JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

Supramolecular Assembly of an Evolved Miniprotein Host and Fluorogenic Guest Pair

Bi Xu, Xinqi Zhou, and Cliff I. Stains*

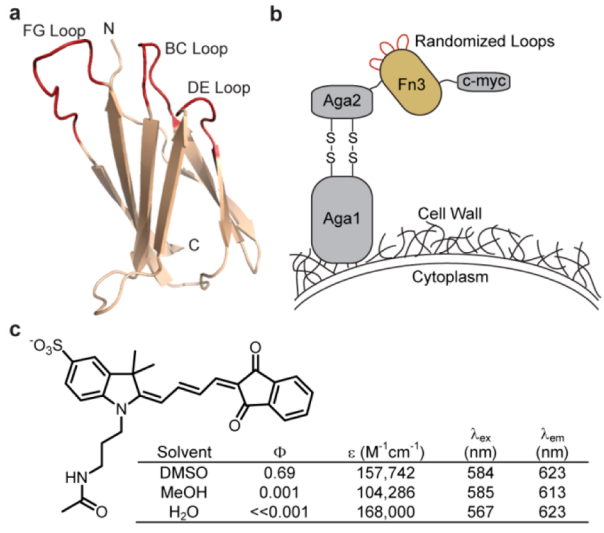
Department of Chemistry, University of Nebraska—Lincoln, Lincoln, Nebraska 68588, United States

Supporting Information

ABSTRACT: Small-molecule-induced assembly of defined protein structures could have broad implications for the fabrication of new materials as well as biological signaling pathways. However, the design of new host−guest pairs capable of small-molecule-induced assembly in a biologically relevant context remains a significant challenge. Herein, we report a series of miniprotein hosts, evolved from the tenth type III domain of fibronectin (Fn3), that display remarkable binding affinity toward a red-shifted environment-sensitive merocyanine derivative, termed sI-Pht. Importantly, the consensus binder isolated from directed evolution experiments (6.2.18) forms a higher order assembly in response to addition of sI-Pht, as assessed by analytical ultracentrifugation. sI-Pht-induced assembly of 6.2.18 results in a 570-fold increase in fluorescence compared to free dye. This property enables the direct visualization of host−guest assemblies by fluorescence microscopy. As a demonstration, we show that supramolecular assembly of the 6.2.18-sI-Pht system can be visualized on the surface of living yeast cells. This new host−guest pair provides a tool for the potential development of new materials as well as pathway engineering. In a broader context, this work details a new design paradigm for the discovery of host−guest systems that function in the context of living cells.

Supramolecular chemistry allows for the controlled, noncovalent assembly of molecular architectures,[1] providing new materials with novel properties.[2] However, the design and implementation of synthetic host−guest pairs in biological systems has been limited by the difficulty of delivering synthetic host−guest molecules into living cells as well as interference from biological molecules that perturb noncovalent assembly.[3] One notable exception is the use of the β-cyclodextrin/lithocholic acid host−guest pair to control protein assembly in living cells after delivery by microinjection.[4] Recent work has also described the use of the cucurbit[n]uril family of hosts to induce protein dimerization, tetramerization, and enzymatic activity using small-molecule and peptide-based guests.[5] Indeed, cucurbit[n]uril hosts possess remarkable binding affinities and specificities for tripeptide sequences containing an aromatic residue at the N-terminus.[6] While these approaches clearly represent significant advances, delivery of these reagents into living systems remains a potential barrier for routine use. As a consequence, naturally occurring protein domains that assemble in response to a small-molecule input have commonly been used to provide control over protein assembly in the biological context.[7] Although these platforms clearly represent a powerful means for manipulating biological responses,[8] more work is required to identify design principles that have the potential to afford non-native host−guest systems. Accordingly, we asked whether miniprotein hosts could be evolved to selectively recognize a small-molecule guest, allowing for inducible assembly of a noncovalent complex. The work described herein provides a proof-of-concept in which an evolved miniprotein host assembles into a higher order structure upon addition of a small-molecule guest. By leveraging the ability to genetically encode the host molecule, guest-induced assembly on the surface of living yeast is achieved. This approach provides a new design paradigm for host−guest systems that are compatible with living cells. In the long term, these reagents may find utility in the design of new materials as well as artificial signaling pathways.

