$_6$-tag at the C-terminus to allow comparison with the most widely used purification method of nickel-nitrilotriacetic acid (Ni-NTA). For SpySwitch, we eluted with pH 5.0 buffer at 4°C. For Ni-NTA, we followed a standard procedure of eluting with 200 mM imidazole.

We stored SpySwitch in Tris-phosphate (TP) buffer with 20% (v/v) ethanol at 4°C, to stop microbial growth. SpySwitch maintained good performance over time, based on purification of SpyTag-sfGFP following SpySwitch storage for 7 months (Supplementary Fig. 2b).

To test SpySwitch purification of proteins expressed in eukaryotic cells, we transiently expressed an anti-HER2 Fab antibody fragment from the human embryonic kidney-based Exp293F cell line (Fig. 3e, f). The construct was tagged with a C-terminal SpyTag003 on the heavy chain, as well as a His$_6$-tag. SpySwitch purification from the cell supernatant was efficient, with higher purity observed for SpySwitch (95%) than either SpyDock (89%, using 2.5 M imidazole elution, Supplementary Fig. 3a) or Ni-NTA (88%, using 200 mM imidazole elution, Supplementary Fig. 3b). These different purification methods also gave a product just below 28 kDa, which is likely to be Fab that has not formed a disulfide, based on reducing SDS-PAGE (Fig. 3e, f). SpySwitch purification of anti-HER2 Fab with a C-terminal SpyTag was also effective (Supplementary Fig. 3c). We have not focused on SpyTag002 (Supplementary Fig. 1) 27, because of the superior reactivity of SpyTag003 12, but pH elution from SpySwitch was also successful in purifying anti-HER2 Fab (Fig. 3d).

Overall we have shown that both N- and C-terminally SpyTagged proteins could efficiently be purified by SpySwitch using mild pH elution from bacterial or mammalian expression systems at high purity.

**Biophysical characterization of SpySwitch and its interactions.** To get insight into this successful purification behavior, we performed biophysical validation of our SpySwitch construct. We confirmed the molecular identity of SpySwitch by electrospray ionization mass spectrometry (ESI-MS) (Supplementary Fig. 4a). We then used isoform titration calorimetry (ITC) to find out the binding affinity of SpySwitch. We tested the interaction between monomeric SpySwitch and SpyTag-MBP as well as SpyTag003-MBP, at pH 7.5 and 5°C (Supplementary Fig. 4b, c). Both SpyTag-MBP and SpyTag003-MBP bound with 1:1 stoichiometry. SpyTag-MBP (K$_D$ = 1.1 ± 0.1 μM) (Supplementary Fig. 4b) bound SpySwitch slightly tighter than SpyTag003-MBP (K$_D$ = 1.5 ± 0.2 μM) (Supplementary Fig. 4c). No binding between SpySwitch and either SpyTag-MBP or SpyTag003-MBP was observed by ITC at pH 5.0 and 5°C (Supplementary Fig. 5), suggesting that any interaction present had negligible ΔH or was weaker than the detection limit of ITC.

**SpySwitch is also temperature-responsive.** It would be beneficial to have an orthogonal method for purification of certain SpyTagged proteins. Important viral antigens can be activated to switch conformation by endosomal pH28,29. Therefore, for these proteins pH-dependent elution would not be suitable. We wondered if SpySwitch might allow temperature-dependent elution. Differential scanning calorimetry (DSC) was run on SpySwitch at different pH values (Fig. 4a). At pH 8.0, SpySwitch had a melting temperature of 36.9°C, suggesting that SpySwitch might release its interaction with SpyTag- or SpyTag003-fusions upon heating up to 37°C. We also determined how the melting temperature related to pH. Strikingly, SpySwitch’s melting temperature increased by 5–9 °C per pH unit as the pH decreased from 8.0 to 5.0, with the highest melting temperature at pH 5.0 (Tm = 57.7°C) (Fig. 4a). To see if this temperature-dependence was an intrinsic feature of the scaffold, we also tested the unfolding of the parental SpyDock. SpyDock’s melting temperature was well above 37°C at all pH values and showed less pH-sensitivity than SpySwitch (Fig. 4b). The ΔH and width for each peak is provided in Supplementary Table 1.

We then tested whether we could utilize the temperature-dependence in SpySwitch for purification (Fig. 4c). We dropped low levels of SpyTag003-sfGFP into *E. coli* lysate and bound the protein to SpySwitch resin, as previously, in pH 7.5 buffer at 4°C. We eluted with buffer at 37°C. This elution buffer had a pH of 8.0, to take advantage of the lower Tm of SpySwitch at pH 8.0 than 7.0 (Fig. 4a). The SpyTag003 fusion was purified from the lysate at 98% purity (Fig. 4d). The SpyTag and SpyTag002 fusions were similarly purified by temperature elution from *E. coli* lysate with 98% purity (Supplementary Fig. 6a, c). Temperature elution from SpyDock using the same conditions yielded only a small amount of elution, consistent with the melting temperature of SpyDock (Supplementary Fig. 6b, d). We compared SpySwitch versus SpyDock2.0 or SpyDock for temperature elution of SpyTag-sfGFP and SpyTag003-sfGFP (Fig. 4e). For SpyTag003-sfGFP, temperature elution worked well for SpyDock2.0. However, binding of SpyTag-sfGFP to SpyDock2.0 was severely compromised (Fig. 4e). Very little temperature elution of SpyTag-sfGFP and SpyTag003-sfGFP was seen from SpyDock (Fig. 4e).

To test purification of a pH-sensitive vaccine antigen by SpySwitch, we expressed tagged trimeric influenza hemagglutinin (HA) in mammalian cells and purified from the supernatant, utilizing temperature-switch elution at 37°C. HA-SpyTag was isolated at 99% purity by temperature elution (Fig. 4f), while...
HA-SpyTag003 was isolated to 96% purity (Supplementary Fig. 6e). SpySwitch resin recovery was 17.7 mg protein per mL of packed resin when eluted from SpySwitch using pH 5.0 elution. Recovery increased to 20.4 mg when using 37 °C elution (Supplementary Fig. 7). We also showed that the resin could be successfully regenerated. Initially we used SpySwitch resin to purify SpyTag003-sfGFP. Then we regenerated SpySwitch resin with washes of glycine pH 2.0, then 8.0 M urea, before a final wash in 0.1 M sodium hydroxide. We applied the regenerated resin for purification of HA-SpyTag with temperature elution. HA-SpyTag was successfully purified and we were unable to detect any residual SpyTag003-sfGFP, based on Coomassie stain or Western blotting against sfGFP (Supplementary Fig. 8a, b). To assess how the resin withstands multiple rounds of regeneration, we performed purification of SpyTag-sfGFP, regenerated the resin five times, and re-performed purification of SpyTag-sfGFP. No
impact on purity of SpyTag-sfGFP was observed, while maximum recovery decreased by 28% from 15.8 mg per mL resin for fresh resin (after 7 months storage in 20% ethanol) to 11.4 mg per mL resin after five regeneration cycles (Supplementary Fig. 8 c, d).

SpySwitch to purify antigens for broad coronavirus protection. Given the application of SpyCatcher-bearing virus-like particles to facilitate vaccine assembly, we applied SpySwitch for purification of vaccine antigens of interest. We recently showed that a mosaic vaccine co-displaying antigens from different sarbecoviruses elicits antibodies that cross-react with the targets across the various viruses. Sarbecoviruses represent the coronavirus sub-genus containing the human pathogens SARS and SARSCoV-2.

