Development of light-responsive protein binding in the monobody non-immunoglobulin scaffold

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Monobodies are synthetic non-immunoglobulin customizable protein binders invaluable to basic and applied research, and of considerable potential as future therapeutics and diagnostic tools. The ability to reversibly control their binding activity to their targets on demand would significantly expand their applications in biotechnology, medicine, and research. Here we present, as proof-of-principle, the development of a light-controlled monobody (OptoMB) that works in vitro and in cells and whose affinity for its SH2-domain target exhibits a 330-fold shift in binding affinity upon illumination. We demonstrate that our αSH2-OptoMB can be used to purify SH2-tagged proteins directly from crude E. coli extract, achieving 99.8% purity and over 40% yield in a single purification step. By virtue of their ability to be designed to bind any protein of interest, OptoMBs have the potential to find new powerful applications as light-switchable binders of untagged proteins with the temporal and spatial precision afforded by light.

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Monobodies\textsuperscript{1} and other synthetic non-immunoglobulin protein-binding scaffolds, such as affibodies, anticalins, and DARPin, bind to their targets with affinities and selectivities typically found in antibodies\textsuperscript{3–5}, yet they are much simpler in structure. Because of their ability to be designed to bind a variety of proteins of interest, monobodies have become invaluable tools for biomedical research and biotechnology\textsuperscript{6–9}. Monobodies also show great promise as diagnostic tools and future therapeutics, including for autoimmune diseases\textsuperscript{10,11}, cancer\textsuperscript{11} and most recently SAR-CoV-2\textsuperscript{12}. The impact of monobodies stems from their very tight binding to highly specific targets. However, their repertoire of applications could be greatly expanded if they were engineered with optogenetic control over their binding affinities, such that they could bind their targets instantly and reversibly depending on light conditions, while maintaining their characteristically high affinity and selectivity.

Monobodies are synthetic proteins derived from the 10th domain of human fibronectin type III. While they were originally designed to functionally resemble nanobodies\textsuperscript{6,13}, monobodies feature unique structural advantages such as a reduced size (20–25% smaller) and a compact protein core without disulfide bridges\textsuperscript{1,7}. Their small size (<100 amino acids), simple structure, and relative stability allows them to be expressed in many cell types and be active inside\textsuperscript{13} and outside of cells\textsuperscript{13}. Furthermore, because of their human origins, the use of monobodies as biologic drugs is expected to have a lower risk of unwanted immune responses\textsuperscript{16,17}. As synthetic proteins, they offer flexibility in the design and configuration of their binding surfaces, thereby offering alternative paratope-like regions. This has allowed the development of several monobody libraries that vary particular combinations of loops and strands to produce monobodies with different binding modes, including the original loop-binding\textsuperscript{1,8} and alternative side-binding modes\textsuperscript{7,8}. Because of all these advantages we identified monobodies as preeminent synthetic protein binders and ideal candidates in which to engineer light-switchable binding control.

A protein domain widely used for optogenetic tool development is the second light, oxygen, and voltage (LOV) domain from the oat, Avena sativa, photosensor Phototropin 1, called AsLOV2\textsuperscript{18}. This domain elicits its light response through a large conformational change (Fig. 1a) of its C-terminal Jα helix. In the dark, the Jα helix is packed against the core of the protein\textsuperscript{19,20}. However, exposure to blue light (optimally 447 nm) induces the formation of a covalent bond between a photoexcited flavin mononucleotide (FMN) chromophore and a conserved cysteine, which causes the Jα helix to undock, become disordered, and move away from the core domain\textsuperscript{19,20}. Back in the dark, the FMN-Cys covalent bond decays, allowing the Jα helix to fold back into its tightly packed dark-state conformation\textsuperscript{21,22}. Several studies have exploited this light-triggered conformational change to confer light dependence on natural protein functions. Insertion of AsLOV2 into solvent-exposed loops of kinases, phosphatases, guanine exchange factors, and the small CRISPR inhibitor AcrIIA4 results in light-triggered allosteric switches that make these protein activities and their downstream events light controllable\textsuperscript{23,24}. This versatility of AsLOV2 to bestow light-dependent functionalities in a variety of protein contexts encouraged us to use it to engineer light controls in a monobody.

Here we show that by fusing a monobody to AsLOV2 we obtain a light-dependent monobody, or OptoMonobody (OptoMB), whose binding affinity to its cognate protein target is controllable with light. Taking a structure-based protein engineering approach, we inserted AsLOV2 into structurally conserved, solvent exposed, loops of a monobody that binds the SH2 domain of Abl kinase\textsuperscript{25}. We show that one of these chimeras preferentially binds to the SH2 domain in the dark, allowing us to reversibly control its binding both in vitro and in mammalian cells. We then harness the ~330-fold change in binding affinity between lit and dark states to implement light-based protein purification\textsuperscript{26} using an OptoMB resin in what we call “light-controlled affinity chromatography” (LCAC). This work represents the first demonstration of light control over the binding activity of a monobody and the first example of this capability in a synthetic non-immunoglobulin protein binder. This class of light-responsive protein binder, which in principle can be engineered, screened, or selected to bind any protein of interest, has great potential for numerous new applications in biotechnology, synthetic biology, and basic research.

Results

OptoMonobody design and selection. To demonstrate the feasibility of developing a light-sensitive monobody, we chose the HA4 monobody, reported to bind with high affinity ($K_D$ ~ 7 nM) to the SH2 domain of the human Abl kinase, in vitro and in cells\textsuperscript{25}. This is an interesting and valuable target, as many proteins containing SH2 domains in general, and Abi kinase in particular, are involved in human health and disease\textsuperscript{27–29}. In addition, the availability of the crystal structure of HA4 bound to SH2 domain\textsuperscript{25} is a valuable resource for our rational protein engineering approach. Our strategy to develop a light-sensitive HA4 was to design various chimeras of this monobody with AsLOV2 from A. sativa, and test their ability to bind and release the SH2 domain depending on light conditions.

