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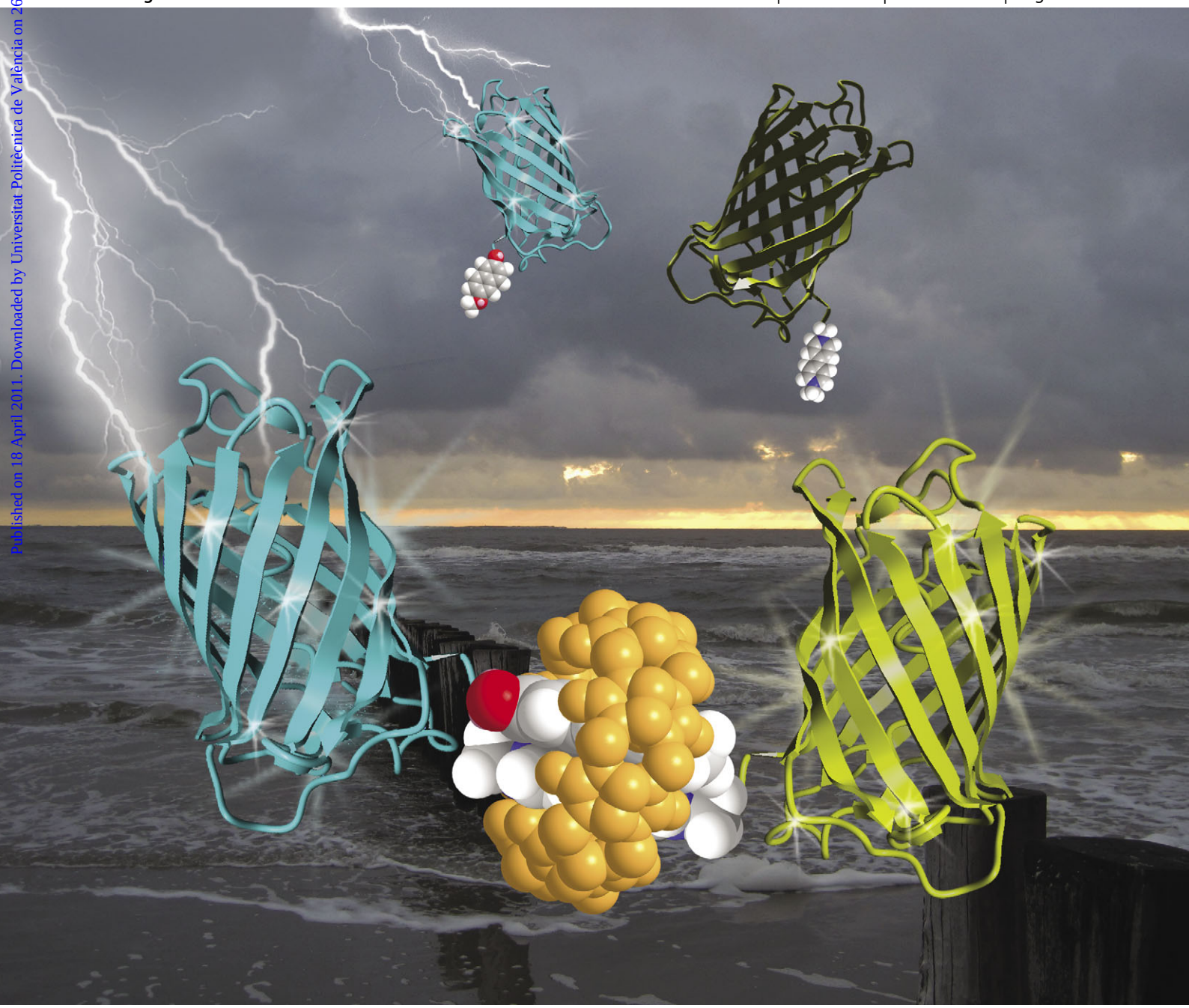