Fig. 4 Temperature-dependence in SpySwitch. a DSC of SpySwitch at pH 5.0, 6.0, 7.0 or 8.0 in succinate-phosphate-glycine (SPG) buffer. Melting temperature (T_m) is given at each pH. b DSC for SpyDock as in (a). c Overview of temperature-based elution. SpyTag interacts with SpySwitch at 4 °C and is eluted at 37 °C. d Reducing SDS-PAGE performed for SpySwitch temperature elution of SpyTag003-sfGFP from bacterial lysate, with capture at 4 °C and elution at 37 °C (pH 8.0). L, doped lysate; FT, flow-through; T, total pooled elution fractions; Resin, protein left on resin following elution. On the right of the gel is the trace from densitometry of lane T (gray, background; orange, bands) plotted by intensity, with percent purity shown. e SpySwitch and SpyDock2.0 but not SpyDock allow temperature elution. SpyTag-sfGFP or SpyTag003-sfGFP was captured by SpyDock, SpyDock2.0 or SpySwitch and eluted by 37 °C incubation at pH 8.0. Fluorescence was determined from flow-through (FT), wash (W), or 37 °C elution (E) fractions (n = 1). Fluorescence in arbitrary units was multiplied by the volume of the fraction, to give arbitrary units*mL. f SpySwitch temperature elution of HA bearing a C-terminal SpyTag from mammalian Expi293F expression, assessed by reducing SDS-PAGE/Coomassie. S, supernatant; FT, flow-through; T, total pooled elution fractions; Resin, protein left on resin following elution. Molecular weight markers represent kDa. Source data are provided as a Source data file.
constructs C-terminally tagged with SpyTag003. We demonstrated efficient purification of each RBD using pH-dependent elution with SpySwitch (Fig. 5b). The band in each case is broad because of heterogeneous N-linked glycosylation.

Thermal stability of antigens is an important characteristic to facilitate robust production and also for future consideration of vaccine cold-chain dependence. We used DSC to validate the integrity of each purified RBD and gain understanding into the different thermal stabilities (Fig. 5c). DSC showed a single homogeneous peak for each RBD. RaTG13 had a similar Tm to SARS-CoV-2 RBD but all the other sarbecovirus RBDs showed substantially higher Tm (Fig. 5c).

A purification method would ideally elute the protein of interest in a buffer where it can be easily applied for downstream applications. Buffer-exchange is time-consuming and leads to sample losses. For fusions to SpyTag or SpyTag003, a major application will be reactions with SpyCatcher variants for nanoassembly. For the RBD antigen from the bat coronavirus Yun11, we performed SpySwitch pH-based elution, neutralized, coupled for multimerization to 5-mer or 60-mer.
and then mixed with 5-mer (SpyCatcher002-oPent) or 60-mer (SpyCatcher003-mi3) scaffolds. We saw almost complete depletion of the 5-mer and the 60-mer in the presence of a molar excess of this Yun11 RBD-SpyTag003 sample, consistent with efficient coupling (Fig. 5d).

It is an important question which antibodies are able to cross-react in their recognition of the different sites of RBD, for vaccine efficacy as well as for therapeutic use against new strains of SARS-CoV-2 or potential new zoonotic viruses. We tested the RBD recognition by antibodies of class 1 to 4 on SARS-CoV-2 RBD (Fig. 5e) by enzyme-linked immunosorbent assay (ELISA) and Western blot. Recognition is presented as a heat map in Fig. 5f, with mean and error bars in Supplementary Table 3. The antibody CR3022, identified from a SARS patient, had been previously recognized as having broad sarbecovirus reactivity. We found that CR3022 indeed recognized a wide set of RBDs, but not RBDs from BM48-31 or FIV viruses (Fig. 5f).

In contrast, we found that the antibody EY6A recognized the complete set of sarbecovirus RBDs that we tested (Fig. 5f). EY6A was isolated from a COVID-19 patient. The contact surface of EY6A, binding at the class 4 site on SARS-CoV-2 RBD, has been previously determined and is shown in Supplementary Fig. 9a, b, along with a sequence alignment of how much this site is conserved through the set of other sarbecovirus RBDs (Supplementary Fig. 9c). The other tested antibodies (FP-12A, F1-3A, FP-8A and FD-5D) derived from COVID-19 patients only bound strongly to SARS-CoV-2 (Fig. 5f). LCA60 is a control antibody to the Middle East respiratory syndrome-related coronavirus (MERS) spike glycoprotein and, as expected, showed no recognition of sarbecovirus RBDs (Fig. 5f).

Display of SARs-CoV-2 RBD on the SpyCatcher003-mi3 virus-like particle (VLP) both enhances immunogenicity and enhances the ability of RBD to be detected in vitro by other proteins (ACE2 or antibodies). We used mi3 for nanoscaffold of the RBD panel analyzed in Fig. 5f. We confirmed that each SpySwitch-purified RBD had reacted efficiently with SpyCatcher003-mi3 (Supplementary Fig. 10a). Considering the potential of these proteins for vaccine assembly, we explored the resilience of their folding to freeze-thaw after VLP reaction. Each RBD on mi3 showed no substantial change in its recognition by the pan-reactive conformation-sensitive monoclonal antibody EY6A, even after 5 cycles of freeze-thaw (Supplementary Fig. 10b).

SpySwitch allowed efficient purification of a panel of sarbecovirus RBDs. This panel revealed the high stability of all sarbecovirus RBDs to unfolding. Additionally, we identified a class 4 antibody with recognition across a wide panel of sarbecoviruses.

**Discussion**

We have created a pH- and temperature-responsive protein for efficient purification of proteins fused to SpyTag or SpyTag003, through rational modification and phage library selection. SpySwitch efficiently purifies proteins from bacterial cell lysates as well as from mammalian cell culture supernatant, using either weak acidic elution at pH 5.0 at 4 °C or temperature elution with neutral pH at 37 °C. These mild elution conditions help maintain the folding of the protein of interest, but are also conducive to the good purity we see (>95% from different targets), with harsher conditions more likely to release proteins non-specifically adsorbed to the resin.

SpySwitch is 10 substitutions away from SpyDock. 5 of these mutations are substitution with a histidine (sites 28, 30, 32, 84 and 85), with their close proximity to each other promoting pH-dependence. Therefore, histidine constitutes a large fraction of the surface-exposed residues surrounding the SpyTag binding site (Fig. 2d), which is likely to lead to substantial alteration of the SpySwitch structure as the pH is decreased. Mutations at positions 91 and 103 arose previously during the generation of SpyCatcher003. A111P was identified here from phage display, but was previously found during the selection of SpyTag002. The other two mutations are distant from SpyTag (residue 39) or are buried (residue 94), which would have been hard to predict, so it was important to have performed phage library selection. We wanted to engineer a SpySwitch system that works well for both SpyTag and SpyTag003, enabling application of a single resin. However, this posed a major challenge because SpyTag003 was engineered to have much stronger binding to its Catcher than SpyTag. Initial SpyDock iterations showed excellent capture of SpyTag003-fusions and mediocre capture of SpyTag-fusions, followed by easy elution of SpyTag-fusions and only partial elution of SpyTag003-fusions. However, after testing the large series of histidine-scanning mutants and multiple mutants arising from phage selection for their performance on both SpyTag and SpyTag003, we gradually identified a successful generally-applicable construct. SpySwitch gave solid purification for both SpyTag and SpyTag003, showing similar ~1 µM affinity for each tag. In contrast, SpyCatcher003 interacts with the non-reactive SpyTag003 D117A fused to MBP with ~10 nM Kd, which suggests that, by optimizing SpyTag003 elutability at acidic pH, we also lowered SpyTag003’s binding affinity for SpySwitch at neutral pH. SpySwitch’s affinity is comparable to common purification resins (e.g. His/Ni-NTA, Strep-tag II/Strep-Tactin), meaning that it is unlikely that all the SpyTagged molecules in solution will be captured by SpySwitch, but quantitative capture is usually not critical for purification of recombinant proteins.

SpyTag002 has threonine at position 112 instead of the histidine of SpyTag and SpyTag003, which might reduce an opportunity for low pH-induced charge-charge repulsion. However, we have seen that pH elution is still successful for SpyTag002. For the sharpest pH-dependent elution profile, we might have introduced further histidines into the Tag, but we avoided that temptation, which would likely compromise the speed of reaction with SpyCatcher003 and would not help the large range of proteins already cloned with SpyTag or SpyTag003. Proteins for secretion in eukaryotes transit through the trans-Golgi network at pH 6.0 as well as secretory vesicles at pH 5.5. Therefore, we anticipate that a large fraction of proteins would tolerate the pH-dependent elution conditions here, but this tolerance will require future testing. Gradual elution does occur from SpySwitch at pH 6.0 (Supplementary Fig. 2a), so this option may be preferred in some cases. Compared to elution with competing ligand, pH elution may allow simple neutralization of the buffer before downstream use, rather than needing any buffer-exchange.

