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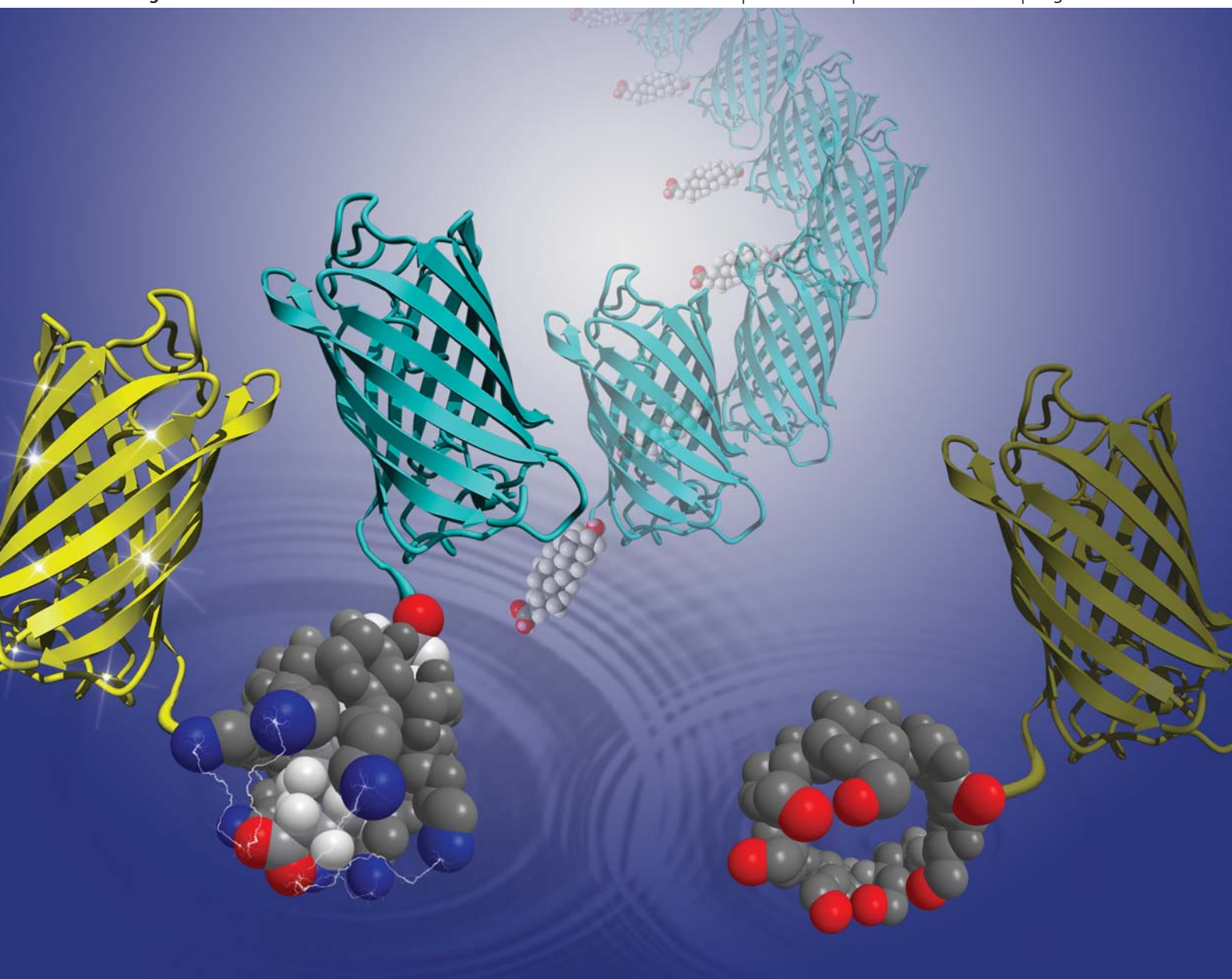

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COMMUNICATION

Strong supramolecular control over protein self-assembly using a polyamine decorated β-cyclodextrin as synthetic recognition element†‡

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The supramolecular host molecule heptakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-cyclodextrin provides strong control over protein self-assembly in synthetic supramolecular protein constructs. Mono-functionalization of this modified β-cyclodextrin with a cysteine residue allows for site-selective synthetic conjugation to a protein and formation of a highly stable synthetic protein complex with a lithocholic acid conjugated protein as the interaction partner.