As a potential host, we chose the well-characterized Fn3 miniprotein domain (Figure 1a).[9] This scaffold is small (10 kDa),



Solvent	Φ	ε ($M^{-1}cm^{-1}$)	λ_{ex} (nm)	λ_{em} (nm)
DMSO	0.69	157,742	584	623
MeOH	0.001	104,286	585	613
H_2O	<<0.001	168,000	567	623

Figure 1. (a) Structure of the Fn3 domain (PDB: 1TTG) with the randomized BC, DE, and FG loops highlighted in red. (b) Schematic of the yeast surface display system. Randomized Fn3 domains are tethered to the yeast surface by a covalent interaction between Aga1 and Aga2. Full-length proteins displayed on the yeast surface can be labeled using the C-terminal c-myc epitope tag. (c) Structure of the merocyanine derivative termed sI-Pht. Inset: photophysical properties of sI-Pht in different solvents.

Received: September 8, 2015
Published: November 2, 2015

DOI: 10.1021/jacs.5b09494
J. Am. Chem. Soc. 2015, 137, 14252−14255

does not contain disulfide bonds, can be expressed in high yield in bacteria to allow for *in vitro* characterization, and has been shown to be capable of binding to a number of proteinaceous targets through directed evolution.[10] We hypothesized that Fn3 domains could also bind to small molecules given their structural homology to antibodies from camelides, which naturally contain a signal variable region termed V_HH.[11] In particular, V_HH domains have been shown to bind to a variety of small molecules with affinities comparable to multidomain antibodies[12] and can form deep clefts that desolvate the bound small molecule to a similar extent as Fab or scFv antibody designs.[13] Building upon these observations, we investigated whether Fn3 domains could be evolved to bind a small-molecule guest. To achieve this goal we chose to utilize yeast surface display[14] in order to identify small-molecule-binding Fn3 domains through affinity maturation (Figure 1b).

As the small-molecule target we chose the merocyanine class of environment-sensitive fluorophores.[15] Importantly, members of this class of dyes display large enhancements in fluorescence in response to changes in the solvent environment, are bright, and exhibit excitation and emission peaks in the red region of the spectrum allowing for suppression of autofluorescence in biological samples. The environment-sensitive nature of these fluorophores enables the direct enrichment of yeast displaying Fn3 domains that are capable of binding and activating dye fluorescence in aqueous solutions by using fluorescence-activated cell sorting (FACS). Accordingly, we chose to synthesize a water-soluble version of the so-called I-Pht merocyanine derivative[15a] containing a sulfate group, sI-Pht (Figure 1c). As expected, the fluorescence of sI-Pht was highly sensitive to the solvent environment (Figure 1c).