Viral fusion proteins, including influenza hemagglutinin, have evolved to be pH-responsive, so it was important to have another option for elution from SpySwitch. Temperature-sensitive proteins have been a mainstay of genetic screens and there is precedent for rational introduction of temperature-sensitivity by introducing cavities in the protein hydrophobic core, through mutating large buried hydrophobic residues to polar ones. For antibody purification, a temperature-sensitive protein A, eluting at 40 °C, has also been developed to avoid the standard pH 3 step in antibody purification, which can promote deamidation and aggregation. Most proteins from mesophilic species are likely to be unaffected by brief exposure to 37 °C, so temperature-based elution should be a widely applicable purification route. Temperature elution has the further important advantage that there is no need to remove the elution buffer, as would often be required for other affinity purification methods, e.g. 200 mM imidazole for Ni-NTA or 10 mM reduced glutathione for glutathione-S-
translase (GST). For vaccine nanosassembly, we previously showed that plug-and-display decoration of virus-like particles is generally applicable for different antigen symmetries. Here, we purified monomers (sfGFP, RBDs), a trimer (hemagglutinin) and a tetramer (β-galactosidase) using SpySwitch (Supplementary Fig. 11), so targets bearing multiple tags can still be efficiently eluted. We purified proteins with predicted isoelectric points (pI) ranging from 5.5 to 9.2, suggesting that SpySwitch is broadly applicable (Supplementary Fig. 11).

SpySwitch has the disadvantage that the resin capacity of 20 mg per ml is lower than Ni-NTA and a protein-based capture agent is likely to remain more expensive than an inorganic matrix. However, we found higher purity than Ni-NTA in our model systems, as well as greater flexibility in elution route. Ni-NTA purification also faces challenges such as the His6-tag promoting dimerization and disruption of various metalloproteins. C-tag purification on a nanobody resin has proven a valuable tool for purification of vaccine antigens, with the advantage over SpyTag003 of only a 4 amino acid tag. However, C-tag can only be used in the C-terminus, whereas SpyTag003 is tolerated at either terminus or in loops, while C-tag elution with 2 M MeSO4 or 50% propylene glycol can disrupt protein conformation. The CL7-tag has excellent binding affinity but uses pH 3 for elution and is >100 residues.

The THETA system uses a temperature-sensitive single-chain Fv fragment for purification of a peptide tag, although includes 50% glycerol in the elution buffer. Split intein-based purification allows removal of tags, but the use of reducing agent to accelerate cleavage and free cysteines in the tag will be a problem for many complex antigens. Apart from the cysteine for resin anchoring, there are no other cysteines in SpySwitch or any SpyTag version, giving applicability in oxidizing or reducing conditions.

SpySwitch shows tolerance of harsh conditions, including urea and sodium hydroxide, facilitating its regeneration. SpyCatcher and SpyCatcher003 are efficient at refolding, and we found that SpySwitch could be efficiently regenerated multiple times following a purification run, which will contribute substantially to the cost-effectiveness of SpySwitch resin. SpySwitch also tolerates storage in 20% (v/v) ethanol, facilitating protection from microbrial growth and its use over at least 7 months at 4 °C. In future work, applications of SpySwitch beyond protein purification may be explored. SpySwitch immobilized to resin or magnetic beads may be employed for co-immunoprecitation, and Gibson assembly was used to perform cloning and site-directed mutagenesis, unless otherwise indicated. All open-reading frames were validated by Sanger sequencing (Source Bioscience). Residue numbers for SpyTag and SpyCatcher variants follow PDB ID: 2X58, pDEST14-SpyCatcher2.1. S49C E77A (SpyDock, Supplementary Fig. 1) (GenBank MK376462, Addgene plasmid ID 124618) was a kind gift from Juha Huiskonen, University of Helsinki, and contains His6-tag, thrombin cleavage site, SpyDock, GGSGE linker, and E. coli β-galactosidase amino acids 1-1024.

Methods

Plasmids and cloning. Standard PCR methods with Q5 High-Fidelity 2x Master Mix (New England Biolabs) and Gibson assembly were used to perform cloning and site-directed mutagenesis, unless otherwise indicated. All open-reading frames were validated by Sanger sequencing (Source Bioscience). Residue numbers for SpyTag and SpyCatcher variants follow PDB ID: 2X58, pDEST14-SpyCatcher2.1. S49C E77A (SpyDock, Supplementary Fig. 1) (GenBank MK376462, Addgene plasmid ID 124618) was a kind gift from Juha Huiskonen, University of Helsinki, and contains His6-tag, thrombin cleavage site, SpyDock, GGSGE linker, and E. coli β-galactosidase amino acids 1-1024.

pOOP-anti-HER2 4D5 Fab.00.11 light chain (GenBank ON131084) has previously been described52. pOOP-anti-HER2-SpyTag 4D5 Fab.00.11 heavy chain (GenBank ON131085) contains SpyTag at the C-terminal end, followed by a His-tag. The heavy chains of pOOP-anti-HER2-SpyTag 4D5 Fab.00.11 (GenBank ON131075) and pOOP-anti-HER2-SpyTag003 4D5 Fab.00.11 (GenBank ON131076) were cloned using restriction enzymes KpnI-HF and DraIII-HF, with ligation performed using T4 DNA Ligase (all New England Biolabs). pcDNA3.1-H3Vic-SpyTag-SpyHis (GenBank ON131077), encoding the H3 Victoria strain of influenza HA, was derived from pcDNA3.1-H3Vic-SpyTag003-His3 (GenBank MT945422)52. Sarbecovirus RBD constructs p3BNC-RBD-His8-SpyTag003 have previously been described by Dr. Alexander Cohen and Prof. Pamela Bjorkman (Caltech)52 (SARS-CoV-2 [GenBank ON131086], SARS-CoV [GenBank ON131087], ReT1G3-CoV [GenBank ON131088], SCoV-CoV [GenBank ON131089], Rs4081-CoV [GenBank ON131090], pangolin17 (pang17)-CoV [GenBank ON131091], RsynY0-CoV [GenBank ON131092], Rf-1-CoV [GenBank ON131093], Rf-1-CoV [GenBank ON131094], Yunnan2011 (Yunn11)-CoV [GenBank ON131095], BM48-31-CoV [GenBank ON131096], and BK7y2-CoV [GenBank ON131097]).

pBAD-Dsb(a)-HA tag-spicyTag002.40 CoV 7 (GenBank ON131078) for phage display was derived from pBAD-Dsb(a)SBTI-plII34 with an N-terminally truncated pII region. Site-directed mutagenesis was performed on the pBAD construct by Gibson assembly to introduce further selected variants. An alternative codon template for SpyDock2.0 R32H C495 S97T A111P (GenBank ON131079) was ordered as a gBlock (Integrated DNA Technologies) and cloned into the phagemid vector. pGEK-2T-GST-BirA was a gift provided by Dr. Chris O’Callaghan (University of Oxford). Expression plasmids for EY6A50, FP-12A, Fl-3A (GenBank MT943493 for V14 and MT943494 for V1454, FP-8A and FD-5D (GenBank MT943497 for V15 and MT943498 for V1545) based on plasmids from either infected or vaccinated donors were a kind gift from Prof. Kuan-Ying Arthur Huang (Chang Gung University). The reverse-transcribed variable heavy (VH) and variable light (VL) regions were present in plasmids bearing the rest of the human IgG1 heavy chain (AbVc-hlgG1)51, human kappa light chain (AbVc1.1-IgKc, Addgene 80796), or human lambda light chain (AbVc1.1-IgLC2, Addgene 99575). The VH1 and VL1 segments of LCA06b and CR3162 (GenBank DQ168596 for VH1 and DQ168570 for VL1) were synthesized as cDNA (GeneArt or IDT) and cloned into the heavy and light chain expression plasmids described above.

