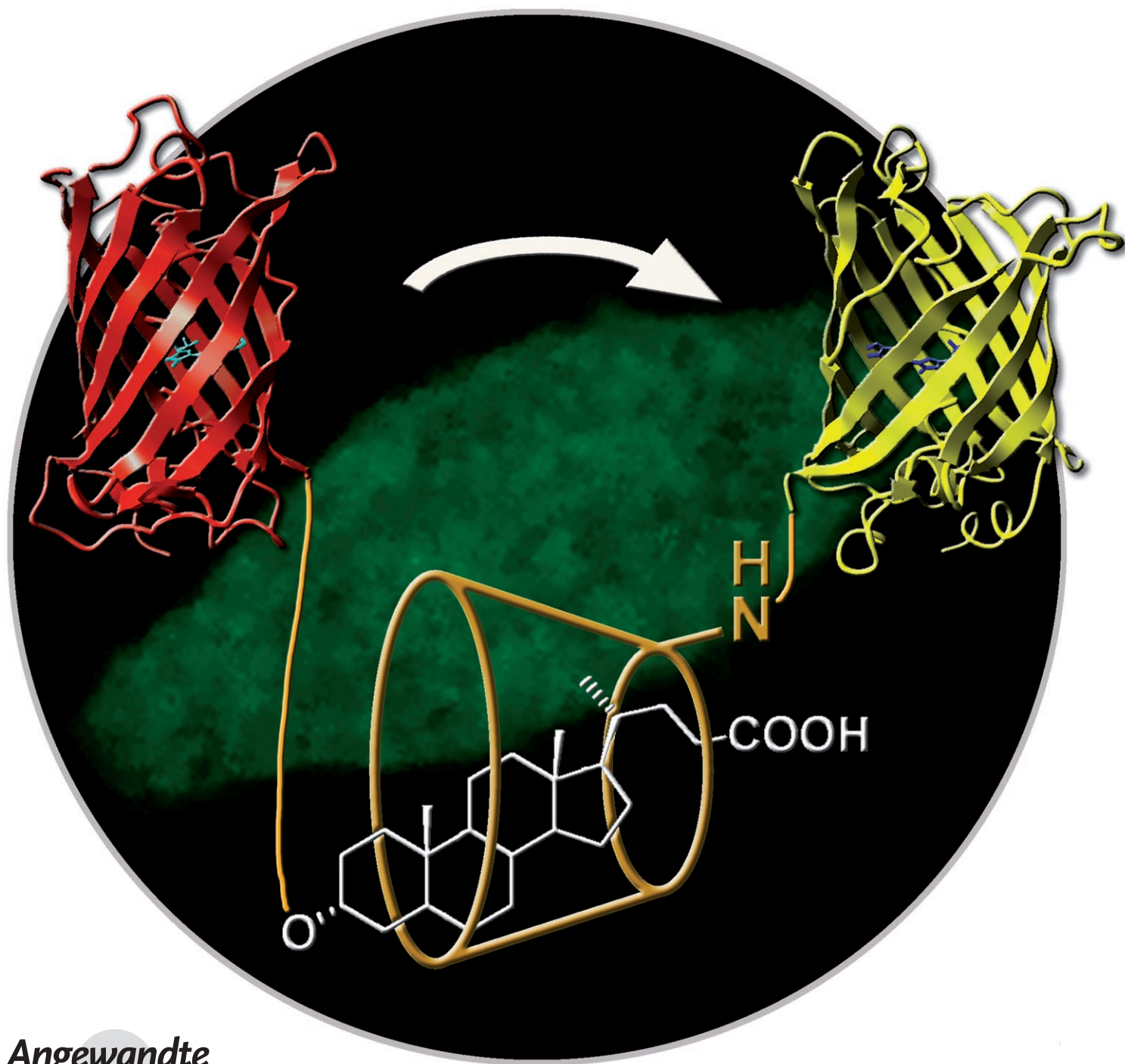


A Synthetic Supramolecular Construct Modulating Protein Assembly in Cells**

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Supramolecular chemistry has allowed the development of self-assembling systems whose drive to assemble and disassemble is controlled by the reversible interactions of specific control elements that are tunable through external factors such as light, environment, and exogenous ligands.^[1] In particular, in the field of materials science, noncovalent interactions in supramolecular switches,^[2] electronics,^[3] and polymers^[4] provide successful entries in modulating and controlling materials' properties and functions. In chemical biology, similar control over protein localization and assembly and the resulting activation and deactivation has been exploited with supramolecular interactions for the study of, for example, signal transduction,^[5,6] tubulin stabilization,^[7] and transcription factors.^[8–10] Typically these studies included the use of small-molecule tools and their interaction with specific protein domains. Synthetic supramolecular constructs have been used to act as binding elements to protein substructures.^[11–15] The use of typical synthetic supramolecular control elements like cyclodextrin hosts and steroid ligands to effect protein localization or function has not been explored. Here we report the use of such supramolecular noncovalent recognition by synthetic supramolecular elements for