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COMMUNICATION

Cucurbit[8]uril induced heterodimerization of methylviologen and naphthalene functionalized proteins†

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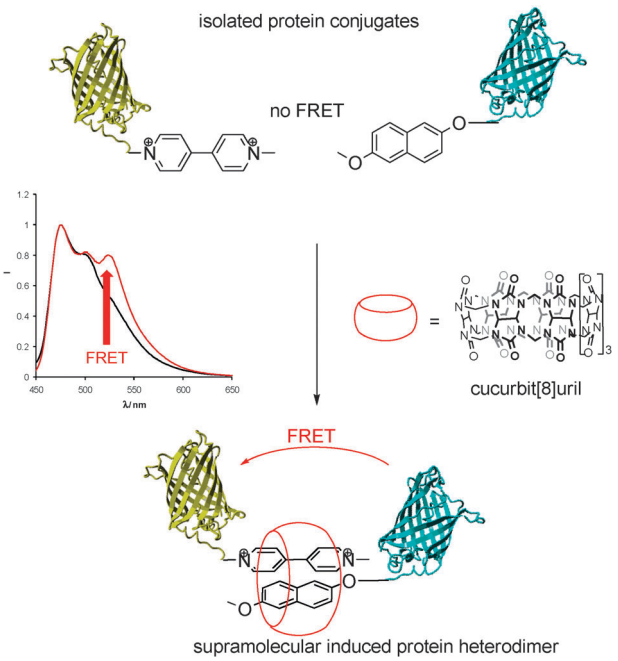
Cucurbit[8]uril is a supramolecular inducer of protein heterodimerization for proteins appended with methylviologen and naphthalene host elements. Two sets of fluorescent protein pairs, which visualize the specific protein assembly process, enabled the interplay of the supramolecular elements with the proteins to be established.

Controlled generation or stabilization of protein complexes is of high interest for applications in for example the biomedical or bionanotechnology fields. Supramolecular approaches are in principle very attractive for this since they allow bioorthogonal control over these interactions,[1] thus complementing the protein engineering and small molecule approaches.[2] A number of supramolecular approaches for the controlled assembly of proteins have been reported in this respect based on, for example, host–guest chemistry[1] or on the combination of natural protein assemblies with synthetic polymer chemistry.[3] Synthetic supramolecular host–guest systems are very attractive for the generation of small well-defined protein assemblies such as homo- and heterodimers. The strong cyclodextrin–lithocholic acid interaction for example, could be applied for the controlled supramolecular heterodimerization of proteins in solution and in cells, by appending these supramolecular elements to the C-terminus of the proteins.[4,5] The supramolecular host molecule cucurbituril has also found strong application in the protein assembly and immobilization field.[6] Proteins genetically encoded with an N-terminal FGG-motif can for example be assembled into a homodimer *via* two-fold binding of the peptide motif within cucurbit[8]uril (CB[8]).[7] CB[8] is not only known to form 1 : 2 complexes with peptide motifs,[8] but also to form stable 1 : 1 : 1 ternary complexes with an electron deficient supramolecular guest molecule such as methylviologen (MV) and an appropriately corresponding electron rich guest such as alkoxynaphthalene (Np).[9,10] These two guest molecules form a charge transfer complex inside the CB[8] cavity. Here we demonstrate that this specific ternary supramolecular system can be used to induce the selective heterodimerization of two proteins, with CB[8] acting as a supramolecular inducer of dimerization (Scheme 1). Furthermore we show that there is a distinct interplay of the supramolecular host–guest system with the proteins. Two sets of fluorescent protein FRET pairs allowed establishing the specific characteristics of the CB[8]–MV–Np system with respect to protein assembly and unspecific protein aggregation.



Scheme 1 Supramolecular induced protein dimerization by formation of a ternary complex between methoxynaphthol and methylviologen–CB[8].

