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Construction of Functional Solid Biomaterials Using In-cell Protein Crystals

Thuc Toan PHAM

2023

Preface

This doctoral thesis was completed at the Tokyo Institute of Technology's School of Life Science and Technology from September 2020 to August 2023 under the direction of Prof. Takafumi Ueno. The author wishes to express heartfelt gratitude to Prof. Uno for his focused supervision and guidance in study and research and his comprehensive support in daily life to the author throughout the entire study term.

The author would also like to thank Assistant Professor Satoshi Abe for his successive training in conducting experiments and writing research articles.

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The author also thanks all collaborators for their hospital assistance, training, and suggestions.

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Last but not least, the author wishes to express his heartfelt gratitude to his parents, Pham Thi Thu Nguyet and Pham Van Dung, his siblings, Pham Hien Khanh, Pham Dang Nguyen, soulmate Nguyen Thi Mai Phuong, and tutor Dr. Izumi Takeuchi for their unwavering support and encouragement throughout his life.

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Survey of this thesis

Protein crystals have been utilized to construct solid materials with various functions. Protein crystals provide vast assembly patterns and highly porous solvent networks that allow us to modify the solvent exposure interface to create functional solid materials. Different methods have been developed to immobilize metal ions, metal complexes, small molecules, and proteins or enzymes in the porous crystal channels for storage, catalyst, drug delivery system, and structural analysis. In addition, the protein-protein interface interactions within protein crystals also are modified for constructing novel supramolecular assemblies. In-cell protein crystals provide such advantages in preparation and stabilities among the protein crystals platform. Herein, I focused on using in-cell protein crystal platforms to establish novel methods for constructing solid materials from two opposite perspectives: dissolving modified in-cell protein crystal to obtain the construct assembly structure and encapsulating protein assembly into the in-cell protein crystal platform for constructing solid materials.

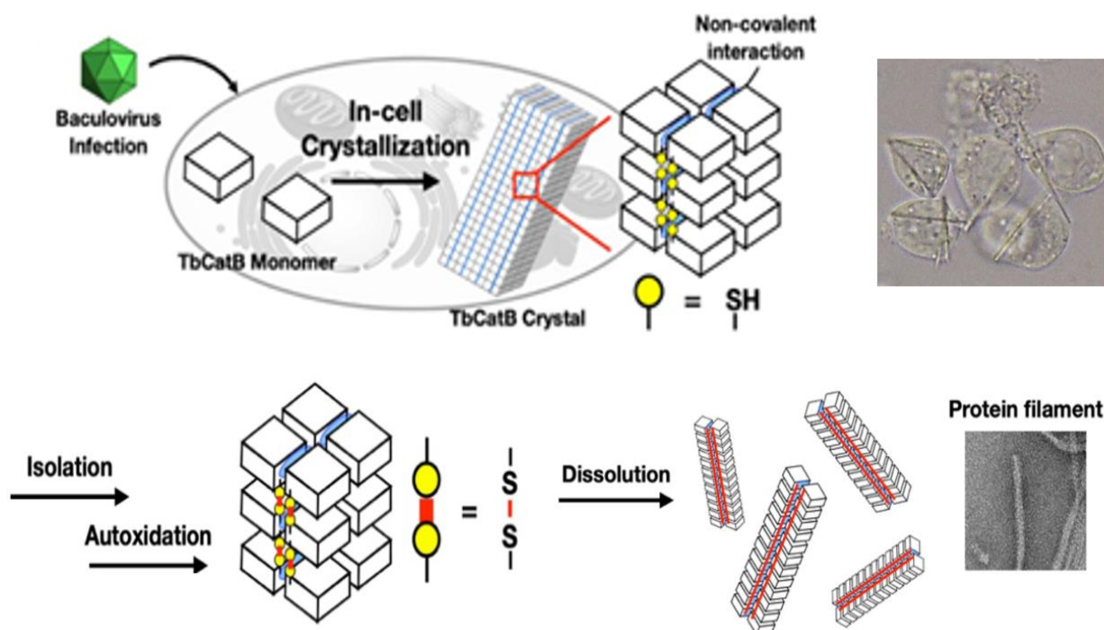

Figure 1. Schematic representation of the construction of supramolecular protein bundled filament via spontaneous disulfide bond formation in protein crystals and dissolution of the crystals. Transmission electron microscopy was used to examine the bundled filament.

