Construction of an energy transfer system in the bio-nanocup space by heteromeric assembly of gp27 and gp5 proteins isolated from bacteriophage T4†

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Protein assemblies, such as viruses and ferritins, have been employed as useful molecular templates for the accumulation of organic and inorganic compounds to construct bio-nanomaterials. While several methods for conjugation of heterofunctional molecules with protein assemblies have been reported, it remains difficult to control their fixation sites in the assemblies. In this article, we demonstrate the three-dimensional arrangement of different types of fluorescent probes using the heteromeric self-assembly of $(gp27\text{-}gp5)_3$ which is the component protein of bacteriophage T4 (gp: gene product). The composites exhibited fluorescence resonance energy transfer from fluorescein to tetramethylrhodamine dyes immobilized in the bio-nanocup space. The alternation of the donor and acceptor positions induced fluorescence self-quenching by the formation of ground-state complexes of the acceptors. These results indicate that the site-specific conjugation method using the bio-nanocup space of the heteromeric protein assembly has potential for the integration of several types of functional molecules in protein nanospaces.

Introduction

Conjugation of protein assemblies with synthetic molecules is a subject that has drawn much interest in the field of chemical biology.[1,2] Protein assemblies with robust structures such as viral coat proteins and ferritins are useful templates for the deposition of organic molecules and inorganic materials for preparation of catalysts, imaging reagents and drug delivery systems.[3,4] The organization of multiple functionalities on a single protein assembly is necessary for the construction of such integrated protein systems. Several promising protein assemblies conjugated with heterofunctional molecules have been prepared with the objective of creation of artificial protein composites.[5–14] One of the pioneering approaches involves independent modification of lysine and cysteine residues with various types of molecules on the exterior surface of a spherical viral cage.[5,6] An alternative method includes re-assembly of differently-labeled subunits of homomeric protein assemblies such as α-hemolysin, TMV and CCMV.[7–10,12] Although the latter method, which employs self-assembly reactions of protein subunits, is effective for introducing desired functions, it remains difficult to control the relative locations of different types of molecules on a single protein assembly due to the occurrence of random self-assembly of the labeled subunits.[8–10] We describe herein a novel strategy for site-specific conjugation of heterofunctional molecules in a bio-nanocup space consisting of the heteromeric protein assembly from bacteriophage T4.

We have employed a heteromeric protein assembly designated $(gp27\text{-}gp5)_3$ where the term "gp" means "gene product". The components of the assembly are overexpressed and isolated from bacteriophage T4 (Fig. 1a).[15–17] Three gp27 monomers spontaneously assemble on a bio-nanotube composed of three gp5 proteins, $(gp5)_3$, to afford a bio-nanocup structure (Fig. 1a). We have previously reported that the $(gp27\text{-}gp5)_3$ bio-nanocup provides a potent reaction space for the conjugation of iron porphyrins.[17,18] Oxidation reactions catalyzed by the iron complexes were found to proceed within the cage. We expected that the heteromeric assembly reaction should enable us to modulate the three-dimensional arrangement of different functional molecules and their alternation in the protein nanospace. In order to validate this idea, we therefore selected stepwise conjugation of fluorescein (donor) and tetramethylrhodamine (acceptor) in the heteromeric $(gp5\text{-}gp27)_3$ assembly to control the fluorescence resonance energy transfer and self-quenching by the alternation of donor and acceptor positions (Fig. 1c).[19]

Results and discussion

Design of the conjugation sites for fluorescent probes

Cysteine thiols react selectively with fluorescein-5-maleimide (Fl) and tetramethylrhodamine-5-maleimide (TMR). These probes

