Construction of a closed regular-triangle trimer of helix-linked cytochrome c_{555} using sortase A

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Protein-based supramolecules require precise arrangement of building blocks for controlling the protein assembly. The building block regular-triangle trimer (cp-c_{555})$_3$ has previously been constructed from cp-c_{555}, which is an α-helix-linked circular permutant of *Aquifex aeolicus* cyt c_{555}.[1] However, (cp-c_{555})$_3$ may dissociate to monomers. To stabilize the triangle structure, a closed regular-triangle of three cp-c_{555} molecules is constructed by covalently connecting terminal regions using sortase-mediated ligation (SML). Comparing SML using sortase A for six (cp-c_{555})$_3$ variants, the variant with GGG at the N-terminus and LPETG at the C-terminus reacted most efficiently. OP-(c_{555})$_3$, a genetically connected molecule of three cp-c_{555} molecules containing the optimized sequence for SML, was designed to increase the SML product yield. OP-(c_{555})$_3$ was expressed in *E. coli* cells and the terminal regions were connected by SML, generating a closed regular-triangle CL-(c_{555})$_3$ (Fig. 1A). CL-(c_{555})$_3$ showed higher thermostability than (cp-c_{555})$_3$ and OP-(c_{555})$_3$. The structural stability of CL-(c_{555})$_3$ was confirmed by high speed-AFM observation. The crystal structure of CL-(c_{555})$_3$ revealed two stacked CL-(c_{555})$_3$ triangle molecules (Fig. 1B) with a covalent linkage across the terminal regions (red loops in Fig. 1B). Additionally, the stacked CL-(c_{555})$_3$ triangles packed into a nanoporous supramolecular structure (Fig. 1B), constructing two pores with diameters of approximately 16 and 30 Å. These results provide a method to stabilize building block proteins, enabling a unique nanoporous assembly with fixed pore sizes. By adjusting helical lengths, assemblies with tunable pore sizes may be constructed for capturing large target molecules.

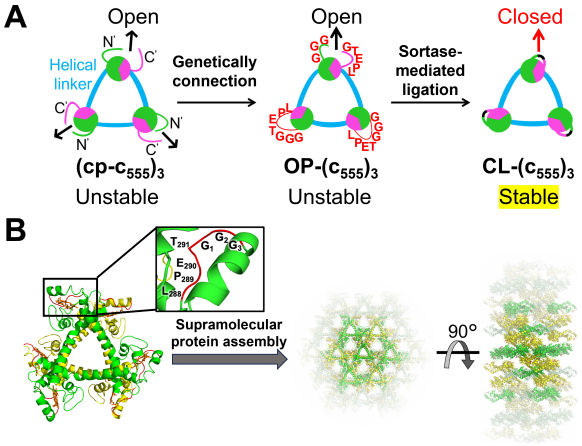

Fig. 1. (A) Schematic representation of construction a closed regular-triangle CL-(c_{555})$_3$ by SML. (B) Crystal structures of CL-(c_{555})$_3$ and its nanoporous supramolecular assembly (PDB ID: 9L08).

[1] A. Oda, et al., *Chem. Asian J.* **2018**, 13, 964.