Protein assemblies can be designed for the development of nano-bio materials. This has been achieved by modulating protein-protein interactions. However, the fabrication of highly ordered protein assemblies remains challenging. Protein crystals, which have highly ordered arrangements of protein molecules, provide useful source matrices for synthesizing artificial protein assemblies. **Chapter 2** describes constructing a supramolecular filament structure by engineering covalent and non-covalent interactions in a protein crystal. Performing in-cell crystallization of Trypanosoma brucei cysteine protease cathepsin B (TbCatB), we achieved a precise arrangement of protein molecules while suppressing random aggregation due to disulfide bonds (Figure 1). After isolating the crystals from living cells, we synthesized bundled filament from the crystals by autoxidation of cysteinyl thiols.

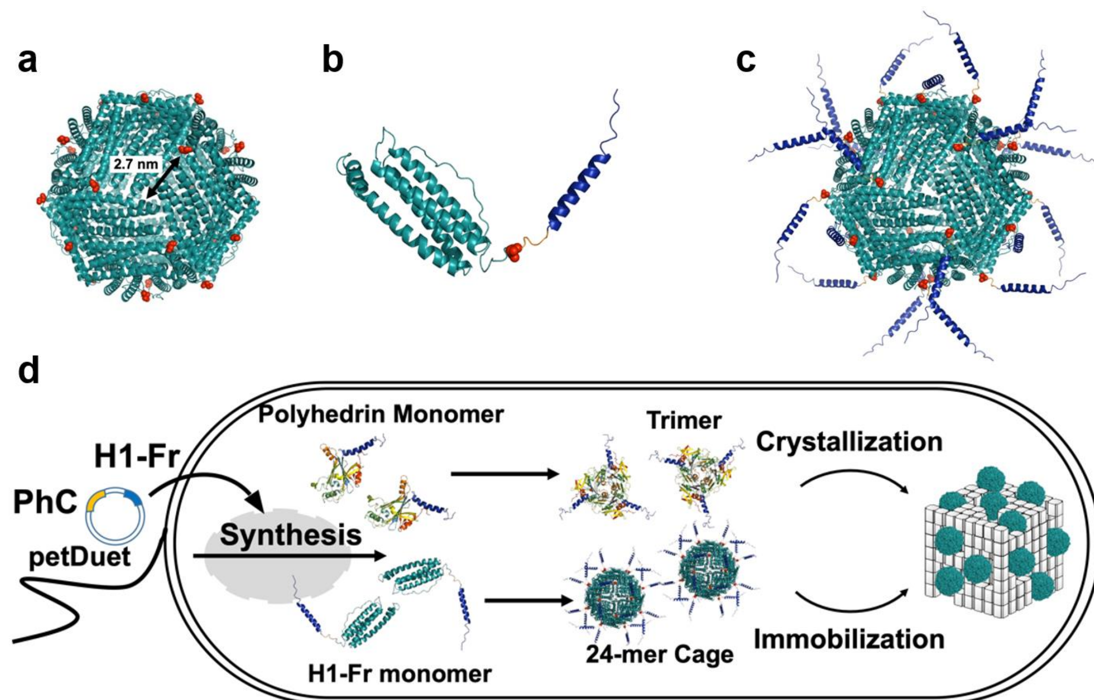

Figure 2.) Crystal structure of ferritin cage (PDB: 1DAT). A red spherical model indicates the locations of the N-terminus of ferritin. Model structure of fusion protein of H1-helix and ferritin (H1-Fr); (b) monomeric structure, and (c) 24-subunit cage structure. (d) Schematic illustration of the preparation of PhC immobilized H1-Fr in *E.coli*.