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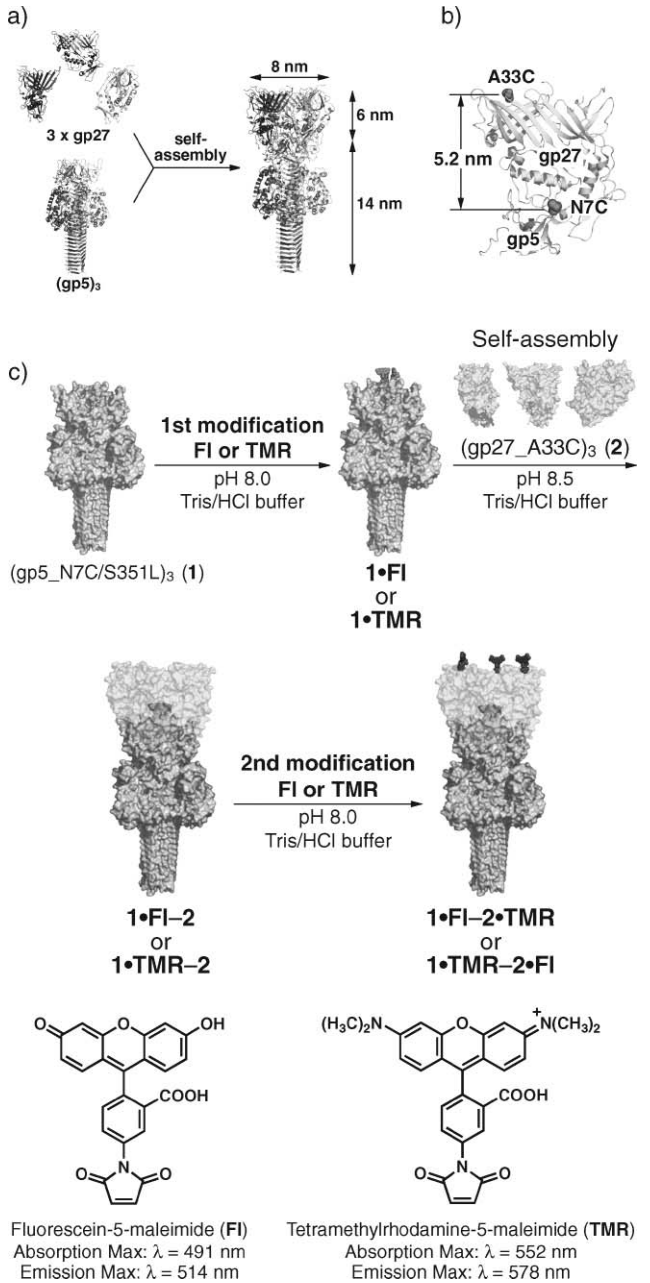

Fig. 1 (a) Self-assembly of $(gp5)_3$ and three gp27 protein subunits (PDB: 1K28)[15] and (b) the mutation sites on $(gp27\text{-}gp5)_3$. (c) Synthetic route for the insertion of hetero-fluorescent probes (fluorescein and rhodamine) into $(gp27\text{-}gp5)_3$.

were inserted stepwise into the cup space of an engineered version of $(gp27\text{-}gp5)_3$ where Asn7 in gp5 and Ala 33 in gp27 were replaced with cysteine (Fig. 1b).[20] We have already reported the preparation of a gp5 mutant, gp5_N7C/S351L, and the crystal structure of $(gp27\text{-}gp5\_N7C/S351L)_3$ conjugated with Fe(III) porphyrin complexes.[17] This previous study indicated that the modification of N7C with Fe(III) porphyrin did not cause a structural change at the N-terminal region of $(gp5)_3$. The introduction of fluorescent probes into $(gp5\_N7C/S351L)_3$ (**1**) is not also expected to prevent assembly of $(gp5)_3$ with three gp27 monomers (Fig. 1b). Moreover, Ala33 of gp27 located at the top edge of the $(gp27)_3$ cage was replaced by a cysteine residue in order to be able to modify it with a second fluorescent molecule (Fig. 1b). The distance of three sulfur atoms of A33C in $(gp27)_3$ was found to be 5.2 nm on the basis of the crystal structure.[17] Thus, the fluorescent probes conjugated with the A33C residues are expected to exhibit fluorescence emission without quenching caused by aggregation of fluorophores.

Preparation of 1·Fl–2·TMR and 1·TMR-2·Fl

For the modification of $(gp5\_N7C/S351L)_3$ (**1**), excess Fl was added to a buffer solution of **1** at pH 8.0. To assemble $(gp27\_A33C)_3$ (**2**) with **1**·Fl, a cell lysate of gp27_A33C was added to a solution of purified **1**·Fl at pH 8.5 to accelerate the formation of **1**·Fl–**2**.[21] **1**·Fl–**2** was purified by His-trap and size-exclusion column chromatography. **1**·Fl–**2** was then treated with TMR at pH 8.0 to yield **1**·Fl–**2**·TMR. The elution profile of **1**·Fl–**2**·TMR by size-exclusion chromatography (Sephadex G-200) showed that the main product eluted at the same volume as that of unmodified system $(WTgp27\text{-}gp5\_N7C/S351L)_3$ which was employed as a reference (see ESI†). The result suggested that **1**·Fl–**2**·TMR was formed as an intact $(gp27\text{-}gp5)_3$ assembly structure. **1**·TMR-**2**·Fl was also prepared under the same conditions except for the reverse order of the reactions with TMR and Fl.