Rational selection of histidine mutants. The crystal structure of SpyTag-SpyCatcher (PDB ID: 4MLI)54 was visually inspected for potential histidine mutagenesis sites. Interface residues contributing to the binding between SpyTag and SpyCatcher (PDB ID: 4MLI)54 was visually inspected for potential histidine mutagenesis sites. Interface residues contributing to the binding between SpyTag and SpyCatcher (PDB ID: 4MLI) was visually inspected for potential histidine mutagenesis sites. Interface residues contributing to the binding between SpyTag and SpyCatcher (PDB ID: 4MLI) was visually inspected for potential histidine mutagenesis sites. Interface residues contributing to the binding between SpyTag and SpyCatcher (PDB ID: 4MLI) was visually inspected for potential histidine mutagenesis sites. Interface residues contributing to the binding between SpyTag and SpyCatcher (PDB ID: 4MLI) was visually inspected for potential histidine mutagenesis sites.
 SpyCatcher were determined using the InterfaceResidues script in PyMOL 2.0 (Jason Vertrees, https://pymolwiki.org/index.php/InterfaceResidues). Two binding interfaces were identified in SpyCatcher and residues therein were computationally substituted to positively charged histidine residues. These sites were inspected for potential electrostatic charge-charge repulsion with adjacent partially or fully positively charged residues on SpyTag and SpyCatcher within a ~5 Å radius. To target the positively charged N-terminus of SpyTag003, for which no crystal structure is currently available, the N-terminus of SpyTag in PDB ID MLI was computationally extended in PyMOL using the Builder. We added the residues RGV (based on SpyTag003) to the terminal A111 of SpyTag whilst maintaining backbone β-sheet hydrogen bonding to ~3 Å SpyTag residues within a ~5 Å radius (using the amino acid R108 side-chain in SpyDock2.0). These sites were computationally inspected for interfaces that were identiﬁed in SpyTag003 by ﬂuorescence assay. The best single histidine mutants were combined to double histidine mutants, which were in turn combined to triple histidine mutants.

**SDS-PAGE and quantiﬁcation.** SDS-PAGE was performed using an XCell SureLock system (Thermo Fisher) with 10%, 12% or 14% polyacrylamide gels. Samples were mixed with 6x SD loading buffer (234 mM Tris-HCl pH 6.8, 24% (v/v) glycerol, 120 μM bromophenol blue, 234 mM SDS, supplemented with 60 mM dithiothreitol (DTT) for reduced samples). Samples were heated at 95 °C for 5 min in a Bio-Rad C1000 thermal cycler. Gels were run in 24 mM Tris base, 192 mM glycine, 3.5 mM SDS at 200 V and stained with Coomassie Brilliant Blue G-250. After destaining in MilliQ water, gels were imaged using a ChemDoc XRS + imager with ImageLab version 5.2.1, and analyzed with ImageLab version 3.0 (both Bio-Rad). For the histidine mutant screen performed in Fig.1c, the histidine detection was performed. Additional bands were added manually where the automatic detection had failed to detect faint bands. 1 mm background subtraction was applied. The percentage purity is quantiﬁed as (band intensity of protein of interest)/(sum of the band intensities from all bands in lane)×100. The lane proﬁle was plotted by intensity.

**Bacterial protein expression.** pET28a-AviTag-SpyTag003-MBP, pET28a-SpyTag-sGFP and pET24a-SpyTag003-sGFP were transformed into chemically competent E. coli BL21 (DE3) RILP. (Agilent Technologies). For pET28a-SpyTag-MBP, pET28a-SpyTag003-MBP and pET28a-SpyTag003-sGFP, the expression was transformed into chemically competent E. coli BL21 (DE3) (Lucigen), pDEST14-SpySwitch (and variants thereof), pDEST14-SpyDock2.0, pDEST14-SpyDock and pDEST14-SpyTag003-0pEnt were transformed into chemically competent E. coli C41 (DE3), a gift from Anthony Watts (University of Oxford). Colonies were picked and grown in LB supplemented with 50 μg/mL kanamycin (pET28a) or 100 μg/mL ampicillin (pET28a) at 37 °C for 200 rpm for 4-16 h. 1 L LB containing 0.8% (v/v) glucose and appropriate antibiotic was inoculated in 1/10 dilution with starter culture and incubated at 37 °C and 200 rpm in ultra-yield baffled flasks (Thermo Fisher). At A600 0.5, the cultures were induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Fluorochem) and cultured for 4 h at 30 °C and 200 rpm. For pDEST14-SpyTag003-0pEnt, cultures were incubated for 16 h with shaking at 200 rpm at 22 °C after induction. For pET28a-SpyTag003-β-galactosidase, cultures were induced with 0.84 mM IPTG and incubated at 37 °C and 200 rpm for 24 h. After centrifugation and either directly processed or washed with PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4) and cell pellets were stored at −80 °C until purification.

SpyTag003-Mbp was expressed in E. coli BL21 (DE3) RILP cells (Agilent) and purified by precipitation with 170 mg ammonium sulfate per mL of cell lysate. The resuspended protein was further puriﬁed by size-exclusion chromatography on a HiPrep Sephacyr S-400 10-600 column, which was equilibrated with 25 mM Tris-HCl pH 8.5 and 150 mM NaCl and eluted at an A280 of 0.85, using an ÄKTA Pure 25 system (GE Healthcare). Fractions were then concentrated using a VivaPure 20 100 kDa molecular weight cut-off spin concentrator.

**IgG1 expression and puriﬁcation.** ExpC-HO-S cells (Thermo Fisher, A29133) were cultured in an Erlenmeyer flask with vent cap (Corning) at 37 °C with 125 rpm shaking and were transferred with 1 μg/mL of each of the heavy and light chain expression plasmids using the ExpFectamine CHO Reagent (Thermo Fisher). Transfection mix was prepared in OptiPro Serum Free Media (Thermo Fisher) according to the manufacturer’s instructions with some modiﬁcations. Briefly, the SpySwitch variant was concentrated to at least 20 mg/mL using a Vivaspin 20 5 kDa MWCO centrifugal filter at 4 °C and reduced with 1 mM TCEP in coupling buffer (50 mM Tris-HCl + 5 mM EDTA, pH 8.5) for at least 30 min at 25 °C. Resin incubation was performed at 25 °C throughout, with incubations protected from light. 20 mg reduced SpySwitch variant was added per 1 mL pET28a-Stuffer Link Coupling Resin Buffer (Millipore Sigma) and 100 μL 1 M NaCl, then additionally TP buffer (25 mM orthophosphoric acid adjusted to pH 7.0 with Tris base) were performed to remove non-covalently-bound SpySwitch variant. Coupled resin was stored in 20% (v/v) ethanol in TP buffer.

**Protein binding by Ni-NTA.** Purifications were performed at 4 °C. Bacterial cell pellets were thawed and resuspended in Ni-NTA buffer (50 mM Tris-HCl pH 7.8 + 300 mM NaCl) supplemented with complete Mini EDTA-free Protease Inhibitor Cocktail (Roche) and 1 mM phenylmethylsulfonyl ﬂuoride (PMSF, Sigma-Aldrich). The culture was filtered and total cell lysate was harvested by centrifugation at 4000 × g for 4 °C for 5 min, then passed through a 0.45 μm syringe ﬁlter (Thermo Fisher). Supernatants were stored at 4 °C for short periods or frozen at −80 °C before puriﬁcation.

**Resin coupling.** SpySwitch variants were coupled to SulfoLink Coupling Resin (Thermo Fisher, 20402) according to the manufacturer’s instructions with some modiﬁcations. Briefly, the SpySwitch variant was concentrated to at least 20 mg/mL using a Vivaspin 20 5 kDa MWCO centrifugal filter at 4 °C and reduced with 1 mM TCEP in coupling buffer (50 mM Tris-HCl + 5 mM EDTA, pH 8.5) for at least 30 min at 25 °C. Resin coupling was performed at 25 °C throughout, with incubations protected from light. 20 mg reduced SpySwitch variant was added per 1 mL pET28a-Stuffer Link Coupling Resin Buffer (Millipore Sigma) and 100 μL 1 M NaCl, then additionally TP buffer (25 mM orthophosphoric acid adjusted to pH 7.0 with Tris base) were performed to remove non-covalently-bound SpySwitch variant. Coupled resin was stored in 20% (v/v) ethanol in TP buffer.