To build our chimeras, we inserted a shortened AsLOV2 domain\textsuperscript{30} in all seven structurally conserved, solvent-exposed loops of HA4 (Fig. 1b). Given the large conformational change of AsLOV2 triggered by light (Fig. 1a), our hypothesis was that the native conformation of the monobody domain in some chimeras would be preserved in the dark, allowing it to bind to SH2, but disrupted in the light, causing it to release its target. Guided by the crystal structure of HA4 bound to SH2 (PDB ID: 3k2m)\textsuperscript{25}, and considering the side-binding mode of the HA4-SH2 interaction (Fig. 1b), we explored potential sites within the seven solvent-exposed loops in HA4 where we could insert AsLOV2. We selected as many positions as possible in each loop, avoiding those where we have reasons to believe the dark-state conformation of AsLOV2 would disrupt the core $\beta$-sheets of the monobody or interfere with its side-binding mode of interactions with SH2. We also excluded positions where the light-triggered conformational change of the AsLOV2 Jα helix might be impeded by clashes with the monobody core. After this structural analysis, we selected 17 AsLOV2 insertion sites across all solvent-exposed loops of HA4, as well as N- and C-terminal fusions (Fig. 1b, Supplementary Table 1).

To find chimeras that can bind the SH2 domain in the dark but not in the light, we screened our constructs using an in vitro pull-down assay (Fig. 1c). First, we produced an N-terminally Histagged fusion of yellow fluorescent protein (YFP) and SH2 domain (His$_6$-YFP-SH2) in Escherichia coli, and immobilized it onto cobalt-charged agarose beads. We then incubated the beads with crude extracts of E. coli expressing each of the different AsLOV2-HA4 chimeras, in either blue light or darkness. After washing the beads under the same light conditions (see Methods), we eluted with imidazole and resolved the products with denaturing polyacrylamide gel electrophoresis (SDS-PAGE) to analyze the binding of each chimera in different light conditions (Fig. 1c, d, Supplementary Fig. 1). We anticipated that chimeras that bind to SH2 preferentially in the dark would show a more intense band on SDS-PAGE for samples that were incubated and washed in the dark, relative to the samples treated in the light (Fig. 1c).
We found that AsLOV2 insertions in two different HA4 loops produce chimeras with the expected behavior in our pull-down assays (Fig. 1d). One promising chimera has AsLOV2 inserted between residues Met29 and Ser30 (a site we call MS29, following a naming system for sites used in this study), located in Loop BC of HA4 (Fig. 1b, d, Supplementary Fig. 1). We only saw an effect involving this loop when AsLOV2 was inserted at position MS29 and Loop BC was shortened by removing the three surrounding amino acids (Ser30, Ser31, and Ser32, see Supplementary Note 1). Another chimera with positive results has AsLOV2 inserted between Ser58 and Ser59 (site SS58) located in Loop DE of HA4 (Fig. 1b, d). Insertions at other positions within Loop DE (57YSSS60) show smaller degrees of variation in band intensity between beads treated in the light versus in the dark (Fig. 1d, Supplementary Fig. 1a). Pull-downs were repeated at least twice observing similar results.
Supplementary Fig. 1). This suggests that Loop DE is a “hot spot” for favorable orientations between AsLOV2 and the monobody to produce light-responsive chimeras that switch between a conformation that allows target binding (in the dark) and one that promotes target dissociation (in the light). Compared to AsLOV2 insertions at other positions in Loop DE, the insertion at SS58 shows the largest difference between light and dark conditions, with a band that is approximately 2.1 times more intense in dark compared to light (Fig. 1d, Supplementary Fig. 1). This suggests that Loop DE is a hot spot for favorable orientations between AsLOV2 and the monobody to disfavor SH2 binding (see “Discussion”).

**In vitro characterization of OptoMB.** We next set out to test whether the light-dependent interaction of our OptoMB and SH2 domain (Fig. 2a) could be directly visualized by fluorescence microscopy. We cloned and purified His-tagged OptoMB and a variant harboring a mutation in AsLOV2 (V416L) that extends the lifetime of the photoactivated state from 55 to 4300 s$^{31}$ (OptoMB$_{V416L}$) and immobilized them separately onto Ni-charged agarose beads. We also prepared a dark-state OptoMB mutant with the well-characterized C450V mutation in AsLOV2 that prevents light-induced conformational switching (OptoMB$_{C450V}$)$^{32}$. Immobilized OptoMB$_{C450V}$ and parental HA4 monobodies were used as controls. The monobody-coated beads were then incubated with a fusion of YFP and SH2 (YFP-SH2) in the dark until they reached equilibrium and imaged over time using confocal microscopy as we changed conditions from darkness to blue light (450 nm). For both OptoMB and OptoMB$_{V416L}$-coated beads, light exposure induces a pronounced decrease in YFP fluorescence on the surface of the bead (Fig. 2b, c; Supplementary Fig. 2a, Supplementary Movie 2), whereas identical illumination and imaging conditions produced only slow photobleaching for beads coated with OptoMB$_{C450V}$ (Fig. 2b, c, Supplementary Movie 2) or parental HA4 monobodies (Supplementary Fig. 2a). Localized illumination could also be used to restrict SH2 unbinding to a single OptoMB-coated bead in a crowded field. In this case, YFP fluorescence was rapidly and reversibly controlled for the illuminated bead but not a nearby unilluminated bead (Fig. 2d, e, Supplementary Movie 3). These results demonstrate that OptoMBs provide spatiotemporal control over protein binding.

To quantify the changes in OptoMB-SH2 binding, we determined the kinetic rate constants and binding affinity in different light conditions. In these assays we took advantage of the different classes of mutations in AsLOV2 mentioned above to
vary properties of the OptoMB binding switch. Bio-layer interferometry (BLI) uses visible light for measuring changes in binding, so we used the light-insensitive OptoMB<sub>450V</sub> variant for assessing dark-state-binding kinetics and affinity. Conversely, we ensured that illumination could drive efficient conversion to the lit state by using the OptoMB<sub>v416L</sub> variant. We fit the resulting binding and dissociation data to a mass-action kinetic-binding model (Fig. 3a, b, Supplementary Fig. 2b), from which we obtained estimates of the rate constants of binding ($k_{on}$) and unbinding ($k_{off}$) as well as the overall dissociation constant ($K_d$) of the OptoMB-SH2 interaction in different light conditions (Table 1; Supplementary Table 2, Supplementary Fig. 2c, d).