In order to identify mutant Fn3 hosts capable of binding the sI-Pht guest, we affinity matured a previously described library of Fn3 domains[14a] against sI-Pht. After each round of selection, enriched Fn3 domains were diversified by error-prone PCR and loop shuffling.[14a,b] During early rounds of selections, low affinity binders were enriched using bead-based affinity pull-downs with biotin tagged sI-Pht derivatives (Figure S1). Subsequent rounds of selections were performed by directly assaying the fluorescence enhancement of untagged sI-Pht (Figure 1c) bound to displayed Fn3 domains using FACS. After six total rounds of evolution, a population of yeast displaying Fn3 domains with clear sI-Pht binding capacity were isolated (Figure S2). One hundred and five single colonies from this population were individually screened for enhancement of sI-Pht fluorescence on the surface of yeast. From these experiments, 17 colonies were discovered that displayed >8-fold fluorescence enhancements compared to cells displaying wild-type Fn3 (wtFn3, Figure S3). Sequencing of these clones revealed a strong consensus for the mutant sequence termed 6.2.18, which represented 47% of the sequenced clones (Table 1). In mutant 6.2.18, a two-amino-acid shortening of the BC loop relative to wtFn3 and a clear preference for aromatic residues is observed. The DE loop maintained a preference for Thr at position 5, consistent with wtFn3. Positions 1 and 2 of the DE loop displayed a preference for Ser, while Val and Lys were preferred at positions 3 and 4, respectively. Lastly, the FG loop displayed a preference for aromatic residues at the N-terminus and hydrophilic residues at the C-terminus. Overall, the preference for hydrophobic residues in the presumed sI-Pht binding pocket reinforces the general utilization of hydrophobic binding surfaces by supramolecular architectures in water.[3]

Table 1. Selected Sequences of Evolved Fn3 Hosts

clone	amino acid sequences				instances
	BC loop	DE loop	FG loop	framework[a]	
wtFn3	DAPAVTVR	GSKST	GRGDSPASSK	N/A	N/A
6.2.2	YYPGVH	SSVKT	YYGFGVSSSR	E9G	1
6.2.3	YYPGVH	GSFST	YYGFGVSSSR	D7G, T39A, V45A, Q46R	1
6.2.18	YYPGVH	SSVKT	YYGFGVSSSR	N/A	8
6.2.22	YYPGVH	GAWST	YHGFGVSSSR	N/A	1
6.2.83	YYPGVH	SSVRT	YYGFGVSSSR	N/A	1
6.2.85	YYPGVH	SSVKT	YYGFGMSSSR	N/A	1
6.2.89	HYPGVH	SSVKT	YYGFGVSSSR	D3N, V4G	1
6.2.92	RYPGVH	SSVKT	YYGFGVSSSR	N/A	1
6.2.95	YYPGVH	SSVKT	YYGFGVSSSR	K98R	1
6.2.101	YYPGVH	GAWST	YYGFGVSSSR	N/A	1

[a]Mutations refer to residues outside the loop regions.

The consensus mutant, 6.2.18, was chosen for further *in vitro* characterization. A maltose binding protein (MBP) fusion of 6.2.18 (MBP-6.2.18) was expressed in bacteria; fractions corresponding to monomeric protein were isolated by FPLC and characterized by MALDI-MS (Figure S4). We also expressed an MBP fusion to wtFn3 (MBP-wtFn3) as a control. CD spectrometry experiments indicated that MBP-6.2.18 maintained a similar fold to MBP-wtFn3 (Figure 2a). Since efforts to obtain