**pH analysis assay by ﬂuorescence.** To test binding and elution properties of SpyDock variants, 30 μL packed SpyCatcher003 S49C E77A histidine mutant resins were incubated with 50 μg SpyTag003-sGFP in a total volume of 300 μL. For the histidine mutant screen performed in Fig. 1c, 50 μg SpyTag003-sGFP, 1 mM 150 μM NaCl was used for binding and washing. Samples were incubated for 45 min at 4 °C with end-over-end
end rotation. The resin was collected by centrifugation at 1,000 g for 5 min at 4 °C, then transferred to wells of a pre-wetted AcroPrep Advance 5.0 (Agilent) by centrifugation at 500 g and 4 °C for 30 s. The resin was washed four times with 250 μL 50 mM HEPES pH 8.0 or 150 mM NaCl by centrifugation at 500 g and 4 °C for 30 s. For stepwise elution, resin was sequentially incubated in 2 × 50 μL 2×TY containing 20% (v/v) glycerol at 4 °C and 650 rpm shaking in the presence of 7.5 μM free SpyTag003 peptide. The phage were eluted by incubating in 200 μL 50 mM acetic acid/sodium acetate pH 4.0 or 150 mM NaCl for 10 min at 4 °C. Elution fractions were neutralized by addition of 1 M Tris-HCl pH 8.0 to the collector plates (23 μL for pH 6.0, 10 μL for pH 5.0, 20 μL for pH 4.0). 20 μL of each sample was transferred to black, flat-bottom half area 96-well plate (Göring) and the plate was incubated at 4 °C for 30 min. Fluorescence was then measured using a CLARIOstar plate reader with CLARIOstar software version 5.20 RS (both BMG Labtech). The resin was collected by centrifugation at 1,000 g for 5 min at 4 °C, then transferred to wells of a pre-wetted AcroPrep Advance 0.45 μm (Whatman) and data were analyzed using MxPro qPCR software version 4.10 (Agilent).

**Phage display.** AviTag biotinylation of AviTag-SpyTag003-MBP was performed with GST pulldown. 100 μL GST column was incubated with 25 μM BirA and 1.5 mM biotin in the presence of 5 mM MgCl2 and 1 mM ATP for 1 h at 30 °C and 300 rpm. The concentrations of BirA and ATP were doubled and incubation continued for 1 h at 30 °C and 300 rpm. Excess biotin was removed by dialysis into PBS pH 7.4 in three dialysis steps. Phage display with the first library was performed on the pBAD construct using the GeneMorph II Random Mutagenesis Kit (Agilent, containing Mutasyme II) for PhageCatcher003 S30H E77A Y84H E85H 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.5 or 150 mM NaCl with binding for 1 h at 4 °C, and wash steps carried out in 300 μL 50 mM Tris-HCl pH 8.0 or 300 μM NaCl containing 0.8% (w/v) glucose and 100 μg/mL chloramphenicol. Cultures were centrifuged at 4,000 g for 15 min at 4 °C and phage was precipitated by incubating in 50 mM HEPES pH 7.0 at 4 °C with 15% glycerol and neutralized with 2 M Tris-HCl pH 8.5. Phage were recovered in 1 mL recovery medium (Lucigen), pooled and incubated for 1 h at 37 °C in the presence of 7.5 μM free SpyTag003 peptide. Bead capture was performed for 30 min in the presence of 0.5% (v/v) Tween 20 and then five washes with regular Phage Buffer, with mixing at 700 rpm for 90 s each. Phage were eluted by incubation with 50 mM MES pH 6.0 or 150 mM NaCl with shaking at 700 rpm for 15 min at 4 °C. In the fourth round, 1012 cfu phage in 500 μL were incubated with 75 μM biotinylated AviTag-SpyTag003-MBP at 4 °C for 16 h with shaking at 500 rpm in Phage Buffer 2 (25 mM Tris-HCl pH 8.0, 300 mM NaCl 0.05% (v/v) Tween 20) supplemented with 3% (w/v) bovine serum albumin (BSA). Phage bound to biotinylated AviTag-SpyTag003-MBP were captured by incubating in 150 μL BSA-blocked Dynabeads Biotin Binder (Thermo Fisher) magnetic beads per 500 μL reaction for 90 min at 4 °C and 700 rpm. Five washes were performed with Phage Buffer, then phage were eluted by incubating in 200 μL 50 mM acetic acid/sodium acetate pH 4.0 or 150 mM NaCl for 10 min at 4 °C for 30 min. The supernatant was transferred to a fresh BSA-blocked tube and neutralized with 2 M Tris-HCl pH 8.5. Phage were recovered by infecting 2 mL of TG1 cells at A595 of 0.5 and produced using M1K07 helper phage super-infection as described above. The following rounds were performed with the below changes. In the second round, 1011 cfu phage in 200 μL were incubated with 0.1 μM biotinylated AviTag-SpyTag003-MBP for 90 min at 4 °C and 600 rpm. Bead capture with 50 μL magnetic beads was performed with 60 min shaking at 700 rpm at 4 °C. Five washes were performed at 4 °C with Phage Buffer supplemented with 0.5% (v/v) Tween 20 and five washes with Phage Buffer. Phage were eluted and recovered as above. In the third round, 1014 cfu phage in 200 μL was incubated with 0.1 μM biotinylated AviTag-SpyTag003-MBP for 90 min at 4 °C and 600 rpm. Bead capture with 50 μL magnetic beads was performed for 15 min with shaking at 700 rpm. Washes were performed as in round 2. Phage was eluted with 50 mM MES pH 6.0 or 150 mM NaCl with shaking at 700 rpm for 15 min at 4 °C. In the fourth round, 1012 cfu phage in 500 μL were incubated with 75 μM biotinylated AviTag-SpyTag003-MBP at 4 °C for 16 h with shaking at 500 rpm in Phage Buffer 2 (25 mM Tris-HCl pH 8.0, 300 mM NaCl 0.05% (v/v) Tween 20) supplemented with 3% (w/v) BSA. Phage bound to biotinylated AviTag-SpyTag003-MBP were captured by incubating in 150 μL BSA-blocked Dynabeads Biotin Binder (Thermo Fisher) magnetic beads per 500 μL reaction for 90 min at 4 °C and 700 rpm. Five washes were performed with Phage Buffer 2 supplemented with 0.5% (v/v) Tween 20, and five washes with Phage Buffer 2, all in the presence of 7.5 μM free SpyTag003 peptide. The phage were eluted by incubating in 200 μL 50 mM MES pH 6.5 or 150 mM NaCl 4 °C for 5 min with shaking at 700 rpm. The sample was then neutralized with 2 M Tris-HCl pH 8.5. Phage were recovered by infecting 2 mL TG1 cells at A595 of 0.5. The following rounds were performed with the below changes. In the second round, 2 × 1011 cfu phage were incubated with 50 μM biotinylated AviTag-SpyTag003-MBP for 30 min at 4 °C and 650 rpm shaking in the presence of E. coli lysate and the reaction was stopped with 5 μL SpyTag003 peptide. Bead capture was performed for 30 min in the presence of 5 μM SpyTag003 peptide. Five washes were performed with Phage Buffer 2 supplemented with 0.5% (v/v) Tween 20 and then five washes with Phage Buffer 2, all in the presence of 5 μM SpyTag003 peptide. Phage were eluted by incubating in 50 mM HEPES pH 7.0 or 150 mM NaCl 4 °C with shaking at 700 rpm for 5 min. In the third round, 2 × 1011 cfu phage were incubated with 50 μM biotinylated AviTag-SpyTag003-MBP for 15 min at 4 °C and 650 rpm shaking in the presence of E. coli lysate. Then the reaction was stopped with 5 μM free SpyTag003 peptide. Bead capture was performed for 30 min in the presence of 5 μM SpyTag003 peptide. Washes were performed as in round 2. Phage were eluted by incubating in 50 mM HEPES pH 7.0 or 150 mM NaCl 4 °C with shaking at 700 rpm for 2.5 min.

**Sanger sequencing of library clones was performed after each panning round for quality control. A final ten clones were sequenced for library 1 after round 4, five of which are shown in Fig. 2c. A further ten clones were sequenced for the original and for the alternative codon template after round 2 and 3 from library 2. Mutations that occurred at least three times were prioritized for investigation.**

**Purification by SpyTag.** For bacterial lysis doping, untransformed E. coli BL21 (DE3) RILP lysate for 15 min at 4 °C and 600 rpm shaking in the presence of E. coli lysate and the reaction was stopped with 5 μL SpyTag003 peptide. Bead capture was performed for 30 min in the presence of 5 μM SpyTag003 peptide. Five washes were performed with Phage Buffer 2 supplemented with 0.5% (v/v) Tween 20 and then five washes with Phage Buffer 2, all in the presence of 5 μM SpyTag003 peptide. Phage were eluted by incubating in 50 mM HEPES pH 7.0 or 150 mM NaCl 4 °C with shaking at 700 rpm for 5 min. In the third round, 2 × 1011 cfu phage were incubated with 50 μM biotinylated AviTag-SpyTag003-MBP for 15 min at 4 °C and 650 rpm shaking in the presence of E. coli lysate. Then the reaction was stopped with 5 μM free SpyTag003 peptide. Bead capture was performed for 30 min in the presence of 5 μM SpyTag003 peptide. Washes were performed as in round 2. Phage were eluted by incubating in 50 mM HEPES pH 7.0 or 150 mM NaCl 4 °C with shaking at 700 rpm for 2.5 min.
The supernatant was then loaded onto SDS-PAGE. At 37 °C, incubating each fraction for 5 min in a 37 °C incubator, before removing was eluted with 6× 1.5 CV of pre-warmed 50 mM HEPES pH 8.0, neutralization volumes were doubled. To assess maximum capacity of the SpySwitch resin, elution and constructs) as shown on SDS-PAGE, equal amounts of each elution fraction were mixed. To assess the maximum capacity of the SpySwitch resin, elution and neutralization volumes were doubled.