We selected methoxynaphthol (Np) and methylviologen (MV) as the supramolecular guest pair to form a ternary complex with CB[8]. For the site-selective attachment of these supramolecular groups to recombinantly expressed proteins *via* expressed protein ligation, the elements were to be provided with a cysteine moiety. The proteins we selected are two sets of cyan and yellow fluorescent protein (CFP and YFP) FRET pairs. The two sets of fluorescent proteins differ in their

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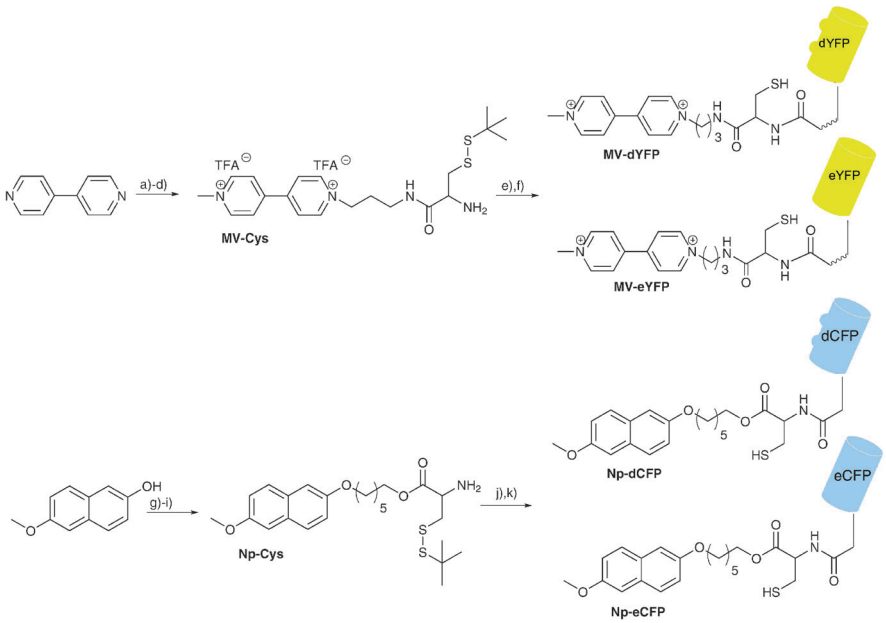

Scheme 2 Synthesis of cysteine modified guest elements methylviologen (MV) and naphthalene (NP) and their ligation to two sets of fluorescent proteins: enhanced CFP and YFP (eCFP, eYFP) and dimerizing CFP and YFP (dCFP, dYFP). (a) MeI in CH_3CN, RT, 24 h; (b) 3-bromopropylamine·HBr in CH_3CN, reflux, 24 h; (c) BocCysS*t*Bu–OSu, DIPEA, CH_3CN, 40 °C, 4 h; (d) 30% TFA, CH_2Cl_2; (e) TCEP, MeOH/phosphate buffer pH 7.5, RT, 30 min; (f) eYFP–thioester or dYFP–thioester in phosphate buffer pH 7.5, RT, 12 h; (g) NaH, 6-bromo-hexan-1-ol, 0 °C, DMF, RT; (h) BocCysS*t*Bu–OH, EDC·HCl, DMAP, DMF; (i) 20% TFA, CH_2Cl_2; (j) TCEP, MeOH/Tris–HCl buffer pH 8.5, Triton, RT, 2 h; (k) eCFP–thioester or dCFP–thioester in Tris–HCl pH 8.5, Mesna, RT, 12 h.

intrinsic affinity for dimerization due to hydrophobic mutations. These were chosen as they allow the supramolecular assembly process to be followed using fluorescence techniques that specifically visualize the interaction between the proteins. This will provide information of the assembly process on the protein level, in contrast to, for example, optical techniques that visualize host–guest assembly in the CB[8] cavity.[11] This allows the interplay of the supramolecular elements with the protein assembly process to be studied and to distinguish from unspecific protein assembly induced by, for example, hydrophobic clustering.