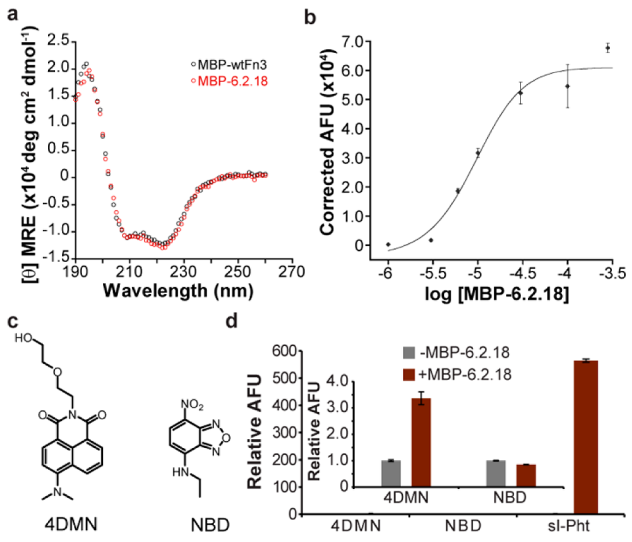


Figure 2. (a) Overlay of the CD spectrum of MBP-wtFn3 and MBP-6.2.18 (2 μM each). (b) Fluorescence of sI-Pht (500 nM) as a function of MBP-6.2.18 concentration. Excitation = 575 nm, emission = 619 nm. (c) Structures of off-target environment-sensitive dye analogues 4DMN and NBD. (d) Fluorescence of environment-sensitive dyes (500 nM) in the absence or presence of MBP-6.2.18 (10 μM). Excitation wavelengths were 460, 485, and 575 nm, and emission maxima were 545, 565, and 615 nm, for 4DMN, NBD, and sI-Pht, respectively.

sufficient quantities of His-tagged protein for CD studies proved unsuccessful, we cannot rule out subtle differences between the structures of wtFn3 and 6.2.18 that may be obscured by the MBP fusion. However, the similarity of the CD spectra of the MBP fusions along with the bias of yeast surface display for folded proteins,[14c] argues against substantial structural differences between monomeric wtFn3 and 6.2.18. Initial titration experi-

DOI: 10.1021/jacs.5b09494
J. Am. Chem. Soc. 2015, 137, 14252−14255

ments using increasing concentrations of sI-Pht produced significant fluorescence quenching due to dye aggregation at concentrations above 1 μM (Figure S5), a phenomena that is commonly observed for this class of dyes.[16] Consequently, the fluorescence of varying concentrations of purified MBP-6.2.18 in the presence of 500 nM sI-Pht was assessed (Figure 2b). These experiments demonstrated that purified MBP-6.2.18 maintained the ability to bind sI-Pht with an EC_{50} of 14 μM. Importantly, MBP-wtFn3 showed no appreciable binding to sI-Pht at equivalent concentrations, ruling out off-target binding from the MBP tag (Figure S6). The excitation and emission maxima of sI-Pht bound to MBP-6.2.18 were determined to be 591 and 612 nm, respectively (Figure S7). These values are similar to those obtained in organic solvents (Figure 1c) and indicate that sI-Pht is bound in a hydrophobic pocket on 6.2.18. Assessment of the fluorescence activation of sI-Pht showed a 570-fold increase in fluorescence upon binding, yielding a bright complex with ε = 78 000 $M^{-1}cm^{-1}$ and Φ = 0.26. To test the specificity of MBP-6.2.18 for sI-Pht, we incubated MBP-6.2.18 with equivalent concentrations of two structurally diverse environment-sensitive fluorophores known as 4DMN[17] and NBD[18] (Figure 2c). No significant off-target binding was observed in these assays, indicating that 6.2.18 is capable of selective binding to sI-Pht (Figure 2d). These results definitively demonstrate the ability of the Fn3 host to selectively recognize small-molecule guests as well as robustly activate the fluorescence of environment-sensitive fluorophores. To the best of our knowledge, 6.2.18 is the first evolved Fn3 domain with small-molecule-binding activity.

Titration of sI-Pht with MBP-6.2.18 indicated positive cooperativity in this system (Hill slope = 19, Figure 2b). To further address binding stoichiometry in this system a Job's plot assay was performed (Figure 3a). Interestingly, this analysis

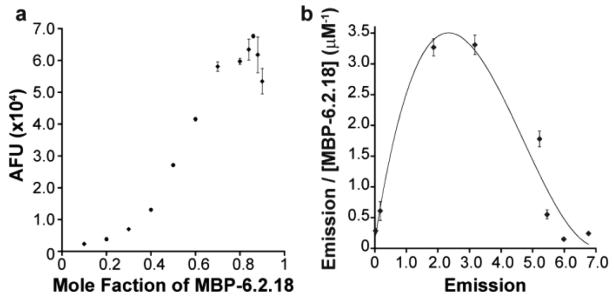


Figure 3. (a) Job's plot analysis, indicating that more than one molecule of MBP-6.2.18 is involved in formation of a fluorescent complex. The remaining mole fraction in this experiment corresponds to sI-Pht. (b) Scatchard plot, demonstrating that complex formation proceeds through positively cooperative binding.