We found that the binding affinity of OptoMB to SH2 changes dramatically when switching from dark to light conditions. The average dissociation constant of the OptoMB-SH2 interaction in the dark (OptoMB<sub>450V</sub>) is $K_d = 0.19 \pm 0.11 \mu M$ (mean ± SD), which is comparable to our measurements for the HA4 monobody (Table 1, Supplementary Fig. 2c, d). However, in the light (OptoMB<sub>v416L</sub>) it is drastically increased to $K_d = 63 \pm 23 \mu M$ (Table 1, Supplementary Fig. 2c). This amounts to an ~330-fold-change in binding affinity between light conditions (Fig. 3c), which explains the light-dependent behaviors observed in the bead-imaging experiments above. The change in $K_d$ of the lit state arises equally from a decrease in the binding rate constant ($k_{on}$) and an increase in the unbinding rate constant ($k_{off}$) (Table 1, Supplementary Table 2, Supplementary Fig. 2c, d). These data are consistent with a light-induced change in the conformation of the OptoMB that disrupts the binding interface, substantially

### Table 1 Rate and dissociation constants from BLI experiments.

| Variant       | State measured | $k_{on}$ (μM<sup>-1</sup>s<sup>-1</sup>) | $k_{off}$ (s<sup>-1</sup>) | $K_d$ (μM) |
|---------------|----------------|----------------------------------------|-----------------------------|------------|
| Monobody HA4  | -              | 0.0631                                 | 0.0145                      | 0.23       |
| OptoMB        | Light conformation | <0.001                              | 0.21 ± 0.09                 | >100       |
| OptoMB<sub>C450V</sub> | Dark conformation | 0.071 ± 0.033                      | 0.01 ± 0.004               | 0.19 ± 0.11 |
| OptoMB<sub>v416L</sub> | Light conformation | 0.004 ± 0.001                      | 0.23 ± 0.02               | 63 ± 23    |

*Average of three individual measurements ± SD.*

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**Fig. 3** OptoMB binding kinetics and SEC profile for lit and dark states. **a, b** Representative BLI measurements of binding (left) and unbinding (right) of YFP-SH2 to immobilized OptoMB. **a** Dark-state measurement using the light-insensitive OptoMB<sub>C450V</sub>, and no external light source. **b** Lit-state measurement using the light-stabilized OptoMB<sub>v416L</sub> and exposing the sample to light. The calculated rate ($k_{on}$, $k_{off}$) and dissociation ($K_d$) constants are shown below the BLI data. **c** Change in calculated $K_d$ values between all dark-state (OptoMB<sub>C450V</sub>) and lit-state (OptoMB<sub>v416L</sub>) measurements. Error bars indicate mean ± SD for $n = 3$ independent measurements (see Table 1). **d** Size exclusion chromatography profiles of SUMO-tagged OptoMB<sub>v416L</sub> and YFP-SH2 interactions in light (blue line) and dark (black line), compared to pure OptoMB<sub>Triple</sub> (red) or YFP-SH2 (yellow) controls. Source data are provided as a Source Data file.
YFP-SH2 and HA4, as well as YFP-SH2/HA4 mixtures run under different light conditions as controls (Supplementary Fig. 3b, c). In contrast, the illuminated OptoMBTriple mixture displays a peak corresponding to the monomeric YFP-SH2 as well as a longer average retention time for the OptoMB–SH2 complex (Supplementary Fig. 3c). Finally, after light elution, beads were eluted with imidazole to recover any remaining protein bound to the beads in order to estimate the capacity and yields of the resin. With these initial LCAC purification trials, we achieved 95–98% purity in a single step, with yields ranging from 18 to 30%, and binding capacities from 112 to 145 nmol (4.5–6 mg) of SH2-tagged YFP per mL of OptoMB resin, depending on the OptoMB variant used (Table 2).

**Light-controlled affinity chromatography**. We reasoned that the substantial change in OptoMB binding affinity in light could open the door to purifying a protein of interest simply by shifting illumination conditions (Fig. 4a), a procedure that we termed “light-controlled affinity chromatography” (LCAC). We immobilized two variants of His-tagged OptoMBs harboring either the wild-type AsLOV2 or the triple mutant (SUMO-tagged OptoMBTriple) described above, onto CoCl2-OptoMB affinity resin. We then incubated these resins with crude lysate from *E. coli* overexpressing YFP-SH2. After washing thoroughly in the dark (see “Methods”), we eluted with blue light either in batch (Fig. 4b) or in a column (Supplementary Fig. 4). Finally, after light elution, beads were eluted with imidazole to recover any remaining protein bound to the beads in order to estimate the capacity and yields of the resin. With these initial LCAC purification trials, we achieved 95–98% purity in a single step, with yields ranging from 18 to 30%, and binding capacities from 112 to 145 nmol (4.5–6 mg) of SH2-tagged YFP per mL of OptoMB resin, depending on the OptoMB variant used (Table 2).

**Methods**

We prepared mixtures of purified YFP-SH2 and OptoMBTriple (SUMO tagged to boost expression, see “Methods”), in which the OptoMBTriple (or HA4 monobody, as control) was added in excess (see “Methods”). Each mixture was then run in a gel filtration column under continuous darkness or blue light (Supplementary Fig. 3a), with pure samples of OptoMBTriple, YFP-SH2 and HA4, as well as YFP-SH2/HA4 mixtures run under different light conditions as controls (Supplementary Fig. 3b, c). When run with OptoMBTriple in the dark or with the parental HA4 monobody, YFP-SH2 elutes as a monobody-bound complex without exhibiting a peak for unbound YFP-SH2 protein (Fig. 3d, Supplementary Fig. 3c). In contrast, the illuminated OptoMBTriple mixture displays a peak corresponding to the monomeric YFP-SH2 as well as a longer average retention time for the OptoMB–SH2 complex (Fig. 3d). Together, the light-triggered reduction in OptoMB–SH2 binding observed in solution and protein-coated surfaces is consistent with the two-order of magnitude change in binding affinity between dark and light conditions measured with BLI, laying the ground for the following in vitro application.

The light dependency of OptoMB interactions with YFP-SH2 can also be analyzed in solution using size exclusion chromatography (SEC). For this demonstration, we used an additional OptoMB variant with a V416I mutation in AsLOV2, which extends the lifetime of the lit state up to 821 s33, as well as two other mutations, G528A and N538E, reported to stabilize the dark-state conformation34. This triple mutant (OptoMBV416I_G528A_N538E) has stabilized lit and dark states, lower leaky activation in the absence of illumination, and thus better overall dynamic range of photoswitching33,34, which we expected would improve the performance of OptoMB in packed chromatography columns. BLI experiments confirmed that this mutant has a reduced binding affinity in the lit state relative to OptoMBV416I (Supplementary Table 2, Supplementary Fig. 2b, c), and thus at least the same change in *Kd* in different light conditions as this variant (Fig. 3c).