Biomolecules such as proteins interact *via* reversible supramolecular interactions, controlling localization and function. Gaining control over these self-assembly mechanisms is key to understanding and targeting (dysfunctional) biological processes in the disease state. Synthetic supramolecular materials chemistry offers a key entry point to generate completely novel synthetic biological systems with orthogonal biomimetic functions.[1–7] In the field of supramolecular chemical biology, the usage of synthetic supramolecular host–guest systems is especially attractive in this respect, due to the biochemical origin of many of these systems and their concomitant bioapplicability.[8] Synthetic host–guest systems can for example be applied to the controlled and reversible dimerization of proteins, by chemical appendage of supramolecular elements to proteins in a site-selective manner.[9] Such systems enable the induction or stabilization of protein-protein interactions in a manner, which is orthogonal to existing protein domain-based approaches.[10] Similarly, supramolecular host molecules can be used to recognize specific protein elements,[11–13] and applied as supramolecular inducers of protein dimerization.[14,15] Here, we demonstrate the novel use of heptakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-cyclodextrin as a strong chemical building block for the controlled assembly and disassembly of synthetic biological architectures.

Previously, we showed how the host–guest complex formed between lithocholic acid and β-cyclodextrin (**1**) can be used for the controlled self-assembly of protein heterodimers in buffered solution[16] and in cells.[17] With a $K_d \approx 1$ μM[18] and given the ease with which mono-functionalized derivatives of these host–guest molecules can be prepared, this supramolecular complex is thus well suited to protein dimerization at micromolar concentrations. However, since multiple protein-protein interactions occur at sub-micromolar concentrations, host–guest complexes with higher affinity are required to enable the controlled formation of supramolecular protein dimers at even lower concentrations than has thus far been possible. Recently, Nitz *et al.* reported that lithocholic acid and heptakis-[6-deoxy-6-(2-aminoethyl-sulfanyl)]-β-cyclodextrin (**2**) form a host–guest complex with $K_a = 5.5 \times 10^7$ M^{-1} ($K_d = 0.02$ μM) in buffered solution at pH 7.5.[19] Applying this strong synthetic complex for protein assembly would in principle provide an entry point for supramolecular control over protein dimerization at concentrations lower than has been achieved using the 'naked' β-cyclodextrin-based system.

In a first experiment, **2**[20] was evaluated as an inhibitor of the supramolecular protein complex formed between a monomeric lithocholic acid-modified monomeric cyan fluorescent protein (mCFP) and a β-cyclodextrin-modified monomeric yellow fluorescent protein (mYFP).[16,17] Monomeric fluorescent protein variants of this kind are mutated at the amino acid position 206 – from an alanine to a lysine (A206K) – in order to suppress intrinsic protein dimerization.[21,22] The interaction between mCFP and mYFP can be conveniently followed by fluorescence spectroscopy, measuring Förster Resonance Energy Transfer (FRET).[23] Thus, the addition of increasing equivalents of **2** led to the progressive inhibition of the synthetic supramolecular protein dimer, which could be observed by a decrease in the FRET signal at 527 nm (Fig. 1). Encouragingly, **2** was found to induce a loss of FRET signal in a concentration range 10 times *lower* than that measured for the normal β-cyclodextrin **1** (ESI, Supporting Figure 10). Through an enhancement of binding affinity, **2** thus competes more effectively than **1** with the protein dimer, and provides an entry for exploration in synthetic protein conjugates.

The above reported result prompted us to selectively modify the mYFP protein with this synthetic supramolecular building block to strongly induce biological self-assembly. While the syntheses of C7-symmetric cyclodextrin analogues derived from **2** are already known, including a number of impressive applications,[20,24,25] mono-functionalized variants have hardly been addressed.[26] Here, we describe the novel synthesis and purification of a mono-functionalized heptakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-cyclodextrin cysteine