clearly showed that binding in this system was not 1:1 and instead pointed to a higher order binding mode in which sI-Pht binds to multiple copies of the host protein. A Scatchard plot also demonstrated positive cooperativity in this system, indicating that additional protein binding events are more favorable during formation of the complex (Figure 3b). To further investigate the molecular architecture of this system, we performed sedimentation velocity experiments before and after addition of sI-Pht (Figure 4). In the absence of sI-Pht, MBP-6.2.18 (50 μM) exists as a primarily monomeric species (74.8%). The equilibrium between dimer and monomer is shifted toward dimer at higher concentrations of protein (Figure S8). However, upon addition of sI-Pht, the appearance of a third higher molecular weight

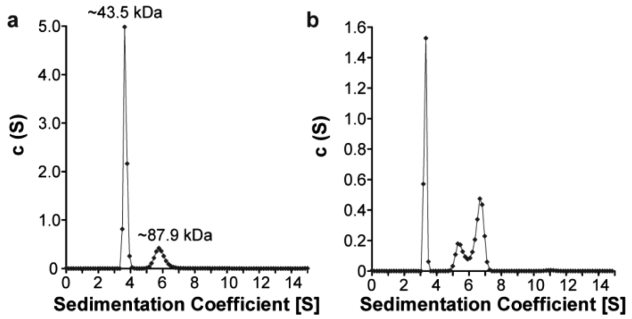


Figure 4. Sedimentation velocity experiments containing MBP-6.2.18 (50 μM) alone (a) or in the presence (b) of sI-Pht (5 μM) monitored at 295 nm. The calculated molecular weight of MBP-6.2.18 is 53.8 kDa.

species is clearly observed (Figure 4b). This new complex (~264 kDa) could be directly observed by monitoring samples using the absorbance of sI-Pht (Figure S9). These experiments indicated that the higher order complex corresponds to the assembly of approximately five MBP-6.2.18 proteins. Together with ITC data (Figure S10) and the Job's plot assay (Figure 3a), we estimate a stoichiometry of 5:1 (MBP-6.2.18:sI-Pht) for supramolecular complexation. Although these data provide clear evidence for complex formation our determination of binding stoichiometry should be viewed as an estimate due to inherent errors associated in molecular weight calculations as well as binding ratios in these experiments. Future work will be focused on high-resolution structural studies in order to more definitively resolve the molecular architecture of this complex. Nonetheless, these data demonstrate the ability to utilize the sI-Pht guest to control the oligomeric state of the 6.2.18 host, producing a highly fluorescent complex upon assembly. We propose that supramolecular assembly provides an evolutionary advantage during FACS enrichment, since greater desolvation of the sI-Pht fluorophore would lead to corresponding enhancements in fluorescence.

In order to demonstrate sI-Pht-induced supramolecular complexation of 6.2.18 in a cellular context, 6.2.18 or wtFn3 was displayed on the surface of living yeast cells by fusion to Aga2 (Figure 1b) and imaged using confocal microscopy. Labeling of the C-terminal c-myc epitope tag with an Alexa Fluor 488 conjugated antibody allowed for the visualization of protein expressed on the cell surface (Figure 5). Addition of sI-Pht to both samples resulted in a clearly observable turn-on red fluorescence signal on the surface of cells expressing 6.2.18, which colocalized with c-myc labeling. Importantly, cells