Using the temperature switch, all steps were as above except that bound protein was eluted with 6× 1.5 CV of 50 mM acetic acid/sodium acetate pH 5.0 + 150 mM NaCl at 4 °C, incubating each fraction for 5 min at 4 °C, with the column capped to prevent flow. The cap was removed, the protein elutes into microcentrifuge tubes containing 0.3 CV 1 M Tris-HCl pH 8.0 + 150 mM NaCl and 0.1 M imidazole in TP buffer. This spent at acidic pH. The resulting buffer contained the eluted protein is termed neutralized SpySwitch pH elution buffer. Elution was repeated six times. For total elution (denoted as T or E for RBD constructs) as shown on SDS-PAGE, equal amounts of each elution fraction were mixed. To assess the capacity of the SpySwitch resin, elution and neutralization volumes were doubled.

Using the temperature switch, all steps were as above except that bound protein was eluted with 6× 1.5 CV of pre-warmed 50 mM HEPEs pH 8.0 + 150 mM NaCl at 37 °C, incubating each fraction for 5 min in 37 °C incubator, before removing the cap to allow flow. To assess protein remaining on the resin after elution, 50 μL packed resin was resuspended with 50 μL PBS pH 7.4. 10 μL of this slurry was mixed with 4 μL 6× SDS loading buffer, before heating at 95 °C for 5 min in a PCR machine. 7 μL from the supernatant was then loaded onto SDS-PAGE.

Protein concentrations were determined by A280 measurement on a NanoDrop 2000c spectrophotometer operated in positive ion mode and utilizing a jet-stream electrospray ion source (Mass Spectrometry Research Facility, Department of Chemistry, University of Oxford). Data were analyzed in Mass Hunter Qualitative Analysis software B.07.00 (Agilent) and protein ionization data were deconvoluted using the maximum entropy algorithm. The mass of reduced SpySwitch without N-terminal formylmethionine was predicted using ExPaSy ProtParam.

**Western blot.** For phage display validation, 3×10^11 cfu of phage were loaded and lane protein and volumes from SDS-PAGE were transferred onto nitrocellulose membranes by wet transfer in 12 mm Tris base. 96 mM glycine, 20% (v/v) methanol at 30 V for 2 h using a XCell SureLock system with XCell II Blot Module. Membranes were blocked for 16 h at 4 °C in 5% (w/v) skimmed milk in PBST [PBS pH 7.4 + 0.05% (v/v) Tween 20]. Primary antibodies were diluted in 2.5% (w/v) skimmed milk in PBST, with rabbit anti-HA tag (Rockland Immunocolors, 600-401-384) at the concentration of 1:2000 and mouse anti-Catalytic domain IgG (Sigma-Aldrich, A14416) at 15,000 h for 1 h at 25 °C in 25% (w/v) skimmed milk in PBST. In the fourth step, membranes were developed by 5 min incubation at 25 °C with SuperSignal West Pico Plus Chemiluminescent Substrate (Thermo Fisher) and imaged using a ChemiDoc XRS + imager.

**Mass spectrometry.** SpySwitch was dialyzed into 50 mM Tris-HCl pH 7.5 + 1 mM TCEP and diluted to 10 μM, then acidified at a final concentration of 0.9% (v/v) formic acid. Samples were loaded onto a C4 solid phase extraction cartridge and washed with 1% (v/v) formic acid, then eluted with 85% (v/v) acetonitrile and 0.1% (v/v) formic acid in deionized water. Samples were analyzed using an Agilent 6550 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer operated in positive ion mode and utilizing a jet-stream electrospray ion source (Mass Spectrometry Research Facility, Department of Chemistry, University of Oxford). Data were analyzed in Mass Hunter Qualitative Analysis software B.07.00 (Agilent) and protein ionization data were deconvoluted using the maximum entropy algorithm. The mass of reduced SpySwitch without N-terminal formylmethionine was predicted using ExPaSy ProtParam.

**Carbamidomethylation of SpySwitch and SpyDock.** SpySwitch or SpyDock were reduced in 50 mM Tris-HCl pH 8.5, 5 mM EDTA, 1 mM TCEP. Carbamidomethylation was performed by adding a final concentration of 20 mM iodoacetamide and incubating for 30 min at 25 °C in the dark. Subsequently, proteins were dialyzed into PBS pH 7.4 in the dark with three changes of dialysis buffer. For DSC and ITC, SpySwitch and SpyDock were dialyzed into succinate-phosphate-glycine (SPG) buffer (2.86 mM succinic acid, 10 mM Na2HPO4, 150 mM NaCl) at pH 5.0, pH 6.0, pH 7.0, pH 7.5, and pH 8.0 or 20 mM Na2HPO4 pH 8.0 or 20 mM NaCl, as appropriate. SPG was chosen because of its ability to buffer pH over a wide pH range. Samples were centrifuged at 15,000 g at 4 °C for 10–20 min, prior to concentration measurement by A280 in triplicate.

**Isothermal titration calorimetry.** Experiments were carried out using a Microcal PEAQ-ITC calorimeter with Microcal PEAQ-ITC Software version 1.3 (both Malvern) at 5°C in 20 mM NaH2PO4 pH 7.5 + 150 mM NaCl or SPG buffer pH 5.0. Before the experiment, proteins were dialyzed in at least two dialysis steps into the respective buffer, with the last dialysis step at 50% data collection step at 10% of total protein at 150 mM NaCl or SPG buffer. SpyTag03-SPG or SpyTag03-MBP or SpyTag03-MBP were titrated into 30 μM carbamidomethylated SpySwitch in the cell with 19 injections. Analysis was carried out using a 1:1 binding model with Microcal PEAQ-ITC Analysis software version 1.1.0.1262. Error estimates represent the uncertainty of the fit to the binding curve, calculated using Microcal PEAQ-ITC Analysis software version 1.1.0.1262. The molar ratio refers to the concentration of SpyTagged construct divided by the concentration of carbamidomethylated SpySwitch. The data are representative of two experiments.
Differential scanning calorimetry. DSC experiments were performed using a MicroCal PEAQ-DSC with MicroCal PEAQ-DSC measurement software version 1.53 (both Malvern). 45 μM carbamidomethylated SpyCatcher or carbamido-methylated SpyDock in SPG buffer at pH 5.0, 6.0, 7.0 and 8.0 or 27 μM of sarbecovirus RBD constructs in PBS pH 7.4 were analyzed. Scans were performed from 10°C to 110°C at 200°C/h and 3 atm. Data were analyzed using MicroCal PEAQ-DSC analysis software version 1.53. Buffer and baseline subtraction were performed. Thermal transitions were fitted to obtain the enthalpy of unfolding ΔH and the melting temperature Tm. The full width at half maximum was determined using Origin2021b (OriginLab).

RBD multimerization. 2, 4, or 6 μM Yn11 RBD-SpyTag03 was incubated with 2 μM (monomer concentration) of either SpyCatcher003-m3i (60-mer) or SpyCatcher022-oPent (5-mer) for 16 h at 4°C in neutralized SpySwitch pH elution buffer. Samples were mixed with 6x SDS loading buffer, supplemented with 1 mM DTT, incubated at 95°C for 10 min, and resolved by 12% SDS-PAGE, before Coomassie staining.