The viologen based host molecule was synthesized from commercially available bipyridine (Scheme 2). Methylviologen was obtained by slow addition of methyliodide to 4,4′-bipyridine. The mono-methylated product was further reacted with 3-bromopropylamine hydrobromide to give an asymmetric substituted viologen derivative[12] which was coupled to a Boc- and S*t*Bu-protected cysteine–succinimide ester in aceto-nitrile. The cysteine conjugate was purified by preparative HPLC and subsequently the Boc group was removed with TFA/DCM to yield **MV–Cys**. The naphthalene based host molecule was synthesized from commercially available 6-methoxy-naphthalen-2-ol. First, an alcohol spacer was attached to the phenolic functionality of the naphthalene using 6-bromo-hexan-1-ol and subsequently the primary alcohol group was coupled to a protected cysteine derivative. After purification *via* preparative HPLC, the resulting compound was Boc-deprotected to give **Np–Cys**. Deprotection of the cysteine S*t*Bu disulfide functionalities of **MV–Cys** and **Np-Cys** was performed immediately prior to protein ligation by treatment with TCEP. The four protein thioesters were expressed and purified analogously as previously described,[5] and ligated to the cysteine-modified host molecules. The YFP–thioester variants were reacted with an excess of **MV–Cys** in the presence of thiophenol for 12 hours to give complete conversion. The naphthalene based host molecule **Np–Cys** showed limited solubility in the ligation buffer and ligations to the CFP variants were therefore performed in the presence of detergents to enhance the solubility and reaction speed.

The CB[8] controlled protein assembly of the two protein FRET pairs was studied using fluorescence spectroscopy. Fluorescence spectra were recorded using an excitation wavelength of 410 nm which is optimized for selective CFP excitation. The supramolecular-induced protein heterodimers were prepared by addition of CB[8] to the specific methyl-viologen-modified YFP, followed by the addition of the naphthalene-modified CFP. A complete set of experiments including all possible reference samples was thus made for both protein pairs (Fig. S1 and S2, ESI†).

The normal variants of the fluorescent proteins were initially investigated (Fig. 1). As envisioned in our design, CB[8] indeed selectively induces the heterodimerization of **MV–eYFP** with **Np–eCFP**. This becomes clearly apparent from the increase in the YFP emission at 527 nm (Fig. 1, blue line), which indicates the occurrence of FRET upon the addition of CB[8]. This results in a YFP/CFP ratio (I(527 nm)/I(475 nm)) of 0.79, in contrast to a ratio of 0.51 for the non-assembled proteins. This CB[8]-induced high energy transfer between the proteins is only observed in the presence of all three supramolecular components, allowing the formation of the ternary complex. In all other cases, such as for protein pairs lacking either one or two of the supramolecular units or in the absence of CB[8],

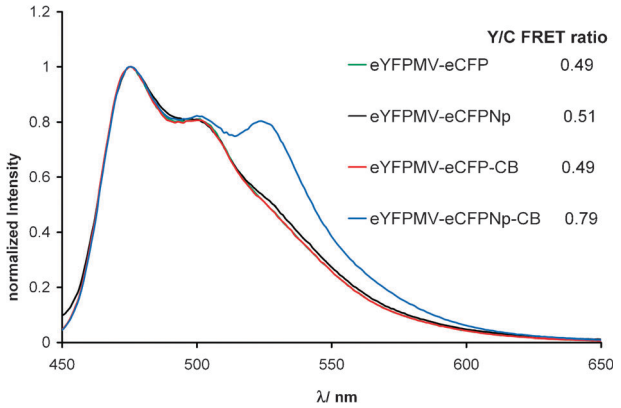

Fig. 1 Fluorescence spectra of normal enhanced fluorescent protein pairs, normalized at the CFP emission maximum and FRET ratio I(527 nm)/I(475 nm). Protein concentration 1 μM each, CB[8] concentration 10 μM.

no FRET was observed. Also the formation of protein homodimers upon addition of CB[8] could be excluded *via* homo-FRET studies (see ESI†). This demonstrates that the chosen supramolecular host–guest system is specific and selectively induces supramolecular heteroassembly of the two proteins.