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To test whether LCAC could be applied to larger and more complex proteins, we used it to purify the main pyruvate decarboxylase from *Saccharomyces cerevisiae*, Pdc1p. This enzyme catalyzes the decarboxylation of pyruvate to acetaldehyde for ethanol fermentation, and is composed of a homotetramer of 61 kDa monomers, significantly larger than YFP. We fused the SH2 domain to the N-terminus of Pdc1p (SH2-Pdc1p1) and performed LCAC to purify it from crude *E. coli* lysate, as described above, using a resin coated with OptoMBTriple. This procedure enabled purification of Pdc1p to 96% purity with a 39% yield (Fig. 4c, Table 2). It is noteworthy that this purification works well despite the potential binding avidity of Pdc1p tetramers, which would be predicted to increase the protein’s apparent affinity for the resin in both the light and dark. These results demonstrate that LCAC can be applied to purify relatively large proteins with quaternary structures of up to at least 300 kDa (including the fused SH2 domain), achieving a high degree of purity and an acceptable yield. Our results with YFP-SH2 and SH2-Pdc1p further demonstrate that OptoMB-assisted purification is compatible with both N- and C-terminal SH2 tags.

While metal-affinity beads are effective at immobilizing OptoMB for LCAC (Fig. 4, Supplementary Fig. 4), they may be incompatible with some protein purification methods. Thus, to determine whether an alternative resin could be used for LCAC, we immobilized our OptoMBTriple onto cyanogen bromide-activated sepharose beads (CNBr beads), which immobilizes proteins by making covalent bonds with their primary amines (see "Methods"). Following the same purification protocol as above, we found that CNBr beads are also effective at purifying both YFP and Pdc1p (Supplementary Fig. 5). A single step of CNBr-based purification achieved yields above 40% and purity of 96.7–99.8%, surpassing any other LCAC method tested (Table 2). These gains are likely related to the lower non-specific binding of *E. coli* proteins to CNBr-sepharose relative to Co2+-agarose beads, and the covalent attachment of OptoMB, which allows for more efficient and extensive washing at higher salt concentrations. However, the total loading capacity of the CNBr-OptoMB-sepharose beads is not as high as that of Co2+-OptoMB-agarose (Table 2), probably because random crosslinking to the CNBr beads inactivates a substantial fraction of the OptoMB by occluding its binding surface to SH2. Although LCAC could be further improved by optimizing the resin (including for reusability), method, or amino acid sequence of the OptoMB, our results demonstrate the feasibility of a practical in vitro application of light-responsive monobodies for protein purification. This approach would make it possible to use buffer conditions that are optimal for protein stability throughout the purification process without needing to elute with a buffer exchange, which may damage the protein of interest (such as the low pH commonly used in antibody-based purification), or require lengthy and expensive subsequent dialysis. It also opens the possibility of using protein-specific OptoMBs to purify proteins that are difficult to fuse to affinity tags.

**Light-dependent OptoMB binding in cells.** We have shown that OptoMB-SH2 binding can be controlled with light in vitro; as a final test of this system, we assessed whether similar control can be achieved in live mammalian cells. We transduced HEK293T cells with lentiviral vectors encoding a membrane-localized, fluorescent SH2 target protein (SH2-mCherry-CAAX) and cytosolic fluorescent OptoMB (OptoMB-irFP), reasoning that a light-dependent change in SH2-OptoMB binding would cause the OptoMB to redistribute between the cytosol and plasma membrane (PM) (Fig. 5a), as has been observed for conventional optogenetic protein–protein interactions in previous studies. As a control, we expressed irFP-labeled HA4 monobody instead of OptoMB, which would be expected to bind to the membrane-localized SH2-mCherry-CAAX regardless of illumination conditions.

Fluorescence imaging confirmed the PM localization of SH2-mCherry-CAAX (Fig. 5b, c, left panels) as well as constitutively PM-bound HA4-irFP (Fig. 5b). In contrast, we observe a light-dependent shift in OptoMB localization (Fig. 5c), with PM enrichment in the dark and rapid redistribution to the cytosol upon light stimulation. Applying cycles of light and darkness further revealed that light-controlled binding is fully reversible intracellularly (Fig. 5d, Supplementary Movie 4). We also compared the extent of OptoMB cytosol-to-membrane redistribution to the well-established iLID-SSPB optogenetic heterodimerization tool. This analysis showed that our OptoMB displays a change in cytosolic intensity between light conditions that is equal in magnitude (both close to 60%) to that of iLID-SSPB, but with opposite sign (Fig. 5e). These experiments demonstrate functional photoswitching of OptoMB binding in cells to a level comparable to existing optogenetic tools, opening the door to their application in this context.

**Discussion**

Here we show that by taking a rational protein engineering approach it is possible to develop a light-switchable monobody (OptoMB). By fusing the light-responsive AsLOV2 domain to a structurally conserved loop of the H4A monobody that binds the SH2 domain of human Abl kinase, we developed an OptoMB that shows an ~330-fold drop in binding affinity when changing conditions from darkness to blue light. In comparison, the well-established iLID optogenetic switch displays an ~58-fold change in binding affinity between light conditions. Furthermore, the light responsiveness of OptoMB is reversible and effective at controlling binding to proteins fused to SH2 (at either their N- or C-terminus), both in vitro and in cells. OptoMBs, along with light-switchable nanobodies (OptoNBs), belong to a class of

Table 2 Purification parameters of LCAC batch experiments.

| Purified protein | OptoMB version | Resin type | Purity (%) | Resin capacitya (mg/mL) | Yield (%) of recovery from the resin |
|------------------|----------------|------------|------------|-------------------------|-------------------------------------|
| YFP-SH2          | OptoMB         | Talonb     | 98.25 ± 2.26 | 5.85 ± 0.67              | 17.8 ± 2.18                        |
| YFP-SH2          | OptoMBTriple   | Talonb     | 95.32 ± 0.46 | 4.5 ± 0.33               | 29.79 ± 2.28                       |
| SH2-Pdc1p        | OptoMBTriple   | Talonb     | 95.53 ± 0.05 | 5.24 ± 0.52              | 39.01 ± 2.61                       |
| YFP-SH2          | OptoMBTriple   | CNBrd      | 99.78 ± 0.22 | 2.55 ± 0.22              | 40.29 ± 3.02                       |
| SH2-Pdc1p        | OptoMBTriple   | CNBrd      | 96.69 ± 1.24 | 1.55 ± 0.18              | 42.28 ± 4.34                       |