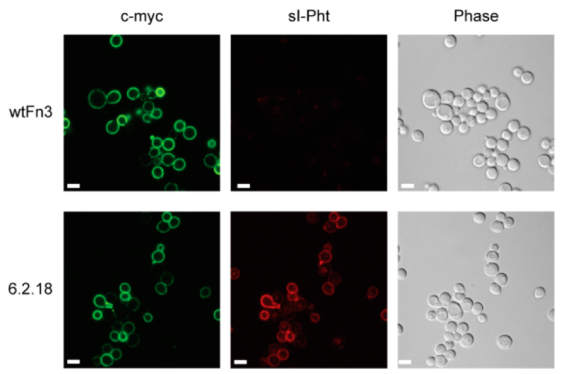


Figure 5. Confocal imaging of wtFn3 (top) and 6.2.18 (bottom) displayed on the surface of living yeast cells. Images are false colored for c-myc or sI-Pht (200 nM) labeling where indicated. Scale bars = 5 μm.

DOI: 10.1021/jacs.5b09494
J. Am. Chem. Soc. 2015, 137, 14252−14255

expressing wtFn3 did not display red fluorescence under these conditions, demonstrating the selectivity of the small-molecule guest. Moreover, due to the turn-on signal generated from supramolecular assembly in this system, washing was not necessary in order to obtain images of the bound species.

In conclusion, we have detailed an approach for the evolution of a miniprotein host capable of assembling into a higher order complex in response to addition of an environment-sensitive small-molecule guest, producing a fluorogenic readout of complex formation. In the long term, this system may allow for the construction of artificial materials as well as signaling pathways that are dependent upon an external small-molecule input. Utilizing this system, supramolecular complexation can be visualized on the surface of living cells. In the long term, the ability to selectively visualize protein assemblies in real time could allow for the fundamental investigation of receptor trafficking dynamics upon activation. Our laboratory is also currently pursuing Fn3 domains capable of binding spectrally orthogonal environment-sensitive dyes as well as modifying the current system in order to obtain cell-permeable derivatives of sI-Pht.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b09494.

Experimental methods and additional characterization data, including Figures S1−S10 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*cstains2@unl.edu

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the Wittrup laboratory for providing the Fn3 domain library as well as the EBY100 yeast strain, the Morrison Miscopy Facility, the Biophysical Core, the Research Instrumentation/NMR facility, and the Nebraska Center for Mass Spectrometry. We acknowledge Prof. D. Becker for helpful discussions and D. Rajasekhar Reddy for providing biotin-containing linkers as well as helpful advice. Funding was from a Nebraska EPSCoR First Award and the University of Nebraska—Lincoln.

■ REFERENCES

(1) (a) Lehn, J. M. *Science* **1985**, *227*, 849. (b) Lehn, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 4763.

(2) (a) Hernandez-Gordillo, V.; Chmielewski, J. *Biomaterials* **2014**, *35*, 7363. (b) Klarner, F. G.; Kahlert, B.; Nellesen, A.; Zienau, J.; Ochsenfeld, C.; Schrader, T. *J. Am. Chem. Soc.* **2006**, *128*, 4831. (c) Przybyla, D. E.; Rubert Perez, C. M.; Gleaton, J.; Nandwana, V.; Chmielewski, J. *J. Am. Chem. Soc.* **2013**, *135*, 3418. (d) Renner, C.; Piehler, J.; Schrader, T. *J. Am. Chem. Soc.* **2006**, *128*, 620. (e) You, L.; Zha, D.; Anslyn, E. V. *Chem. Rev.* **2015**, *115*, 7840. (f) Anslyn, E. V. *J. Org. Chem.* **2007**, *72*, 687. (g) Miller, R. A.; Presley, A. D.; Francis, M. B. *J. Am. Chem. Soc.* **2007**, *129*, 3104.

(3) Uhlenheuer, D. A.; Petkau, K.; Brunsveld, L. *Chem. Soc. Rev.* **2010**, *39*, 2817.

(4) Zhang, L.; Wu, Y.; Brunsveld, L. *Angew. Chem., Int. Ed.* **2007**, *46*, 1798.