Determination of conjugation sites of fluorescent probes by protein digestion

In order to determine the modification sites of Fl and TMR in **1**·Fl–**2**·TMR, unmodified **1**, **1**·Fl, and **1**·Fl–**2**·TMR were digested with endoproteinase Lys-C and the resulting peptide fragments were isolated by reverse-phase HPLC. The HPLC elution profile of unmodified **1** showed an elution peak at 76 min that was not present in the elution profile of **1**·Fl (Fig. 2a, b). The peak at 76 min was confirmed to be the unmodified N-terminal fragment (1–23) of gp5_N7C/S351L by MALDI-TOF MS (measured to be 2721 Da, calculated to be 2725 Da) (Fig. 2d). A new peak with an absorption of Fl at 500 nm that appeared at 70–73 min in the elution chart of **1**·Fl was assigned to the Fl-modified 1–23 fragment of gp5_N7C/S351L·Fl (measured to be 3166 Da, calculated to be 3152 Da) (Fig. 2b, e). The increase in the measured molecular weights from the calculated value is expected to come from unassignable adducts of the reaction of cysteinyl thiols with maleimide derivatives as found in $(gp27\text{-}gp5\_N7C/S351L)_3$ conjugated with Fe(III) porphyrin.[17] These results showed that the N7C residue of gp5_N7C/S351L·Fl was conjugated with a Fl molecule. Moreover, **1**·Fl was reacted with *N*-ethylmaleimide (NEM) to confirm the modification yield of N7C residues in **1**·Fl. The elution profiles of reverse-phase HPLC of the digested mixture suggested that all three N7C residues of **1**·Fl were modified with Fls and that there were no side-products such as the alkylation of amino and imidazole groups (see ESI†).[20] With respect to **1**·Fl–**2**·TMR, a new peak with an absorption of TMR at 550 nm appeared at 74–76 min in the elution chart of **1**·Fl–**2**·TMR (Fig. 2c) compared with that of **1**·Fl (Fig. 2b). The MALDI-TOF MS spectrum of this digested fragment indicated that the Cys residue at position 33 of gp27_A33C·TMR was modified with a TMR molecule (the TMR-modified 16–58 fragment of gp27_A33C·TMR was measured to be 5554 Da and calculated to

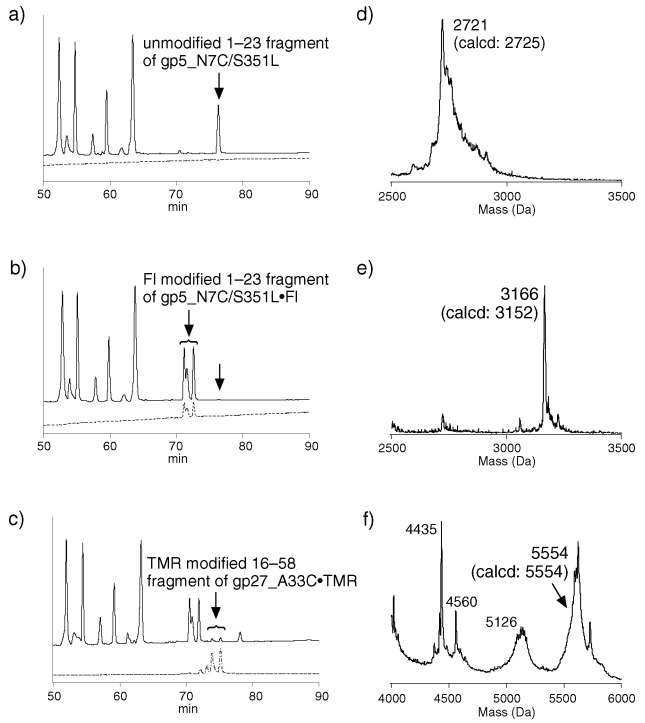

Fig. 2 The elution profiles of reverse-phase HPLC of (a) unmodified **1**, (b) **1**·Fl monitored at 215 nm (solid line) and 500 nm (dashed line) and (c) **1**·Fl–**2**·TMR monitored at 215 nm (solid line) and 550 nm (dashed line). MALDI-TOF MS spectra of the 1–23 fragment of gp5 in (d) unmodified **1**, (e) **1**·Fl and (f) 16–58 fragment of gp27 in **1**·Fl–**2**·TMR.