the control of protein assembly and subsequent inhibition of assembly with an exogenous molecule. Our study includes the site-specific functionalization of model proteins with supramolecular control elements, the detection of protein assembly as a result of their interaction in vitro, and the successful assembly of the functionalized proteins in cellular experiments due to supramolecular control.

For the evaluation of protein assembly through a synthetic supramolecular construct, the supramolecular elements envisaged to modulate the protein assembly were combined with a set of model proteins (Figure 1). Enhanced cyan fluorescent protein (eCFP) and enhanced yellow fluorescent protein (eYFP) were selected as the model proteins, as they are well-characterized and frequently used in the assessment of protein–protein interactions on a cellular level because of their fluorescence resonant energy transfer (FRET) when brought into vicinity.^[16] Lithocholic acid and β -cyclodextrin were chosen as the supramolecular elements for the modu-

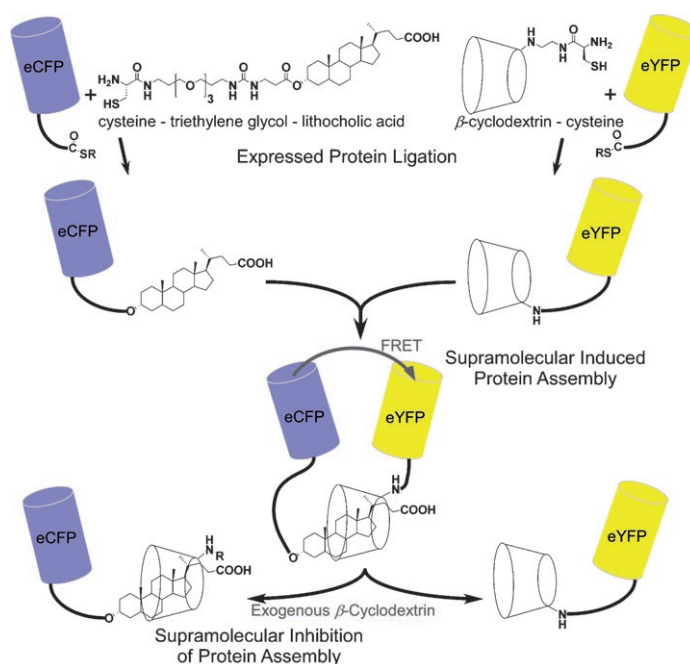


Figure 1. Schematic representation of the synthesis, assembly, and disassembly of eYFP-cyclodextrin and eCFP-lithocholic acid conjugates mediated by cyclodextrin.

lation of the assembly of the proteins, because these elements form strong, unidirectional, and water-stable heterodimers in the submicromolar range as a result of the hydrophobic inclusion of the lithocholic acid into the β -cyclodextrin.^[17]