Values expressed as mean ± SD for n = 3 independent measurements. Source data are provided as a Source Data file.

aSee "Methods" for calculations.
bOptoMB was His-tag bound.
cSUMO tagged.
dOptoMB was covalently conjugated.
The protein fold consists of two antiparallel β-sheets (βS1 and βS2) that interact with each other to form the protein core (Fig. 1b). In our original chimera screens, we inserted AsLOV2 in all intervening loops within (AB, CD, FG, and L7) and between (BC, DE, and EF) these β-sheets, which are accessible in the side-binding mode of monobodies. The only chimeras that show a change in binding affinity in different light conditions are those with AsLOV2 inserted in Loops BC and DE, with DE being the only S2 interaction relative to BC and DE is probably at least a partial disruption of the interactions and angle between them. This in turn would likely change the curvature of βS2 involved in the side-binding mode of monobodies, which defines the shape of the paratope-like surface of H4A and its specific binding interactions with SH2. Interestingly, because the conserved fold of monobodies always includes βS1 and βS2 interactions, this light-triggered disruption of the binding surface may be transferable to other monobodies with a side-binding mode. It also suggests that mutating residues involved in βS1–βS2 interactions may provide some opportunities to tune the photoswitchable behavior of the OptoMB, by stabilizing either the dark- or lit-state conformations.

This model of light-induced disruption of the monobody’s target-binding site is consistent with our measurements of binding kinetics. We originally hypothesized that light-dependent protein binders we call OptoBinders (OptoBNDRs), which offer promising new in vivo and in vitro applications.

A close inspection of the structural model of OptoMB and binding measurements suggest a possible mechanism for the light-dependent binding affinity of OptoMB. The monobody protein fold consists of two antiparallel β-sheets (βS1 and βS2) that interact with each other to form the protein core (Fig. 1b). In our original chimera screens, we inserted AsLOV2 in all intervening loops within (AB, CD, FG, and L7) and between (BC, DE, and EF) these β-sheets, which are accessible in the side-binding mode of monobodies. The only chimeras that show a change in binding affinity in different light conditions are those with AsLOV2 inserted in Loops BC and DE, with DE being the only S2 interaction relative to BC and DE is probably at least a partial disruption of the interactions and angle between them. This in turn would likely change the curvature of βS2 involved in the side-binding mode of monobodies, which defines the shape of the paratope-like surface of H4A and its specific binding interactions with SH2. Interestingly, because the conserved fold of monobodies always includes βS1 and βS2 interactions, this light-triggered disruption of the binding surface may be transferable to other monobodies with a side-binding mode. It also suggests that mutating residues involved in βS1–βS2 interactions may provide some opportunities to tune the photoswitchable behavior of the OptoMB, by stabilizing either the dark- or lit-state conformations.
stirnutation might strain the OptoMB-SH2 interaction causing them to dissociate without necessarily inducing dramatic changes in binding site accessibility, thus predicting mostly a light-induced increase in the OptoMB-SH2 off-rate ($k_{\text{off}}$). However, our BLI measurements revealed that both $k_{\text{on}}$ and $k_{\text{off}}$ are equally affected in the light (~20-fold change in opposite directions, Table 1, Supplementary Table 2, Supplementary Fig. 2b, c). This suggests a significant disruption of the binding surface of the OptoMB in the light, which not only accelerates the dissociation of the bound chimera but also slows down its binding to the target to begin with. Such disruption would be expected from pulling on the β-sheet interactions that make the core of the monobody fold. However, despite what is likely to be a substantial conformational change in the OptoMB, we find that productive binding is reversible in vitro (Fig. 2d, Supplementary Movie 3) and in mammalian cells (Fig. 5d, Supplementary Movie 4). Overall, these data are consistent with a large change in the overall orientation or conformation of the β1–β2 sheets, substantially disrupting binding but without driving irreversible protein misfolding. It is possible that the small size of the monobody domain allows a wide range of interactions between β1 and β2, and the lack of disulfide bonds in the monobody fold facilitate this high efficiency of interconversion between binding states.

Comparison of our binding data to a maximum theoretical value that assumes perfect transmission of energy between AsLOV2 and the monobody's binding interface suggests that light-induced changes in OptoMB could be further improved. Previous studies have measured the free energy available from the dark-to-light conformational change of AsLOV2 using NMR spectroscopy and developed analytical models to study equilibrium constants of the lit and dark states of AsLOV2. These studies predict that 3.8 kcal/mol of energy is transmitted from the absorption of one photon to structural rearrangements in AsLOV2. If all of this energy was transmitted to a change in the binding affinity, it would result in a ~600-fold difference in binding affinity between the lit and dark states. Our BLI experiments measured a change in $K_d$ values of ~30-fold, which is within the same order of magnitude of this maximum theoretical prediction, indicating that the light-induced conformational change of AsLOV2 is efficiently transmitted to disrupt the SH2 binding surface of the monobody domain. Nevertheless, it also suggests that further improvements might be achieved by optimizing the insertion site or linkers between the AsLOV2 and monobody, or by engineering the H4A domain to improve the light-induced allosteric coupling between the AsLOV2 domain and SH2-binding site. It is also possible that performance for particular applications might be improved by tuning the OptoMB-SH2 affinity to either weaker or tighter values. Finally, we note that the AsLOV2 domain might also be altered to tune the efficiency of the light-induced conformational change, for instance by further stabilizing the lit- or dark-state conformations or by altering its photoswitching kinetics (Table 1, Supplementary Table 2). In principle, these changes could increase the overall energy beyond the 3.8 kcal/mol measured for the wild-type AsLOV2 domain. The structural targets set for optimization and the expected results will of course vary depending on the particular application objectives and the individual OptoMB/ligand pair in question.