be 5554 Da) (Fig. 2f), while the native Cys residues (Cys 90 and Cys 310) of gp27_A33C·TMR did not react with TMR. These results are supported by our previous results for $(gp27\text{-}gp5\_N7C/S351L)_3$ conjugated with Fe(III) porphyrin.[17] Thus, only N7C residues of gp5s and A33C residues of gp27s in **1**·Fl–**2**·TMR were modified with Fls and TMRs, respectively. Quantitative analysis with Bradford assay and UV/Vis spectra of **1**·Fl–**2** and **1**·Fl–**2**·TMR showed that **1**·Fl–**2**·TMR contains approximately three Fl molecules (2.7 ± 0.2) and one TMR molecule (1.2 ± 0.1) per $(gp27\text{-}gp5)_3$ (Fig. 3a).

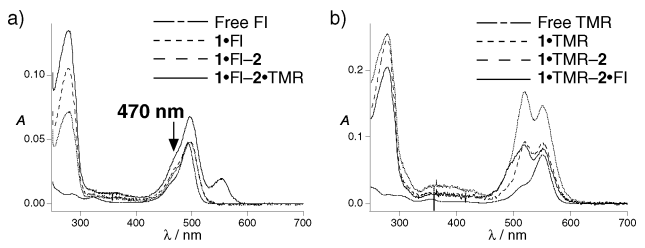

Fig. 3 UV/Vis spectra of (a) Free Fl, **1**·Fl, **1**·Fl–**2** and **1**·Fl–**2**·TMR, and (b) Free TMR, **1**·TMR, **1**·TMR-**2** and **1**·TMR-**2**·Fl in 20 mM Tris/HCl pH 8.3, 0.2 M NaCl.

UV spectra of constructed composites

As shown in Fig. 3a, the UV/vis spectra of **1**·Fl, **1**·Fl–**2** and **1**·Fl–**2**·TMR exhibited shoulder peaks around 470 nm caused by weak ground-state interaction of Fls.[22] It is expected that the three Fl molecules on the top of **1** should be very close to each other, because the distance between each sulfur atom of N7C in the cup space is 1.2 nm, as determined by the crystal structure of $(gp27\text{-}gp5\_N7C/S351L)_3$ conjugated with Fe(III) porphyrin.[17] The absorption spectra of **1**·TMR, **1**·TMR-**2**, and **1**·TMR-**2**·Fl showed two prominent absorption peaks at 519 nm and at 550 nm due to the formation of the ground-state complexes of TMR (Fig. 3b).[23–25] The blue-shifted absorption at 519 nm suggests that TMR molecules retain parallel coordination on the top of **1** (H aggregation).[23,25,26] The association constant of the ground-state complex formation of TMR is known to be about 10^4 times higher than that of Fl due to the delocalized positive charge between the two nitrogens of TMR.[23] Thus, it is expected that ground-state complexes of TMRs in **1**·TMR are preferably formed relative to that of Fls in **1**·Fl.

Examination of fluorescence resonance energy transfer (FRET)

Fluorescence measurements of the composites were conducted to examine FRET from a Fl donor to a TMR acceptor (Fig. 4). **1**·Fl–**2** exhibits fluorescence emission of Fl at 518 nm enough to excite the TMR acceptor (Fig. 4a). The fluorescence spectrum of **1**·Fl–**2**·TMR shows FRET, as judged from the decrease in the fluorescence intensity of the Fl at 518 nm and the increase in the intensity of the TMR at 570 nm relative to **1**·Fl–**2** (Fig. 4a). The FRET efficiency of **1**·Fl–**2**·TMR (0.33) is 33, 7-fold higher than that of **1**·Fl with TMR (0.01), and **1**·Fl–**2** with TMR (0.05), respectively, due to the fixation of TMR on the top edge of **2** (Fig. 4b,c). The FRET of **1**·Fl–**2** with TMR proceeds more effectively than that of **1**·Fl with TMR, because free TMR molecules are accommodated within the hydrophobic cage of $(gp27)_3$ in a manner similar to our previous report that poorly water-soluble Fe(III) porphyrins are accumulated within the $(gp27)_3$ cage.[17] The efficiency (E) correlates with the distance between a donor and an accepter (R), as described by $E = 1/[1 + (R/R_0)^6]$, where R_0 is the Förster distance for 50% efficiency.[27] The R value of **1**·Fl–**2**·TMR (3.3 nm) is consistent with the distance between Fl and TMR molecules of **1**·Fl–**2**·TMR estimated by the crystal structure of $(gp27\text{-}gp5)_3$ (Fig. 1b).