(5) (a) Dang, D. T.; Nguyen, H. D.; Merkx, M.; Brunsveld, L. *Angew. Chem., Int. Ed.* **2013**, *52*, 2915. (b) Dang, D. T.; Schill, J.; Brunsveld, L. *Chem. Sci.* **2012**, *3*, 2679. (c) Nguyen, H. D.; Dang, D. T.; van Dongen, J. L.; Brunsveld, L. *Angew. Chem., Int. Ed.* **2010**, *49*, 895. (d) Uhlenheuer, D. A.; Young, J. F.; Nguyen, H. D.; Scheepstra, M.; Brunsveld, L. *Chem. Commun.* **2011**, *47*, 6798.

(6) (a) Heitmann, L. M.; Taylor, A. B.; Hart, P. J.; Urbach, A. R. *J. Am. Chem. Soc.* **2006**, *128*, 12574. (b) Logsdon, L. A.; Schardon, C. L.; Ramalingam, V.; Kwee, S. K.; Urbach, A. R. *J. Am. Chem. Soc.* **2011**, *133*, 17087. (c) Smith, L. C.; Leach, D. G.; Blaylock, B. E.; Ali, O. A.; Urbach, A. R. *J. Am. Chem. Soc.* **2015**, *137*, 3663.

(7) (a) Banaszynski, L. A.; Wandless, T. J. *Chem. Biol.* **2006**, *13*, 11. (b) Liang, F. S.; Ho, W. Q.; Crabtree, G. R. *Sci. Signaling* **2011**, *4*, rs2. (c) Lin, H. N.; Abida, W. M.; Sauer, R. T.; Cornish, V. W. *J. Am. Chem. Soc.* **2000**, *122*, 4247. (d) Miyamoto, T.; DeRose, R.; Suarez, A.; Ueno, T.; Chen, M.; Sun, T. P.; Wolfgang, M. J.; Mukherjee, C.; Meyers, D. J.; Inoue, T. *Nat. Chem. Biol.* **2012**, *8*, 465. (e) Putyrski, M.; Schultz, C. *FEBS Lett.* **2012**, *586*, 2097. (f) Spencer, D. M.; Wandless, T. J.; Schreiber, S. L.; Crabtree, G. R. *Science* **1993**, *262*, 1019.

(8) (a) Karginov, A. V.; Zou, Y.; Shirvanyants, D.; Kota, P.; Dokholyan, N. V.; Young, D. D.; Hahn, K. M.; Deiters, A. *J. Am. Chem. Soc.* **2011**, *133*, 420. (b) Brown, K. A.; Zou, Y.; Shirvanyants, D.; Zhang, J.; Samanta, S.; Mantravadi, P. K.; Dokholyan, N. V.; Deiters, A. *Chem. Commun.* **2015**, *51*, 5702. (c) Ballister, E. R.; Aonbangkhen, C.; Mayo, A. M.; Lampson, M. A.; Chenoweth, D. M. *Nat. Commun.* **2014**, *5*, 5475. (d) Ballister, E. R.; Ayloo, S.; Chenoweth, D. M.; Lampson, M. A.; Holzbaur, E. L. *Curr. Biol.* **2015**, *25*, R407. (e) Schelkle, K. M.; Griesbaum, T.; Ollech, D.; Becht, S.; Buckup, T.; Hamburger, M.; Wombacher, R. *Angew. Chem., Int. Ed.* **2015**, *54*, 2825.

(9) Koide, A.; Bailey, C. W.; Huang, X. L.; Koide, S. *J. Mol. Biol.* **1998**, *284*, 1141.

(10) Hackel, B. J.; Kapila, A.; Wittrup, K. D. *J. Mol. Biol.* **2008**, *381*, 1238.

(11) Parker, M. H.; Chen, Y.; Danehy, F.; Dufu, K.; Ekstrom, J.; Getmanova, E.; Gokemeijer, J.; Xu, L.; Lipovsek, D. *Protein Eng., Des. Sel.* **2005**, *18*, 435.