The other pair of proteins under study, **MV–dYFP** and **Np–dCFP**, carries specific point mutations[13] which increase their intrinsic affinity for dimerization (both homo- and heterodimerization) due to increased hydrophobic interactions. In general, protein–protein interactions are frequently mediated to a significant extent by hydrophobic and charged regions on their surfaces,[14] allowing for supramolecular recognition.[15] As such, knowledge about the possible interplay of the supramolecular elements with the protein surface is highly important. The weakly dimerizing fluorescent model proteins **MV–dYFP** and **Np–dCFP** are an ideal system to study this.

Control experiments performed on the **Np–dCFP** with unfunctionalized dYFP and CB[8] indeed showed that these supramolecular elements do not lead to unspecific binding or protein interactions (see ESI†). In contrast, experiments with **MV–dYFP** showed that the methylviologen can lead to unspecific protein assembly of the weakly dimerizing proteins (Fig. 2, green and black lines). Protein mixtures of **MV–dYFP** with either **Np–dCFP** or unmodified dCFP feature an increase of the YFP emission in the absence of CB[8]. Interestingly, however, in the presence of CB[8] the unspecific protein assembly induced by the methylviologen is inhibited, as becomes apparent from the absence of YFP emission (Fig. 2, red line). The CB[8] thus shields the methylviologen from making unspecific interactions with hydrophobic protein surfaces. As a result, in the **MV–dYFP**–**Np–dCFP** mixture with CB[8] (Fig. 2, blue line), a specific CB[8] mediated energy transfer is observed, resulting from the supramolecular protein dimer. These results show that the ternary system of CB[8] with MV and Np can also be successfully used for the formation of selective protein heterodimers of more hydrophobic proteins. In these cases, the presence of CB[8] as a host molecule is required to prevent MV induced unspecific dimerization with hydrophobic protein surfaces.

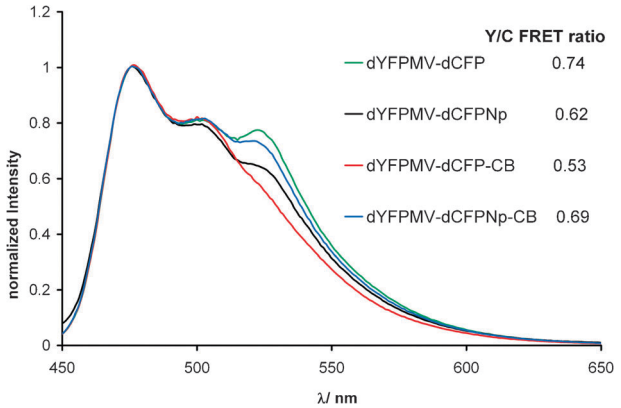

Fig. 2 Fluorescence spectra of dimerizing fluorescent proteins pairs, normalized at the CFP emission maximum and FRET ratio I(527 nm)/I(475 nm). Protein concentration 1 μM each, CB[8] concentration 10 μM.

In conclusion, we have shown that CB[8] constitutes an attractive supramolecular inducer of protein heterodimerization using MV and Np as protein appended host elements. The model system of fluorescent protein pairs allows specific visualization of the protein dimerization event. This in turn allows for detection of potential unspecific interplay of the supramolecular elements with the proteins and provides molecular insights not attainable using experiments solely based on the observation of the charge-transfer complex. This novel system for controlled protein heteroassembly now allows, for example, application in the area of bionanotechnology for the controlled immobilization of proteins on surfaces, or for specific targeting of N-terminal tryptophan motifs in proteins.[16]

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