Even though the demonstrations used in this study rely on fusing SH2 to different proteins, the true potential of this technology is not so much to replace the light-responsive tags used in previous optogenetic systems with SH2 and OptoMB, but in the possibility of engineering other monobodies against different targets of interest to make their specific interaction light dependent. A few lines of reasoning suggest that this extensibility is likely. While our OptoMB design is based on the side-binding mode present in HA4 (involving the Loop FG, one or more β-strands from β2 and, occasionally, Loop CD), and is thus unlikely to be applicable to monobodies that present a loop-binding mode (involving Loops BC, DE, and FG), the majority of monobodies that have been structurally characterized (23 out of 32) bind their targets through the side-binding mode (Supplementary Table 3, Supplementary Fig. 6, see Supplementary Note 2). Further evidence suggests that the side-binding mode is more prevalent, as specimens with this binding mode are often identified from libraries in which only the Loops (BC, DE, and FG) were varied (Supplementary Table 3). As a consequence, the side-binding mode has been exploited to generate the so-called side and loop libraries that specifically vary the Loop FG, BC, and (and Loop CD in some versions), which have yielded monobodies against many targets.

### Methods

**Plasmid construction of chimeras for bacterial expression.** One-step isothermal assembly reactions ( Gibson assembly) were performed using previously described methods. The monobody HA4 and the SH2 domain (codon optimized for *E. coli* expression) were ordered as gBlocks from Integrated DNA Technologies (IDT) containing homology arms. The following vectors from the pCRI system were used: pCRI-7b for constructs without a 6x-histidine tag; pCRI-8b for constructs with a C-terminal His tag (N-terminus), and pCRI-11b for constructs with both SUMO and 6x-histidine tags (N-terminus). As the pCRI vectors contain YFP, the synthesized SH2 domain was inserted into pCRI-7b and 8b (previously linearized with XhoI) by Gibson assembly to construct EZ-L664 and EZ-L703 (see Supplementary Table 4). Monobody HA4 was inserted into pCRI-7b; the vector was digested (opened) with NheI and XhoI and Gibson-assembled to build the template (EZ-L663) used for the AsLOV2 insertions. A stop codon was added before the in-frame (C terminus) 6x-histidine tag of pCRI-7b. The AsLOV2 domain (residues 408–543) was either amplified by PCR from previous constructs or (wt AsLOV2) or synthesized by IDT gblocks (AsLOV2 mutants). To insert AsLOV2 into the monobody, the backbone of the initial construct containing H4A (EZ-L663) was PCR amplified, using Takara HiFi PCR premix, starting from the insertion positions (Supplementary Table 1) that were selected (and adding homology arms to AsLOV2). Next, chimeras were finally assembled mixing each of the amplified products of the backbone PCR from EZ-L663 with the AsLOV2 domain obtained from either PCR amplification (wt AsLOV2) or synthesized by gblocks (AsLOV2 mutants). SUMO tags were added by inserting (with Gibson assembly) the PCR product of the full-length chimeras (with homology arms) into the pCRI-11b plasmid, previously opened with NheI and XhoI. PDC1 was amplified from *S. cerevisiae* (S288C) genomic DNA using PCR, also with homology arms, and the construct (EZ-L886) was built via Gibson assembly (3 fragments) with digested pCRI-7b (NheI and XhoI) and the PCR product of SH2 amplified from EZ-L664 (with homology arms). All constructs (Supplementary Table 4) were sequenced by Geneviz and all protein sequences are available in Supplementary Note 1. We used chemically competent DH5a to clone all vectors. After verifying the plasmid sequence, vectors were used to transform chemically competent BL21 (DE3) or Rosetta strains for protein expression.

**Construction of the structural model of OptoMB.** To build the structural model of the HA4-AsLOV2 chimera (OptoMB) interacting with the SH2 domain (Fig. 1c), a shortened version (residues 408–543) of the AsLOV2 domain (PDB ID: 2V1A) was manually inserted to residues 558 and 559 of the monobody HA4, using the program Coef. The crystal structure of HA4 in complex with the SH2 domain (PDB ID: 3K2M) was used as template. After a manual adjustment, an energy minimization of the HA4-AsLOV2 chimera was carried out with the website version of YASARA (http://www.yasara.org/optimizationserver.htm).

**Plasmid construction for mammalian cells.** Constructs for mammalian cell experiments were cloned using backbone PCR and inFusion (Clontech).
Monobody HA4 or OptoMB variants were PCR amplified and Gibson-assembled from bacterial plasmids (described above) into a pH vector with a C-terminal iFP fusion and metal affinity tag (Addgene #11110). The SH2 for each chimera was affinity-purified EZ-His6 using PCR and Gibson-assembled into a pH vector containing a C-terminal mCherry-CAAX fusion tag (Addgene #50839). Stellar E. coli cells (TaKaRa) were transformed with these plasmids for amplification and DNA storage. All plasmids were sequenced by Genewiz to verify quality.

**Lentivirus production and transduction.** HEK293T cells were plated on a 12-well plate, reaching 40% confluency the next day. The cells were then co-transfected with the corresponding pH plasmid and lentiviral packaging plasmids (pCMV-D) using Fugene HD (Promega). Cells were incubated for ~48 h and virus was collected and concentrated by centrifugation at 7500 x g for 30 min at 4 °C (Sorvall Legend XTR Benchtop centrifuge (Thermo Scientific®)). Virions were pelleted by centrifugation at 70,000 x g for 90 min (Sorvall centrifuge) and supernatant was discarded. Each supernatant was resuspended in between 8 and 12 mL of Binding buffer and frozen droplets were prepared as described above and immediately transferred to a large (LN2-cold) grinding tube. Cryogenic grinding was performed as described above. Broken cells (frozen powder) were thawed in 30 mL Falcon tubes at room temperature with the addition of Lyso buffer up to 5% of the initial cell culture volume. After centrifugation at 1500 x g for 5 min at 4 °C, the supernatant was transferred to collection tubes. Supernatants, the beads were washed 5 x with 1 mL of Buffer A, centrifuging the beads at low speed for 1 min (1000 r.p.m.) before imaging. Mineral oil (50 μL) was added to each of the chimera-containing lysates, and then incubated for 15 min at 4 °C by vertical rotation (at 20 r.p.m. for 15 min) to allow binding.