(12) (a) Spinelli, S.; Frenken, L. G. J.; Hermans, P.; Verrips, T.; Brown, K.; Tegoni, M.; Cambillau, C. *Biochemistry* **2000**, *39*, 1217. (b) Yau, K. Y. F.; Groves, M. A. T.; Li, S. H.; Sheedy, C.; Lee, H.; Tanha, J.; MacKenzie, C. R.; Jermutus, L.; Hall, J. C. *J. Immunol. Methods* **2003**, *281*, 161. (c) Doyle, P. J.; Arbabi-Ghahroudi, M.; Gaudette, N.; Furzer, G.; Savard, M. E.; Gleddie, S.; McLean, M. D.; Mackenzie, C. R.; Hall, J. C. *Mol. Immunol.* **2008**, *45*, 3703. (d) Alvarez-Rueda, N.; Behar, G.; Ferre, V.; Pugniere, M.; Roquet, F.; Gastinel, L.; Jacquot, C.; Aubry, J.; Baty, D.; Barbet, J.; Birkle, S. *Mol. Immunol.* **2007**, *44*, 1680. (e) Tabares-da Rosa, S.; Rossotti, M.; Carleiza, C.; Carrion, F.; Pritsch, O.; Ahn, K. C.; Last, J. A.; Hammock, B. D.; Gonzalez-Sapienza, G. *Anal. Chem.* **2011**, *83*, 7213. (f) Spinelli, S.; Tegoni, M.; Frenken, L.; van Vliet, C.; Cambillau, C. *J. Mol. Biol.* **2001**, *311*, 123.

(13) Holliger, P.; Hudson, P. J. *Nat. Biotechnol.* **2005**, *23*, 1126.

(14) (a) Boder, E. T.; Wittrup, K. D. *Nat. Biotechnol.* **1997**, *15*, 553. (b) Chen, T. F.; de Picciotto, S.; Hackel, B. J.; Wittrup, K. D. *Methods Enzymol.* **2013**, *523*, 303. (c) Colby, D. W.; Kellogg, B. A.; Graff, C. P.; Yeung, Y. A.; Swers, J. S.; Wittrup, K. D. *Methods Enzymol.* **2004**, *388*, 348.

(15) (a) MacNevin, C. J.; Gremyachinskiy, D.; Hsu, C. W.; Li, L.; Rougie, M.; Davis, T. T.; Hahn, K. M. *Bioconjugate Chem.* **2013**, *24*, 215. (b) Toutchkine, A.; Nguyen, D. V.; Hahn, K. M. *Bioconjugate Chem.* **2007**, *18*, 1344. (c) Yapici, I.; Lee, K. S. S.; Berbasova, T.; Nosrati, M.; Jia, X. F.; Vasileiou, C.; Wang, W. J.; Santos, E. M.; Geiger, J. H.; Borhan, B. *J. Am. Chem. Soc.* **2015**, *137*, 1073.

(16) Rosch, U.; Yao, S.; Wortmann, R.; Wurthner, F. *Angew. Chem., Int. Ed.* **2006**, *45*, 7026.

(17) (a) Kollar, J.; Hrdlovic, P.; Chmela, S.; Sarakha, M.; Guyot, G. *J. Photochem. Photobiol., A* **2005**, *170*, 151. (b) Loving, G.; Imperiali, B. *J. Am. Chem. Soc.* **2008**, *130*, 13630. (c) Loving, G.; Imperiali, B. *Bioconjugate Chem.* **2009**, *20*, 2133.

(18) (a) Lancet, D.; Pecht, I. *Biochemistry* **1977**, *16*, 5150. (b) Ghosh, P. B.; Whitehouse, M. W. *Biochem. J.* **1968**, *108*, 155.

DOI: 10.1021/jacs.5b09494
J. Am. Chem. Soc. 2015, 137, 14252−14255