The fluorescence spectrum of **1**·TMR-**2**·Fl, in which the position of two types of fluorescent probe is opposite to that of **1**·Fl–**2**·TMR, shows the emission of Fl at 518 nm but no increase in the emission intensity of TMR at 570 nm (Fig. 4d), because the ground-state complex (H aggregation) of the TMR moieties on the top of **1** causes fluorescence quenching.[23,25] In fact, the fluorescence intensity of TMR at 571 nm in **1**·TMR-**2**·Fl is 6.5-fold lower than that of **1**·Fl–**2**·TMR when just the TMRs of these composites are directly excited at 550 nm (absorption maximum of TMR) (Fig. 4e). These results suggest that the TMRs work as quencher molecules simply by the alternation of TMR and Fl conjugation sites at the top of **1** and the edge of **2** in this system.

Conclusions

We have demonstrated the arrangement of two types of fluorescence molecules in the bio-nanocup space by the heteromeric assembly reaction of gp27 and gp5 protein components isolated from bacteriophage T4. The resulting **1**·Fl–**2**·TMR accelerates FRET from Fls to TMRs, while alternation of TMR and Fl conjugation sites induces significant fluorescence self-quenching

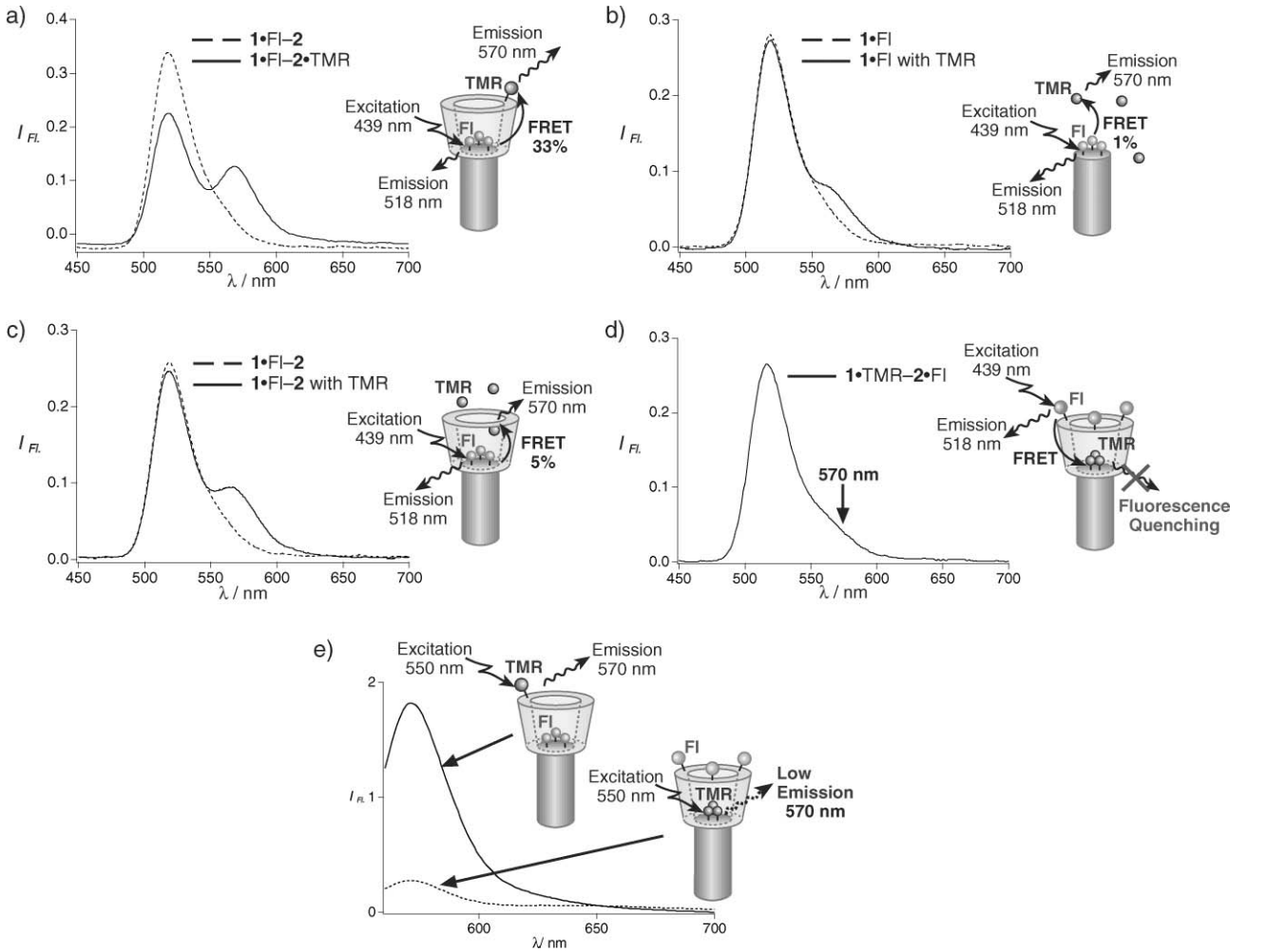

Fig. 4 Fluorescence spectra of (a) **1**·Fl–**2** (broken line) and **1**·Fl–**2**·TMR (solid line), (b) **1**·Fl (broken line) and **1**·Fl with TMR (solid line), (c) **1**·Fl–**2** (broken line) and **1**·Fl–**2** with TMR (solid line) and (d) **1**·TMR-**2**·Fl. The spectra were obtained with 439 nm excitation at 12 °C. The excitation wavelength is shorter than the absorption maximum of fluorescein at 491 nm to avoid direct excitation of TMR and minimize its background fluorescence. The spectrum of **1**·Fl–**2** was corrected for dilution. (d) Fluorescence spectra of **1**·Fl–**2**·TMR (solid line) and **1**·TMR-**2**·Fl (dashed line) obtained with 550 nm excitation at 12 °C.