ELISA. 25 nM RBD-SpyTag03 (or PBS pH 7.4 for the no RBD control) was incubated with 25 nM (monomer concentration) SpyCatcher003-m3i at 4°C for 48 h in neutralized SpySwitch pH elution buffer, before adsorbing on a flat-bottom Immuno Nonsterile 96-Well Plate (Thermo Fisher, 442404) through incubation for 16 h at 4°C. The wells were washed three times with PBS pH 7.4 with 0.1% (v/v) Tween-20, before blocking with 5% (w/v) skim milk in PBS pH 7.4 was added and incubated for 1 h at 37°C. 5% (w/v) skim milk in PBS pH 7.4 was used for the no antibody control. After three washes with PBS pH 7.4 with 0.1% (v/v) Tween-20, the wells were incubated in a 1/1,600 dilution of goat anti-human IgG (Thermo Fisher) was added to the wells and a time-course of A652 measurements was recorded using a FLUOstar Omega plate reader (BMG Labtech) at 25°C. The mean absorbance for triplicates at 16 h was presented as a heat map in Fig. 5f, with the mean and error bars ± 1 s.d. from triplicate measurements presented in Supplementary Table 3. The results are representative of two separate experiments.

Freeze-thaw assay. 2 μM sarbecovirus RBD-SpyTag03 was incubated with 2 μM (monomer concentration) SpyCatcher003-m3i at 6 h at 4°C in neutralized SpySwitch pH elution buffer. The specified number of freeze-thaw cycles was performed by freezing coupled RBD-m3i by placing in a −80°C freezer for 20 min and then thawing by incubation in a Thermomixer C (Eppendorf) at 25°C for 15 min. After the specified number of freeze-thaw cycles was complete, the samples were centrifuged for 30 min at 16,000 g and 4°C. A 1 in 40 dilution of the supernatant was made, neutralized SpySwitch pH elution buffer, and adsorbed on Immuno Nonsterile 96-Well Plates (Thermo Fisher, 442404) through incubation for 16 h at 4°C. PBS pH 7.4 alone was added to the well for the buffer control. The ELISA was performed with 50 nM EYEA as described above, but the 50 μL reaction was stopped after a 2 min incubation in 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher). By adding 50 μL 1 M H2SO4, A652 measurements were performed using a FLUOstar Omega plate reader (BMG Labtech) at 25°C. Individual data points along with mean absorbance values of triplicate measurements are presented, with error bars ± 1 s.d.

Graphs and sequence analysis. Protein structures were visualized in PyMOL version 2.0.6 (DeLano Scientific), using PDB ID: 2X5P for SpyCatcher and PDB ID: 4M1L to represent SpyTag. Antibody classes were defined and contact residues were colored on SARS-CoV-2 RBD from PDB ID: 6M0J, following the Barnes classification. The 1 class 1 binding site was based on PDB ID: 7K8M, class 2 on PDB ID: 7K8X, class 3 on PDB ID: 7K8Z, and class 4 on PDB ID: 6W41. Where class 1 and class 2 binding sites had some overlap, we used the class 1 coloring. We used PDB ID: 8NN2 for the EYEA/SARS-CoV-2 RBD complex, with contact residues on RBD as defined previously. A phylogenetic tree of sarbecovirus RBD sequences was constructed using MEGA X v11.0.8 software. Multiple sequence alignment used Clustal Omega v1.2.4.

Statistics and reproducibility. For representative SDS-PAGE (Fig. 3a–f, Fig. 4d, f, Fig. 5b, d, Supplementary Fig. 2b, Supplementary Fig. 3a, b, Supplementary Fig. 6 a, e, c, Supplementary Fig. 8 a, c, Supplementary Fig. 10a and Supplementary Fig. 11b, c), observations were confirmed at least once with similar or identical conditions. Phage display without arabinose titration was validated by Western blot (Fig. 2b) at least once under similar conditions. Other SDS-PAGE and Western blot (Supplementary Fig. 3c, d, Supplementary Fig. 6b, d and Supplementary Fig. 8b, d) are the results of a single experiment. SpySwitch and SpyDock DSC results (Fig. 4a, b) were confirmed at least once with identical conditions. Assessment of RBD Tm (Fig. 5c, right panel) was performed in two separate experiments. The RBD ELISA (Fig. 5f) was performed in triplicate and confirmed at least once with identical conditions. Mass spectrometry results (Supplementary Fig. 4a) were confirmed once with similar conditions. ITC data were confirmed at least once with identical conditions (Supplementary Fig. 4b, c). SpySwitch capacity (Supplementary Fig. 7) was confirmed once under similar conditions. No statistical method was used to predetermine sample size. No data were excluded from the analyses. The experiments were not randomized. The Investigators were not blinded to allocation during experiments and outcome assessment.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Amino acid sequences of SpyDock and SpySwitch are available in Supplementary Fig. 1. Sequences of other constructs are available in GenBank as described in the section Plasmons and cloning. Plasmons encoding pDEST41-SpyDock, pDEST41-SpySwitch, pET28a-SpyTag-MBP, pET28a-SpyTag-sfGFP, pET28a-SpyTag03-MBP, pET28a-AvTag-SpyTag03-MBP, pET28a-SpyCatcher003-m3i and pDEST41-SpySwitch022-oPent have been deposited in the Addgene repository (https://www.addgene.org/Mark_Howarth/). Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, M.H. Source data are provided with this paper.

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Author contributions
S.K.V. performed all experiments except for Figs. 1 and 2 and some of the RBD analysis. I.N.A.K.A. performed rational His mutagenesis and Fig. 1. R.R. performed phage selections and Fig. 2. R.A.H. carried out RBD analysis. T.K.T. generated antibody resources. S.K.V., I.N.A.K.A., R.R. and M.H. designed the project. S.K.V. and M.H. wrote the manuscript. All authors approved the manuscript.

Competing interests
M.H. is an author on a patent application by the University of Oxford covering SpyTag003/SpyCatcher003 (UK Intellectual Property Office 1706430.4). M.H. is an author on a granted patent from the University of Oxford covering peptide tags forming spontaneous isopeptide bonds (EP2534484) and a SpyBiotech co-founder, shareholder, and consultant. S.K.V., R.R., I.N.A.K.A. and M.H. are authors on a patent application by the University of Oxford covering SpySwitch (UK Intellectual Property Office 2117283.8). M.H. and I.N.A.K.A. are authors on a patent application by the University of Oxford covering Spy&Go (UK Intellectual Property Office 1819850.7). The remaining authors declare no competing interests.

Additional information
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Supplementary Information

SpySwitch enables pH- or heat-responsive capture and release for plug-and-display nanoassembly