We used expressed protein ligation for the site-selective incorporation of the supramolecular elements into the proteins,^[18] thus enabling single labeling of the protein at, in our case, the C terminus of the protein. The eCFP or eYFP was fused with an intein domain followed by a chitin binding domain. The resulted fusion proteins were expressed in *E. coli*, purified using chitin agarose, and cleaved by 2-mercaptoethanesulfonate (MESNA) to generate C-terminal thioesters. The β -cyclodextrin and lithocholic acid were connected by small spacers to cysteine units for ligation purposes by means of standard synthetic techniques. The lithocholic acid D ring is known to enter β -cyclodextrin through the larger rim.^[17] Therefore, the cysteine for protein ligation was introduced selectively on the smaller rim of the β -cyclodextrin to prevent unwanted interference during supramolecular dimerization. Concomitantly, the 3-OH group on the A ring of lithocholic acid was connected to a cysteine moiety by a short triethylene glycol linker. The cysteine-modified β -cyclodextrin was ligated in large excess with the eYFP-Mesna thioester. This resulted in a quantitative reaction of the eYFP, enabling straightforward purification of this construct (eYFP-CD) by gel filtration. The ligation of the cysteine-modified lithocholic acid to the eCFP-Mesna thioester was performed with only a small excess of the supramolecular ligand and an additional detergent to increase the solubility of the lithocholic acid construct. This ligation did not proceed quantitatively and thus required a more sophisticated purification. The ligated protein was separated from the unligated protein by a Triton extraction, in which the

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eCFP-lithocholic acid (eCFP-LA) construct resided in the detergent-rich phase. The detergent was subsequently removed from the protein construct by ion-exchange chromatography to yield the ligated protein in pure form. The protein-supramolecular constructs were characterized with SDS-PAGE, ESI, and MALDI-TOF mass spectrometry. As an example in Figure 2 the purity and integrity of the eCFP-LA construct are documented.

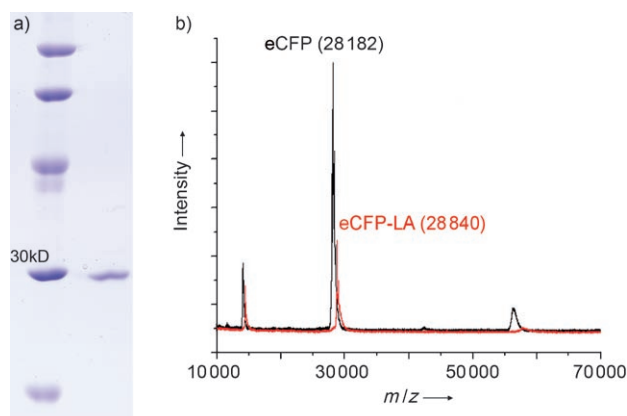


Figure 2. a) SDS-PAGE of the purified eCFP-LA (right lane) and protein size marker (left lane). b) MALDI-TOF mass spectra of eCFP-Mesna thioester (black, calculated m/z 28184) and purified eCFP-LA (red, calculated m/z 28839).

The supramolecular assembly of the proteins was first studied in buffer by fluorescence spectroscopy. At a concentration of roughly $0.5 \mu\text{M}$ and with excitation at 410 nm, both the eCFP-LA and the unmodified eCFP-thioester show a fluorescence signal with an emission maximum at 474 nm (Figure 3a and b, black lines). At this excitation wavelength, the eYFP-CD and the eYFP-thioester feature only weak fluorescence with an emission maximum at 527 nm (Figure 3b, blue line). Mixtures of eYFP-CD and eCFP-LA showed a strong FRET effect as judged from the significant decrease of the eCFP-LA fluorescence intensity at 474 nm and the increase of the fluorescence intensity at 527 nm upon excitation at 410 nm (Figure 3a, red line); this results in an emission ratio (eYFP-CD/eCFP-LA) of 1.13. Corresponding reference experiments with eYFP-CD and eCFP (Figure 3b, red line) and eYFP and eCFP-LA (not shown) did not feature this strong FRET effect and showed an emission ratio (eYFP-CD/eCFP) of 0.72. The decrease in donor intensity at the expense of the acceptor intensity only when both proteins feature a supramolecular element provides strong evidence for a FRET effect induced by the supramolecular interaction of the lithocholic acid of eCFP-LA with the cyclodextrin of eYFP-CD.