**Screening for light-responsive monobodies.** A 6x-histidine tagged fusion of YFP and SH2 (His6-YFP-SH2) was grown in 500 mL of autoinduction media (described above) and SH2 (His6-YFP-SH2) was grown in 500 mL of autoinduction media (described above) and supernatant was discarded. Cell pellets were resuspended in Binding buffer (Tris 100 mM pH 8.0, NaCl 150 mM, glicerol 1%, and 5 mM imidazole) and supernatant was discarded. Each supernatant was resuspended in between 8 and 12 mL of Binding buffer and frozen droplets were prepared as described above and immediately transferred to a large (LN2-cold) grinding tube. Cryogenic grinding was performed as described above. Broken cells (frozen powder) were thawed in 30 mL Falcon tubes at room temperature with the addition of Lyso buffer up to 5% of the initial cell culture volume. After centrifugation at 1500 x g for 5 min at 4 °C, the supernatant was transferred to collection tubes. Supernatants, the beads were washed 5 x with 1 mL of Buffer A, centrifuging the beads at low speed for 1 min (1000 r.p.m.) before imaging. Mineral oil (50 μL) was added to each of the chimera-containing lysates, and then incubated for 15 min at 4 °C by vertical rotation (at 20 r.p.m. for 15 min) to allow binding.

**Binding buffer** with approximately 50 times the resin or column volume. The beads were suspended in Binding buffer (Tris 100 mM pH 8.0, NaCl 150 mM, glicerol 1%, and 5 mM imidazole) and supernatant was discarded. Each supernatant was resuspended in between 8 and 12 mL of Binding buffer and frozen droplets were prepared as described above and immediately transferred to a large (LN2-cold) grinding tube. Cryogenic grinding was performed as described above. Broken cells (frozen powder) were thawed in 30 mL Falcon tubes at room temperature with the addition of Lyso buffer up to 5% of the initial cell culture volume. After centrifugation at 1500 x g for 5 min at 4 °C, the supernatant was transferred to collection tubes. Supernatants, the beads were washed 5 x with 1 mL of Buffer A, centrifuging the beads at low speed for 1 min (1000 r.p.m.) before imaging. Mineral oil (50 μL) was added to each of the chimera-containing lysates, and then incubated for 15 min at 4 °C by vertical rotation (at 20 r.p.m. for 15 min) to allow binding.

**Purification of binders and targets.** HA4, monobody-AsLOV2 chimera (OptoMB and variants), and YFP-SH2 constructs were purified using N-terminal 6x-histidine tag (Ni-NTA) and metal affinity chromatography. Chimera was expressed at 18 °C for 3 days in 1 or 2 L of Autoinduction media (plus Kan 50 μg/mL). HA4 and YFP-SH2 were expressed in 1 or 2 L of Autoinduction media (plus Kan 50 μg/mL) for 16 h at 30 °C. Cells were then harvested by centrifugation at 7500 x g for 20 min at 4 °C in a Lynx 6000 centrifuge (Sorvall™). The supernatant was discarded. Each supernatant was resuspended in between 8 and 12 mL of Binding buffer and frozen droplets were prepared as described above and immediately transferred to a large (LN2-cold) grinding tube. Cryogenic grinding was performed as described above. Broken cells (frozen powder) were thawed in 30 mL Falcon tubes at room temperature with the addition of Lyso buffer up to 5% of the initial cell culture volume. After centrifugation at 1500 x g for 5 min at 4 °C, the supernatant was transferred to collection tubes. Supernatants, the beads were washed 5 x with 1 mL of Buffer A, centrifuging the beads at low speed for 1 min (1000 r.p.m.) before imaging. Mineral oil (50 μL) was added to each of the chimera-containing lysates, and then incubated for 15 min at 4 °C by vertical rotation (at 20 r.p.m. for 15 min) to allow binding.

**OptoMB characterization by size exclusion chromatography.** Purified monobody HA4 (8 μL from a 250 μL sample) or OptoMBv416L_G528A_N538E fused to a SUMO tag (28 μL from a 42 μL sample) dissolved in Buffer A was mixed with YFP-SH2 (20 μL from a 58.8 μL sample in Buffer A) in approximately 1:2.1 molar ratio of binder to target and completed to a total volume of 100 μL of Buffer A. Each 100 μL in Buffer A was incubated for 15 min in dark or dark (wrapped in aluminum foil) and incubated at 4 °C. Tubes were washed 10 x with 1 mL of Buffer A, centrifuged at 7500 x g for 5 min at 4 °C (using an Akta pure from GE Healthcare), which was then run at 1 mL/min with Buffer A at 4 °C (using an Akta pure from GE Healthcare). Pure OptoMBv416L_G528A_N538E samples (28 μL from a 42 μL sample) completed to 100 μL Buffer A were also loaded and run in this condition. To test different light conditions, the column was either illuminated with wrapped blue LED strips (450 nm, with approximate intensity of 30 μmol/m²/s) or covered with aluminum foil for the total duration of the filtration (Supplementary Fig. 3A). For experiments in the dark, we also minimized light sources in the room and covered the chromatography cabinet with a black blanket. The SEC was monitored by UV absorbance at 280 nm.

**Intracellular imaging of HEK293T cells.** For live cell imaging, we used 0.17 mm glass-bottomed wall-less 96-well plates (In Vitro Scientific). Glass was first treated with 10 μg/mL of fibronectin in PBS for 20 min. HEK293T cells expressing both SH2-mCherry-CAAX and either the monobody HA4 or OptoMB were then plated and allowed to adhere onto the plate before imaging. Mineral oil (50 μL) was added on top of each well with cells prior to imaging, to limit media evaporation. The cells were imaged at 27 °C with either light condition. To test different light conditions, the column was either illuminated with wrapped blue LED strips (450 nm, with approximate intensity of 30 μmol/m²/s) or covered with aluminum foil for the total duration of the filtration (Supplementary Fig. 3A). For experiments in the dark, we also minimized light sources in the room and covered the chromatography cabinet with a black blanket. The SEC was monitored by UV absorbance at 280 nm.