of TMR. This fluorescence emission control using a heteromeric protein assembly should enable us to construct not only FRET systems such as biosensors based on the quenching molecules, but also integrated bio-nanosystems including several types of functional molecules.[19]

Experimental

Materials and methods

Reagents were purchased from Molecular Probes, Nacalai Tesque, Wako, Hampton Research, Kishida Reagents Chemicals, and Sigma-Aldrich and used without further purification. Gp5 and gp27 mutant genes were constructed by site-directed mutagenesis. The expression and purification of proteins were performed as reported previously.[28] All the buffer solutions used for purification contained 2 mM dithiothreitol or 0.5 mM tris(2-carboxyethyl)phosphine for preventing the formation of disulfide bonds. All reactions were carried out at 4 °C.

Modification of $(gp5\_N7C/S351L)_3$ (1) with maleimide fluorescent probe

A DMF solution of 30 eq. maleimide fluorescent probe, fluorescein-5-maleimide (Fl) or tetramethylrhodamine-5-maleimide (TMR) was added to a solution of **1** (6 μM) in 20 mM Tris-HCl pH 8.0, 0.2 M NaCl and 0.5 mM TCEP. The reaction mixture was stirred at 18 °C for 2 h in the dark. The mixture was centrifuged and passed through Sephadex G-25 equilibrated with 20 mM Tris-HCl pH 8.5 and 0.1 M NaCl and 0.5 mM TCEP to remove free Fl.

Self-assembly of 1·X (X = Fl or TMR) and $(gp27\_A33C)_3$ (2). A cell lysate of gp27_A33C (Buffer: 0.1 M Tris-HCl pH 8.5, 5 mM Im and 1 mM TCEP) was added to a solution of **1**·X. The mixture was stirred at 4 °C for 90 min in the dark. The solution was loaded onto a His-trap column. The protein eluted from the column with 5 to 200 mM imidazole gradient, as was then purified by size-exclusion column chromatography: Sephadex G-200 on an ÄKTA explorer 10S FPLC system (GE Healthcare) (Buffer:

20 mM Tris-HCl pH 8.5, 0.2 M NaCl and 0.5 mM TCEP). The sample was dialyzed against 20 mM Tris-HCl pH 8.0, 0.2 M NaCl and 0.5 mM TCEP buffer for 6 h. Dye concentration was estimated by measuring the absorption maximum at 495 nm (extinction coefficient 83,000 cm^{-1} M^{-1}) of fluorescein.