To provide further proof for a molecular recognition event and validate the reversible nature of the interaction, β -cyclodextrin was added to the mixture of eYFP-CD and eCFP-LA as a competitor for lithocholic acid. β -Cyclodextrin was added in excess to ensure sufficient inhibition of the complex. Upon addition, the eYFP-CD fluorescence decreased and the eCFP-LA emission returned, leading to a

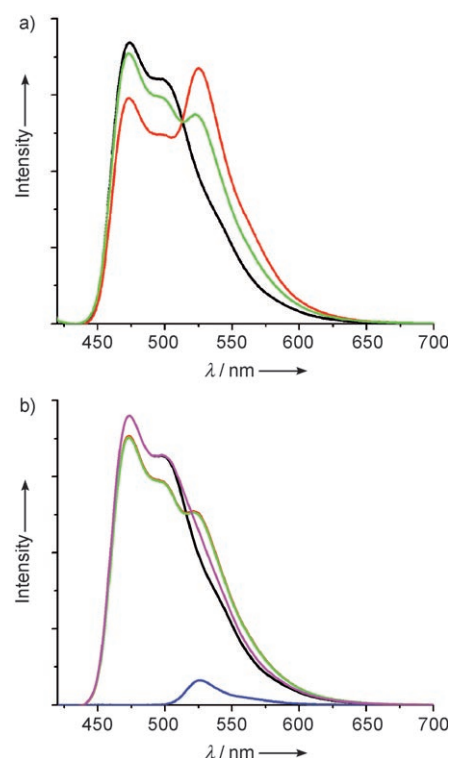


Figure 3. a) Fluorescence spectra of $0.5 \mu\text{M}$ eCFP-LA (black); mixture of $0.5 \mu\text{M}$ eCFP-LA and $1 \mu\text{M}$ eYFP-CD (red); mixture of $0.5 \mu\text{M}$ eCFP-LA, $1 \mu\text{M}$ eYFP-CD, and 1 mM CD (green). b) Reference fluorescence spectra: $0.5 \mu\text{M}$ eCFP (black); $1 \mu\text{M}$ eYFP-CD (blue); mixture of $0.5 \mu\text{M}$ eCFP and $1 \mu\text{M}$ eYFP-CD (red); mixture of $0.5 \mu\text{M}$ eCFP, $1 \mu\text{M}$ eYFP-CD, and 1 mM CD (green); summation of $0.5 \mu\text{M}$ eCFP and $1 \mu\text{M}$ eYFP-CD (magenta). All spectra were recorded in 50 mM sodium phosphate solution, pH 7.5, at 20°C .

fluorescence spectrum comparable to that of the reference proteins without dimerization elements (Figure 3a, green line) with an emission ratio (eYFP-CD/eCFP-LA) of 0.75. Addition of cyclodextrin to the reference mixture of eYFP-CD and eCFP did not change the shape of their fluorescence spectrum and the emission ratio of the two fluorophores (Figure 3b, green and red lines). These results show that the induced protein assembly occurs selectively through the supramolecular elements and is reversible. A titration experiment of eYFP-CD with eCFP-LA could be fitted with a model for heterodimerization, and gave a dimerization constant of $(1.6 \pm 0.2) \times 10^6 \text{ M}^{-1}$, which is similar to the reported binding constant of cyclodextrin and lithocholic acid.^[17] The dimerization of the modified proteins and their reversible inhibition by cyclodextrin could also be shown with native gel electrophoresis (see the Supporting Information).

To provide a proof-of-principle of the supramolecular assembly in physiological media, the supramolecular protein constructs were microinjected in MDCK (Madin-Darby canine kidney) cells and investigated by using confocal fluorescence microscopy. The presence or absence of dimerization was investigated by donor fluorescence recovery after acceptor photobleaching. Cells microinjected with both eCFP-LA and eYFP-CD showed a significant FRET efficiency (Figure 4a, row A): the fluorescence intensity of the