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**Supplementary Fig. 1 SpySwitch sequence and mutation sites.** Amino acid sequence alignment of SpyDock and SpySwitch, as well as SpyTag, SpyTag002 and SpyTag003, numbered according to PDB ID: 2X5P. Residues shown in orange were introduced to generate SpySwitch. Residues in green show mutations present in SpyCatcher003, but not in SpyDock. His$_6$-tag and linker sequence are shown in gray. * = stop codon.
Supplementary Fig. 2 SpySwitch resin can be used for gentler elution and is stable to storage. (a) SpySwitch purification at pH values closer to neutral. SpySwitch was used for purification of SpyTag-sfGFP or SpyTag003-sfGFP doped into *E. coli* lysate, with elution at either pH 5.0, pH 5.5 or pH 6.0. After neutralization, fluorescence was determined from the flow-through (FT), wash (W, pH 8.0), or elution (E) fractions (*n* = 1). Fluorescence in arbitrary units was multiplied by the volume of the fraction, to give arbitrary units*mL*. (b) SpySwitch resin was stored in 20% (v/v) ethanol in TP buffer pH 7.0 at 4 °C for 7 months, then used for purification of SpyTag-sfGFP doped into *E. coli* lysate. Reducing SDS-PAGE/Coomassie results are shown. L, doped lysate; FT, flow-through; T, total pooled elution fractions; Resin, protein left on resin following elution. Molecular weight markers represent kDa. Source data are provided as a Source data file.
Supplementary Fig. 3 Purification of anti-HER2 Fabs. Purification of anti-HER2 Fab bearing a C-terminal SpyTag003 and His$_6$-tag expressed in Expi293F cells using (a) SpyDock with elution in 2.5 M imidazole in TP buffer pH 7.0 or (b) Ni-NTA with elution in 200 mM imidazole in Ni-NTA binding buffer. (c) Purification of anti-HER2 Fab bearing a SpyTag and His$_6$-tag expressed in Expi293F cells using SpySwitch, with elution by pH 5.0 at 4 °C. (d) As in (c) bearing SpyTag002. Samples were analyzed by non-reducing SDS-PAGE with Coomassie staining. % purity in (a) and (b) was calculated by densitometry. S, Expi293F supernatant; FT, flow-through; T, total pooled elution fractions; Resin, protein left on resin following elution. The putative heavy and light chains of the anti-HER2 Fab are marked, based on close mobility to the reduced anti-HER2 Fab heavy and light chain shown in Figure 3f, and these bands are absent in any other purifications from mammalian culture supernatant. Molecular weight markers represent kDa. Source data are provided as a Source data file.
**Supplementary Fig. 4 Biophysical characterization of SpySwitch.** (a) ESI-MS of SpySwitch, showing observed and predicted mass. (b) Isothermal titration calorimetry for affinity of SpySwitch to SpyTag-MBP at pH 7.5 and 5 °C. Error estimates represent the uncertainty of the fit. Data are representative of two experiments. (c) Isothermal titration calorimetry for affinity of SpySwitch to SpyTag003-MBP, measured as in (b). Source data are provided as a Source data file.
**Supplementary Fig. 5 Isothermal titration calorimetry at pH 5.0.** ITC showed undetectable interaction of SpySwitch to (a) SpyTag-MBP or (b) SpyTag003-MBP at pH 5.0 and 5 °C. Source data are provided as a Source data file.
Supplementary Fig. 6 SpySwitch purification from bacterial and mammalian expression systems. (a) SpySwitch temperature elution of SpyTag-sfGFP from bacterial lysate, with capture at 4 °C and elution at 37 °C. L, doped lysate; FT, flow-through; T, total pooled elution fractions; Resin, protein left on resin following elution. (b) SpyDock temperature elution of SpyTag-sfGFP as in (a). (c) SpySwitch temperature elution of SpyTag002-sfGFP as in (a). (d) SpyDock temperature elution of SpyTag003-sfGFP as in (a). (e) SpySwitch temperature elution of HA-SpyTag003 from mammalian Expi293F expression, with capture at 4 °C and elution at 37 °C. Samples were analyzed by reducing SDS-PAGE with Coomassie staining. % purity in (a), (c) and (e) was calculated by densitometry. S, supernatant. Molecular weight markers represent kDa. Source data are provided as a Source data file.
Supplementary Fig. 7 SpySwitch resin capacity. SpySwitch resin capacity was determined by doping SpyTag-sfGFP into bacterial lysate at 18 mg or 36 mg per mL packed resin and purifying by pH switch at pH 5.0 or temperature switch at 37 °C. Protein concentration was measured by $A_{280}$ after elution and the yield was calculated. Source data are provided as a Source data file.
Supplementary Fig. 8 SpySwitch resin can be regenerated multiple times. (a) SpyTag003-sfGFP was bound to SpySwitch resin, before regeneration by sequential washes with 0.1 M glycine pH 2.0, then 50 mM Tris-HCl pH 7.5 + 8 M urea, and finally 0.1 M NaOH. Regenerated SpySwitch resin was used for purification of HA-SpyTag from Expi293F cells with temperature elution at 37 °C. SpyTag003-sfGFP was loaded in varying amounts as a standard to allow for calibration. Samples were analyzed by reducing SDS-PAGE with Coomassie staining. M, molecular weight markers; S, Expi293F supernatant; FT, flow-through; W, wash. (b) Western blot analysis of regeneration, performed as in (a), staining with anti-GFP. (c) SpySwitch purification from fresh resin. SpyTag-sfGFP was doped into E. coli lysate and fresh SpySwitch resin was used for purification by pH elution. Samples were analyzed by reducing SDS-PAGE with Coomassie staining. % purity was calculated by densitometry. L, doped lysate; FT, flow-through; T, total pooled elution fractions. (d) SpySwitch purification from resin that had been regenerated 5 times. Purification was conducted as in (c) after five rounds of regeneration. Molecular weight markers represent kDa. Source data are provided as a Source data file.
Supplementary Fig. 9 EY6A binding to SARS-CoV-2 RBD. (a) EY6A Fab, shown in magenta, binding to SARS-CoV-2 RBD, shown in gray, with the contact site colored in blue. Based on PDB ID: 6ZER. (b) Binding interface of EY6A on SARS-CoV-2 RBD with the interaction residues shown in stick representation, based on PDB ID: 6ZER. Blue indicates residues at the binding site for EY6A conserved in all twelve sarbecovirus RBD constructs. Pink indicates a binding site residue in SARS-CoV-2 not conserved in other sarbecovirus RBD constructs. (c) Sarbecovirus RBD sequence alignment. Residues are colored as in (b). The amino acid numbering is based on the Spike protein of SARS-CoV-2.
Supplementary Fig. 10 SpySwitch-purified RBDs are efficiently coupled to VLPs and resilient to freeze-thaw. (a) VLP coupling. Sarbecovirus RBD constructs were coupled to SpyCatcher003-mi3 by reacting 2 μM of each construct for 16 h at 4 °C in neutralized SpySwitch elution buffer. SDS-PAGE/Coomassie shown after coupling reaction. Molecular weight markers represent kDa. (b) Resilience to freeze-thaw. Recognition of sarbecovirus RBDs coupled to SpyCatcher003-mi3 by the conformation-sensitive antibody EY6A, after the coupled RBDs were subjected to the indicated number of freeze-thaw cycles. Individual data points and the mean absorbance were plotted, with error bars denoting ± 1 s.d. (n = 3). 0 freeze-thaw cycles in blue, 1 freeze-thaw cycle in orange and 5 freeze-thaw cycles in gray. Source data are provided as a Source data file.
**Supplementary Fig. 11 Proteins purified by SpySwitch.** (a) Predicted isoelectric point (pl) of proteins purified by SpySwitch, based on ProtParam values calculated without glycosylation. (b) Purification of SpyTag-MBP by SpySwitch pH elution from bacterial lysate. (c) Purification of SpyTag003-β-galactosidase from bacterial lysate by SpySwitch pH elution. Samples were analyzed by reducing SDS-PAGE with Coomassie staining. % purity was calculated by densitometry. L, lysate expressing protein of interest; FT, flow-through; T, total pooled elution fractions; Resin, protein left on resin following elution. Molecular weight markers represent kDa. Source data are provided as a Source data file.
Supplementary Table 1 DSC parameters of SpySwitch and SpyDock. DSC of SpySwitch or SpyDock at pH 5.0, 6.0, 7.0 or 8.0 in SPG buffer. ΔH, T<sub>m</sub> and FWHM (full width at half maximum) are given at each pH.

| Construct | pH | ΔH (kcal/mol) | T<sub>m</sub> (°C) | FWHM (°C) |
|-----------|----|---------------|--------------------|-----------|
| SpySwitch | 8  | 34.6          | 36.9               | 22.4      |
|           | 7  | 28.7          | 41.8               | 20.9      |
|           | 5  | 14.4          | 57.7               | 17.7      |
|           | 6  | 23.6          | 50.4               | 17.6      |
| SpyDock   | 8  | 15.2          | 50.1               | 23.3      |
|           | 7  | 18.2          | 50.5               | 23.9      |
|           | 6  | 28.0          | 53.4               | 21.5      |
|           | 5  | 22.3          | 59.1               | 22.6      |

Supplementary Table 2 DSC parameters of sarbecovirus RBD constructs. DSC was performed in PBS pH 7.4. ΔH, T<sub>m</sub> and FWHM (full width at half maximum) are given.

| Construct | ΔH (kcal/mol) | T<sub>m</sub> (°C) | FWHM (°C) |
|-----------|---------------|--------------------|-----------|
| SARS      | 101           | 59.8               | 7.3       |
| WIV1      | 104           | 57.5               | 8.5       |
| SHC014    | 140           | 62.7               | 5.9       |
| BM48-31   | 88.7          | 59.9               | 8.0       |
| BtKY72    | 99.9          | 58.4               | 7.6       |
| pang17    | 96.8          | 59.5               | 6.9       |
| SARS-CoV-2| 99.4          | 52.8               | 6.9       |
| RaTG13    | 96.1          | 52.0               | 6.8       |
| Rs4081    | 86.1          | 61.6               | 9.5       |
| Yun11     | 83.5          | 63.5               | 9.8       |
| RmYN02    | 81.4          | 62.5               | 10.3      |
| Rf1       | 73.1          | 62.2               | 10.1      |

Supplementary Table 3 Recognition of sarbecovirus RBDs by a panel of antibodies. Mean absorbance from ELISA ± 1 s.d. (n = 3), based on the data in Fig. 5f.