Modification of 1·X–2 (X = Fl or TMR) with maleimide fluorescent probe. A DMF solution of 30 eq. maleimide fluorescent probe (Fl or TMR) was added to a solution of **1**·X–**2**. The reaction mixture was stirred at 14 °C for 2 h in the dark. The mixture was centrifuged and passed through a Sephadex G-25 equilibrated with 20 mM Tris-HCl pH 8.0 and 0.1 M NaCl to remove the remaining fluorescent probes. The resulting solution was finally purified by Sephadex G-200 column chromatography equilibrated with 20 mM Tris-HCl pH 8.0 and 0.2 M NaCl. The protein concentration of was quantified using a Bradford assay. Dye concentration was obtained by measuring the absorption maximum at 550 nm (extinction coefficient 91,000 cm^{-1} M^{-1}) of TMR.

Digestion analysis of unmodified 1, 1·Fl, 1·Fl–2 and 1·Fl–2·TMR with Lys-C. Digestion analysis was performed by a literature method.[17] To determine the modification sites, unmodified **1**, **1**·Fl and **1**·Fl–**2**·TMR were digested with Lys-C and the resulting peptides were isolated by reverse-phase high-performance liquid chromatography (Vydac C-18 reverse-phase column).

Quantitative determination of unmodified N7C residues in 1·Fl. A DMF solution of 30 eq. *N*-ethylmaleimide was added to a solution of **1**·Fl purified by Sephadex G-200 column chromatography in 20 mM Tris-HCl pH 8.0, 0.2 M NaCl and 0.5 mM TCEP. The reaction mixture was stirred at 18 °C for 2 h in the dark, and then centrifuged and passed through a Sephadex G-25 column equilibrated with 20 mM Tris-HCl pH 8.0 and 0.2 M NaCl to remove free Fls. The eluted protein was then purified by an ÄKTA explorer 10S FPLC system (20 mM Tris-HCl pH 8.0 and 0.2 M NaCl, Sephadex G-200). **1**·Fl reacted with *N*-ethylmaleimide was digested with Lys-C and the resulting peptide fragments were isolated by reverse-phase HPLC, as shown in Fig. S2†.

Fluorescence resonance energy transfer. As described by Förster transfer theory, the efficiency of energy transfer is known correlate with the distance between a donor and an accepter, according to $E = 1/[1 + (R/R_0)^6]$ where R_0 is the Förster distance for 50% efficiency and R is the distance between a donor and an accepter.[27] R_0 is calculated from $R_0 = 9.78 \times 10^3(\kappa^2 n^{-4} \Phi_D J)^{1/6}$. The absolute fluorescence quantum yield (Φ_D) of **1**·Fl–2 was 0.03 which was determined with a Hamamatsu C9920-02 system calibrated by integrating a sphere system with an error of ±3%. R_0 was determined to be 36 Å using $\kappa^2 = 2/3$, $n^{-4} = 1.4$, $\Phi_D = 0.03$ and $J = 3.86 \times 10^{-13}$ M^{-1} cm^3.[29] The efficiency of FRET, $E = (1 - F_{DA}/F_D)/f_A$ (the fluorescence intensity of the Fl in the absence (F_D) and presence (F_{DA}) of TMR, and the fractional labeling with acceptor (f_A)) was used for the calculation of the distance between a donor and an acceptor (R).[27] The value of f_A is 0.33 on the basis of the quantitative analysis of Fl and TMR molecules of **1**·Fl–**2**·TMR.

Physical measurements. UV–vis spectra were recorded on a Shimadzu UV-2400PC UV/vis spectrophotometer. MALDI spectra were recorded on a Voyager DE-PRO (PerSeptive Biosystems). Fluorescence measurements were obtained on a F-7000 fluorescence spectrophotometer (HITACHI) at 12 °C in 20 mM Tris-HCl pH 8.3 and 0.2 M NaCl.

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