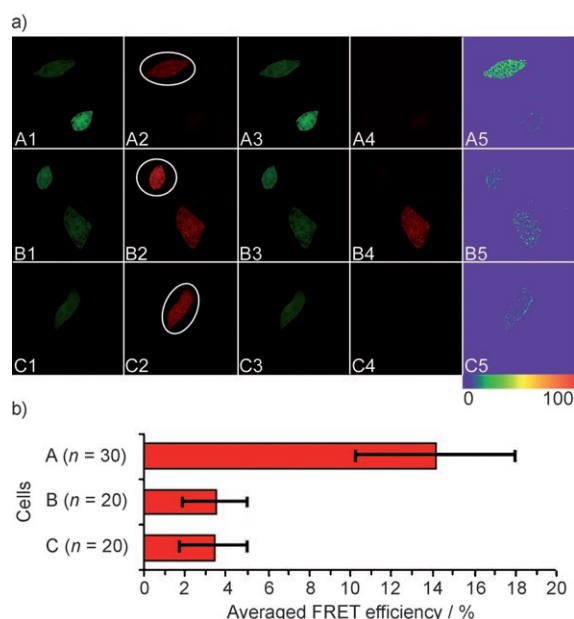


Figure 4. a) Confocal fluorescence microscopy images of MDCK cells microinjected with A) eCFP-LA and eYFP-CD; B) eCFP-thioester and eYFP-CD; C) eCFP-LA and eYFP-CD and subsequently incubated with cell-permeable cyclodextrin. 1) donor before acceptor photobleaching, 2) acceptor before photobleaching, 3) donor after acceptor photobleaching, 4) acceptor after photobleaching, 5) FRET efficiency. Circled cells were used in bleaching experiments, other cells serve as an internal reference. b) Averaged FRET efficiencies with error bars for cells (n) microinjected with A) eCFP-LA and eYFP-CD; B) eCFP-thioester and eYFP-CD; C) eCFP-LA and eYFP-CD and incubated with cell-permeable cyclodextrin.

donor had significantly increased (Figure 4a, A1 and A3) after the acceptor had been photobleached (Figure 4a, A2 and A4) in the circled cell. Similar experiments on reference constructs did not result in an increased fluorescence after photobleaching (Figure 4a, row B). The reversible supramolecular protein assembly could be switched off in the cells by incubation with exogenous β -cyclodextrin conjugated to a cell-penetrating peptide. The FRET efficiency of cells microinjected with eYFP-CD and eCFP-LA returned to low values after incubation for only 5 min with cell-permeable cyclodextrin at 10 μM overall concentration and pyrenebutyrate for good membrane passage^[19] (Figure 4a, row C). These results demonstrate that the supramolecular interaction is not disrupted by either the cellular environment or by the endogenous cholesterol during the time scale of our studies (up to 1 h). An explanation is that most of the cholesterol in cells is located in the membrane, and the affinity of cholesterol for β -cyclodextrin is more than two orders of magnitude lower than that of lithocholic acid.^[17]

The microinjection of the proteins in the cells under the conditions applied leads to protein concentrations in the regime of 1–10 μM . A larger number of these cells featuring average fluorescence intensity were analyzed to obtain statistically relevant data concerning the FRET efficiency of the different mixtures. Cells containing the eYFP-CD and eCFP-LA constructs (A in Figure 4b) have an average FRET efficiency of 14%. We noticed that cells with significantly

higher or lower fluorescence intensity had concomitant higher and lower FRET efficiency. The reason for this may lie in the concentration dependence of the lithocholic acid/ β -cyclodextrin interaction in this concentration regime. The cells featuring unmodified eCFP and eYFP-CD (B in Figure 4b) featured average FRET efficiencies of approximately 3%, which is very close to the background signal of the nonbleached cells. Additionally, for these reference construct microinjected cells the concentration dependence of the FRET efficiency was much less than that of the cells featuring the eCFP-LA and eYFP-CD constructs. The cells containing both eYFP-CD and eCFP-LA that had been treated with the exogenous cyclodextrin for 5 min (C in Figure 4b) showed similar low FRET efficiencies of around 3% and low protein concentration dependence.^[20] Similar assembly inhibition results could be obtained upon treating eCFP-LA and eYFP-CD microinjected cells with a lithocholic acid derivative (see the Supporting Information). These results thus confirm the reversible nature of the supramolecularly induced protein assembly in a cellular environment and point out that synthetic supramolecular architectures might have potential in chemical biology approaches.

In summary, synthetic supramolecular elements can be site-selectively incorporated in proteins. The supramolecular elements allow the modulation of protein assembly with temporal control both in vitro and in cells when an exogenous small molecule is applied. In this proof-of-principle study the proteins were microinjected into the cells. Further studies might focus on circumventing microinjection, for example by selective protein labeling in cells. Additionally, the switching behavior of the system could be improved by providing the supramolecular architectures with a switch for repeated on/off cycles. We believe that such advanced supramolecular architectures hold promise for controlling protein localization and function in cells.

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