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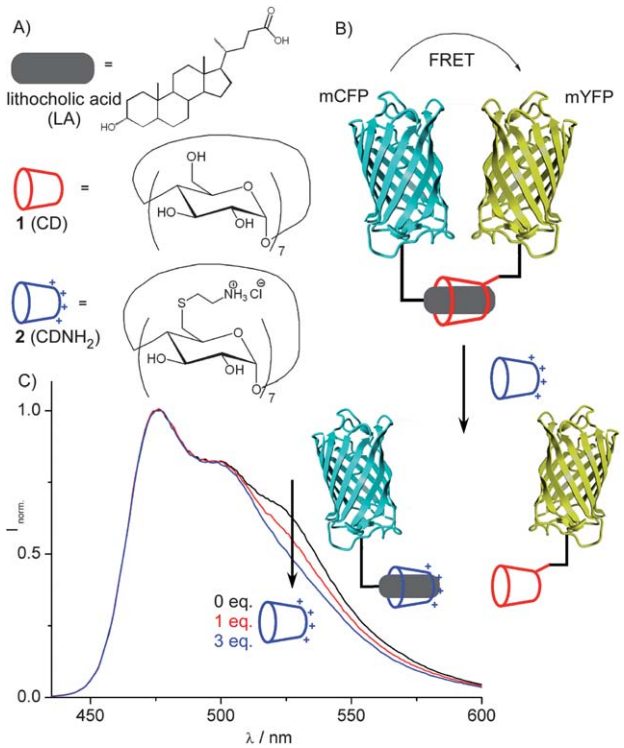
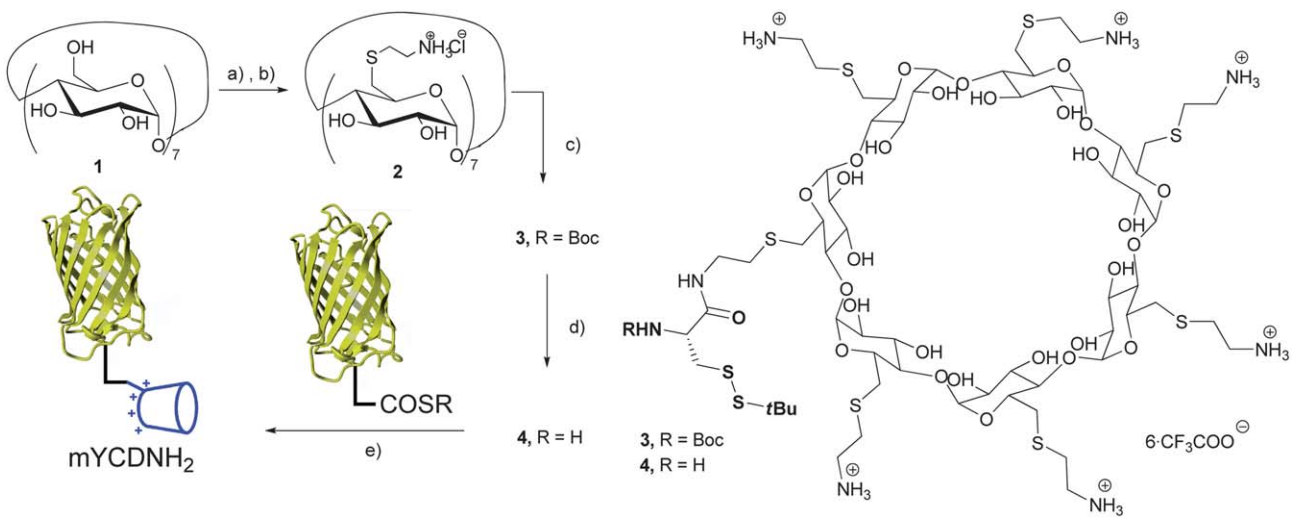


Fig. 1 (A) Used host–guest molecules, (B) reversal of supramolecular protein dimer, monomeric fluorescent proteins represented by backbone folds of cyan (mCFP) and yellow (mYFP) fluorescent proteins, (C) fluorescence emission spectra showing disappearance of FRET signal due to increasing concentration of polyamine cyclodextrin **2**. Fluorescence emission spectra of mCFP-mYFP synthetic protein complex (0.5 μM, $\lambda_{exc}^{(CFP)}$ 410 nm) before (black) and after addition of 0.5 μM (red) and 1.5 μM **2**, showing a disappearance of the mYFP emission at around 527 nm.

derivative for attachment to a fluorescent protein *via* expressed protein ligation[16,17] (Scheme 1). The known hydrochloride salt of **2** can be prepared in two steps starting from commercially available **1**.[19,20,25,27] Therefore, **1** was first converted at the C6 position to the bromide in accordance with literature protocol.[28] The obtained per-6-bromo-β-cyclodextrin was then treated with cysteamine hydrochloride under basic conditions to yield pure **2**,[20] which was to be subsequently reacted with *N*-Boc- and *S*-StBu-protected cysteine. The *S*-StBu protective group is strategically incorporated to enable *in situ* deprotection immediately prior to protein ligation. Unlike the mono-functionalization of β-cyclodextrin, which is a well studied and much used reaction sequence,[29] the mono-functionalization of **2** is now being investigated. We predicted that treating **2** with a suitably pre-activated cysteine derivative would produce a complex mixture. and reasoned, due to the excellent water solubility of **2**, that the desired mono-functionalized derivative (**3**) could potentially be separated using reversed-phase HPLC. In practice, the protected cysteine derivative was thus pre-activated as the hydroxysuccinimide ester[30] and added drop-wise to a DMSO solution of **2** in the presence of excess triethylamine. As expected, formation of mono-substituted product **3** could be observed by LC/MS, along with unreacted **2** and the di- and tri-substituted cyclodextrin, presumably as ill-defined mixtures. Somewhat surprising, however, was the high degree of separation achieved in this case between **3** and the remaining components (ESI, Supporting Figure 2), which formed an excellent basis for further purification. Therefore, using an optimized gradient on a reversed-phase HPLC system, it was possible to separate **3** from the other cyclodextrin components present in >95% purity (ESI). After successful isolation of the mono-functionalized **3**, deprotection of the Boc-group was performed in a quantitative manner using 95% TFA/H_2O to yield the pure trifluoroacetate salt **4** for use in our planned protein ligation studies.