**Imaging of coated agarose beads.** Approximately 200 μL of Ni-NTA agarose slurry (30% suspension) (Qiagen) equilibrated in Buffer A were mixed with 500 μL of 100 μM of either monobody HA4, OptoMB, or OptoMBv416L_G528A_N538E (ASLOV2 V416L) or OptoMBv416L_G528A_N538E fused to a SUMO tag (28 μL from a 42 μL sample) dissolved in Buffer A were mixed with YFP-SH2 (20 μL from a 58.8 μL sample in Buffer A) in approximately 1:2.1 molar ratio of binder to target and completed to a total volume of 100 μL of Buffer A. Each 100 μL in Buffer A was incubated for 15 min in dark or dark (wrapped in aluminum foil) and incubated at 4 °C. Tubes were washed 10 x with 1 mL of Buffer A, centrifuged at 7500 x g for 5 min at 4 °C (using an Akta pure from GE Healthcare), which was then run at 1 mL/min with Buffer A at 4 °C (using an Akta pure from GE Healthcare). Pure OptoMBv416L_G528A_N538E samples (28 μL from a 42 μL sample) completed to 100 μL Buffer A were also loaded and run in this condition. For experiments in the dark, we also minimized light sources in the room and covered the chromatography cabinet with a black blanket. The SEC was monitored by UV absorbance at 280 nm.
with the beads labeled with OptoMonobody (or monobody HA4 as control). The mixture was equilibrated for at least 1 h at room temperature and up to overnight at 4 °C prior to imaging, performed at room temperature. The same microscope setup as described for the cells imaging was used, except for the objective (×20 in this case), to follow YFP fluorescence over time on the surface of the bead. For the spatial control of the OptoMB-SH2 interaction on beads the same setup was used. Two beads in an area of around 200 × 250 μm were imaged at the same time applying a light (450 nm) mask, which uses a square ROI with a dimension of 120 × 120 μm to cover and illuminate only one bead. The YFP fluorescence was recorded over time (for a total of 1 h) for both, the illuminated and the unilluminated bead. Quantification was performed by measuring the change in YFP fluorescence intensity over time in a defined region on the surface of the bead (using ImageJ) and subtracting the background.

**Calculation of binding kinetics by BLI**

Measurements of the binding (k_on) and unbinding (k_off) rate constants, as well as the dissociation (or affinity) constant (K_d) for HA4 monobody and OptoMB (including variants V416L and V416I-G528A-N538E) were performed on Octet RED96 instruments (ForteBio). Ni-NTA sensors were first equilibrated using Buffer A for 10 min prior to the measurement. A volume of 200 μL of Buffer A or protein solutions (previously dialyzed with Buffer A when needed) were added to clear 96-well plates. During the experimental run, the Ni-NTA sensors were first immersed in Buffer A to record the baseline. Protein binders were then loaded by switching to wells with solutions of the desired concentrations (for up to 200 nM in 15 mL) overnight or a variable of OptoMB variants (with or without SUMO) between 100 μg/mL and up to 1 mg/mL at values of ~4 nm were reached (avoiding saturation of the sensors). The sensors were then transferred back into Buffer A to remove unbound protein. To measure the binding rate constant (k_on) the sensors with bound monobody HA4 or OptoMB variants were subsequently shifted to a reference well containing various concentrations of YFP-SH2 (at concentrations indicated in Fig. 3a, b and Supplementary Fig. 2b). To measure the unbinding rate constant (k_off), the sensors were then moved to wells containing Buffer A to trigger dissociation of YFP-SH2. To measure binding kinetics of the light state, the lid of the Octet remained open during the measurement and a blue LED panel was held above the 96-well plate maintaining constant light illumination for the duration of the experiment. For the OptoMB variant with the AsLOV2 variant V416I-G528A-N538E, light states were sufficiently long-lived to remain fully activated in response to pre-illumination with a blue light panel and the constant illumination of the Octet sensors. The raw binding and unbinding data were simultaneously fit to models of the binding and unbinding reactions:

\[ y_{on}(t) = \frac{a_0}{1 - e^{-\text{rate}_{\text{on}}/C0}} + a_t e^{-\text{rate}_{\text{on}}/C1} t \]  
\[ y_{off}(t) = \frac{a_0}{1 - e^{-\text{rate}_{\text{off}}/C1}} + a_t e^{-\text{rate}_{\text{off}}/C2} t \]

This model incorporates the following dependent and independent variables:

- \( y_{on} \) refers to the 5th binding curve: \( y_{on}(t) \) the 5th unbinding curve; \( y_{on} \)
- \( y_{off} \) refers to the concentration of YFP-SH2 used for the 5th binding curve; and \( t \) is the time elapsed since the beginning of the binding/unbinding phase.
- \( \text{rate}_{\text{on}} \) and \( \text{rate}_{\text{off}} \) are the on-rate (enforced to be identical across all binding and unbinding curves) and off-rate (enforced to be identical across all binding and unbinding curves). A global parameter \( k_{\text{off}} \) is the off-rate (enforced to be identical across all binding and unbinding curves); \( k_{\text{on}} \) represents the slow unbinding of His-tagged OptoMB from the probe, leading to a gradual decay of signal throughout the entire experiment.

**LCAC of SH2-tagged proteins using covalently coupled OptoMB**

A total of 1–1.5 g (dry weight) of CNBr-activated Sepharose® 4B (GE Healthcare) was equilibrated in Co-charged (Talon®) STEC buffer (Supplementary Table 4) overnight in a 12 mL Eppendorf. TPB was equal to TPE plus the total residual SH2-tagged protein (eluted with light), we calculated the TRP of each sample on a 12% SDS-PAGE gel. The overall yield (TPB) was calculated as TPB minus the total elution volume (TRPvol). The concentrations of the fractions eluted with light were calculated using absorbance at 280 nm with a spectrometer (Biospectrometer, Eppendorf). TPB was equal to TPE plus the total residual SH2-tagged protein remaining on the column after light elution (TRP). To calculate the TRP, we chose the optimal elution conditions (Co-charged, 4 °C) for eluting and recovered residual protein were calculated by running each sample on a 12% SDS-PAGE gel alongside standards of the same SH2-tagged protein of known concentrations. Band intensities were then computed by densitometry analysis in Fiji (ImageJ). The amount of recovered protein was determined by v/v% of the protein present in that fraction. The overall yield (TPB) was calculated using densitometry analysis in Fiji (ImageJ). The overall yield (TPB) was calculated using densitometry analysis in Fiji (ImageJ).
where \( y \) is the yield of purified protein obtained with the resin; \( TPE \) the total amount of protein eluted with light; \( TPE_{\text{Evol}} \) the concentration of all the fractions that eluted with light; \( \text{TPEvol} \) the total volume of the light-elution fraction; \( \text{TPB} \) the total protein eluted with light; \( C_a \) the protein-binding capacity of the resin; and \( V_r \) is the volume of resin used in the experiment.

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Competing interests

A patent application describing the OptoMB design and applications is currently pending. Inventors: J.E.T., J.L.A., E.M.Z., A.A.G., and C.C.-L. Application number: 62962517, pending. Includes light-responsive monobodies and their uses thereof. The remaining authors declare no competing interests.

Additional information

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