The mono-functional cysteine-cyclodextrin derivative **4** was then ligated to mYFP, which had previously been successfully ligated to the normal β-cyclodextrin (**1**).[16] We chose the monomeric fluorescent protein in this case because, for this variant, the affinity of the supramolecular protein dimer is determined predominantly by the supramolecular host–guest interaction and not by any intrinsic binding affinity between the two proteins.[16] Therefore, **4** was first thiol-deprotected using an excess of TCEP in phosphate buffer and



Scheme 1 Synthesis of mono-functionalized cysteamine-β-CD (**4**) and ligation to the thioester of mYFP: (a) *N*-bromo-succinimide, PPh_3, DMF, 75 °C, 4 h, 60%;[28] (b) cysteamine hydrochloride, Et_3N in DMF, 72 h, rt, 89%; (c) BocCys*St*Bu-OSu,[30] Et_3N, DMSO, 22 h, rt, 13%; (d) 95% TFA/H_2O, 3 h, rt, quantitative; (e) phosphate buffer pH 7.5, TCEP, MPAA, rt, overnight, 60%.

then added directly to the protein thioester. Ligation of the protein was performed overnight at room temperature using 18 eq. of **4**. To remove the excess of ligand, the buffer was exchanged after ligation to TrisHCl (pH 8) containing 50 mM NaCl. In the LC/MS spectrum of the protein ligation product, a small quantity of non-functionalized protein could be detected. For purification of the desired protein-conjugate, ion exchange chromatography was applied. It was reasoned that the ligation of the positive highly-charged cyclodextrin to the protein would facilitate its sequestration from the starting protein thioester based on charge differences. Assuming protein modification with **4** to be equal to the introduction of six unprotected lysine side chains, a difference in isoelectric point (pI) of 1.1 between the modified (pI = 6.8) and the unmodified protein (pI = 5.7) was calculated. A strong anion exchange resin was then selected for purification, and the protein eluted with increasing concentrations of NaCl. The first fractions contained pure modified protein (named $mYCDNH_2$ hereafter) which was followed by increasing amounts of unmodified protein. Analysis *via* SDS-PAGE and LC/MS showed that the purification strategy was successful (ESI).

Protein assembly was next investigated using fluorescence spectroscopy. To study the applicability of the newly-modified cyclodextrin **4** for supramolecular protein dimerization, the ligated protein $mYCDNH_2$ was mixed with mCFP ligated to lithocholic acid (mCLA). The efficiency of the $mYCDNH_2$–mCLA assembly was compared with a synthetic supramolecular protein dimer based on the 'naked' β-cyclodextrin (mYCD-mCLA). Fluorescence spectra were recorded using an excitation wavelength of 410 nm which is optimized for mCFP excitation and performed in phosphate buffer containing 1 mM TCEP. The TCEP was added to ensure that no disulfide bridges were formed between the free cysteine residues remaining after protein ligation.[16] The two synthetic proteins were mixed in a 1 : 1 ratio and fluorescence emission spectra were recorded (Fig. 2). In principle, successful protein heterodimerization results in FRET, which is visible as an increase in mYFP emission at 527 nm and a decrease in mCFP emission at 475 nm. In practice, a very strong FRET signal was observed for the protein mixture containing both functionalized proteins: mCLA and $mYCDNH_2$ (Fig. 2, blue). This strong FRET effect clearly indicated that a supramolecular protein hetero-dimer was formed by virtue of the host–guest elements. In a control experiment, $mYCDNH_2$ was mixed with the non-functionalized mCFP thioester protein. In this case, no increase in FRET was detected (Fig. 2, black), showing that cyclodextrin **4** itself does not induce unspecific protein assembly. Interestingly, direct comparison of the two β-cyclodextrin-based supramolecular protein dimers (mYCD and $mYCDNH_2$) revealed a significantly higher FRET effect for the $mYCDNH_2$ based complex (Fig. 2, blue), with a mYFP/mCFP ratio (I(527 nm)/I(475 nm)) of 1.18 compared to 0.67 for the mYCD based complex. This finding is in agreement with the original design of the host molecule **4**, which is expected to show significantly higher binding affinity for lithocholic acid compared with β-cyclodextrin in the micromolar regime, and possibly facilitate a better orientation for energy transfer. These results show that the supramolecular host–guest complex of lithocholic acid and heptakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-cyclodextrin is a highly efficient synthetic system for the controlled assembly of two proteins.

In conclusion, we have demonstrated that **2** and novel mono-functionalized derivative **4** are two attractive synthetic supramolecular elements for the controlled assembly and disassembly of proteins. A facile synthetic approach to mono-functionalized heptakis-[6-deoxy-6-(2-aminoethylsulfanyl)]-β-cyclodextrin was devised and the resulting synthetic building block successfully conjugated to a fluorescent protein. This optimized synthetic host–guest complex enabled enhanced energy transfer between two proteins at lower concentrations than had been previously achieved, resulting in a roughly two-fold increase in mYFP/mCFP ratio at 0.5 μM. Alternatively, the known β-cyclodextrin derivative **2** is a valuable inhibitor of synthetic supramolecular protein assembly in its own right, capable of complete inhibition of supramolecular protein

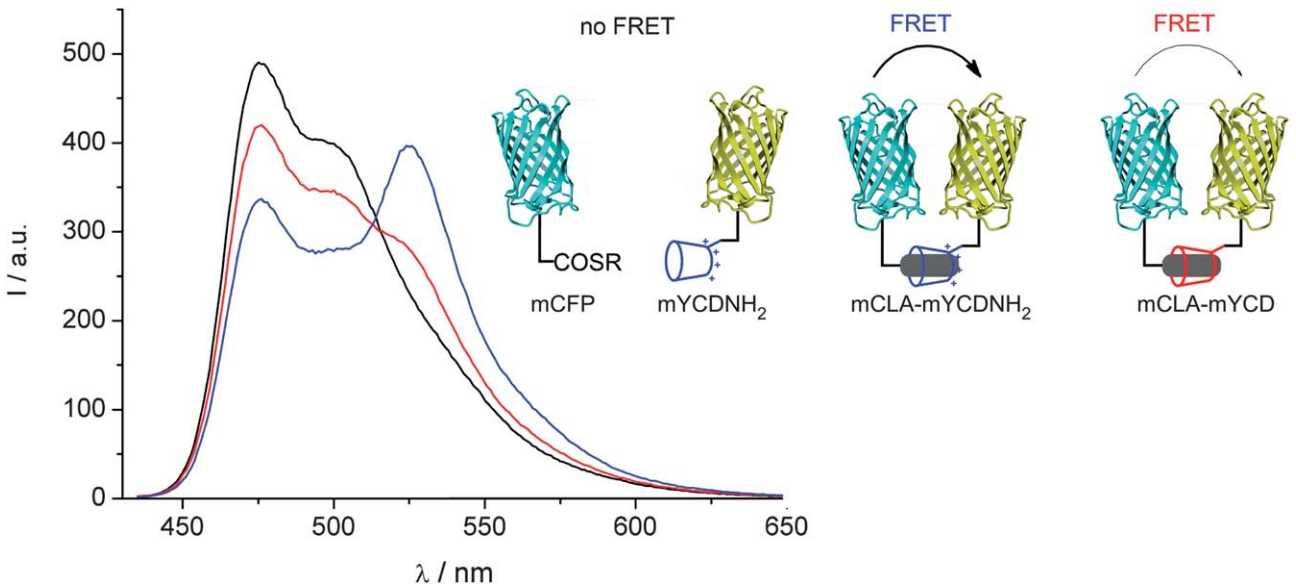


Fig. 2 Fluorescence emission spectra of 1 : 1 mixtures of 0.5 μM for each protein in phosphate buffer containing 1 mM TCEP; mCFP and $mYCDNH_2$ (reference, black), mCLA and mYCD (red), mCLA and $mYCDNH_2$ (blue). Fluorescence spectra were recorded using an excitation wavelength of 410 nm which is optimized for mCFP excitation.

dimerization, over ten-fold more potent than the 'naked' β-cyclodextrin. This well-defined host–guest system is an ideal platform for further application in synthetic biological constructs, addressing for example protein self-assembly or protein surface immobilization[3,31,32] and makes supramolecular protein architectures even more attractive for controlling biological processes.

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