The development of solid biomaterials has rapidly progressed in recent years for applications in bionanotechnology. The immobilization of proteins, such as enzymes, within

protein crystals is used to develop solid catalysts and functionalized materials. However, an efficient method for encapsulating protein assemblies has yet to be established. **Chapter 3** presents a novel approach to displaying protein cages onto crystalline protein scaffold using in-cell protein crystal engineering. The polyhedra crystal (PhC) scaffold, which displays a ferritin cage, was produced by co-expression of polyhedrin monomer (PhM) and H1-ferritin monomer in *E. coli (*Figure 2). The H1-tag is derived from the H1 helix of PhM. The pH-dependent release of H1-Fr cages shows that this hybrid crystal can be a pH-sensitive drug delivery carrier. Our technique represents a unique strategy for encapsulating protein assemblies into in-cell protein crystals and is expected to contribute to various applications in structural biology and biotechnology.

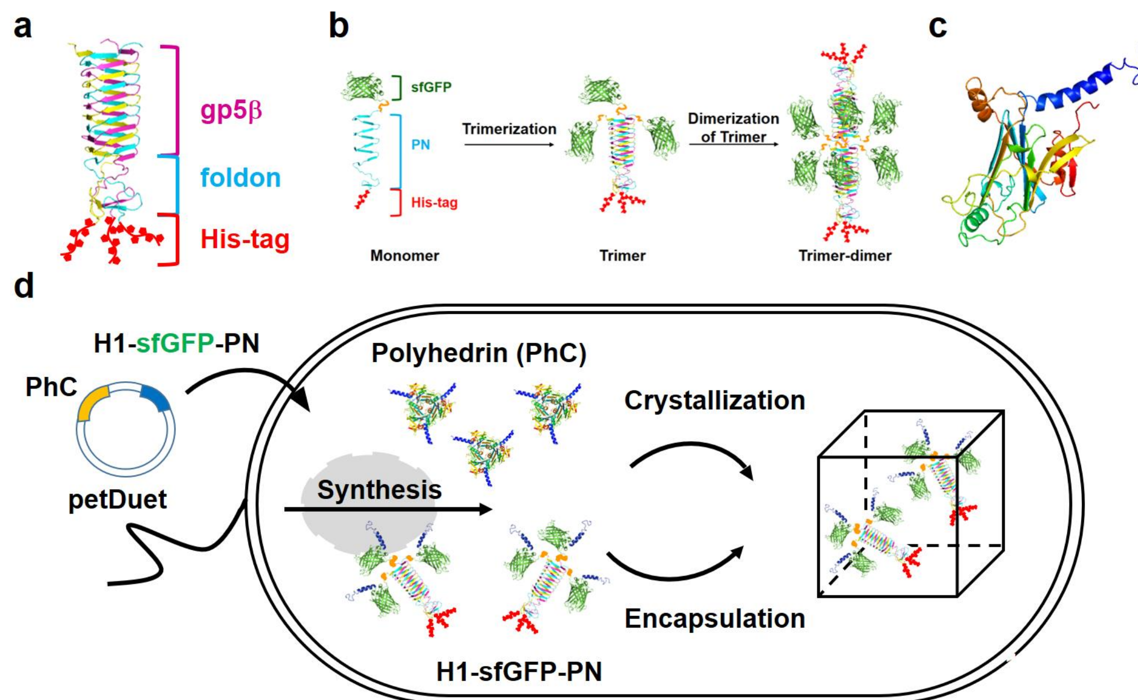

Figure 3. (a) Structure of a trimer of Protein Needle (PN) (PDB ID: 3A1M). (b) A protein needle is an assembler accumulating fused proteins with a high concentration around the PN (ref). (c) Structure of polyhedrin monomer (PDB ID: 2OH5). (d) Schematic representation of the enhancement of encapsulation of sfGFP into polyhedra crystal boosting by PN in *E.coli* system.

In Chapter 3, we found that multiple interactions with PhM are crucial for encapsulating protein assembly into PhC crystal. Get inspired from this point; a method for enhancing protein

encapsulation that stays in a monomeric stage assisting by protein assembly has been developed. **In Chapter 4**, We have established a new method for encapsulating superfold green fluorescent protein (sfGFP) into polyhedra crystal (PhC) with high efficiency using a protein needle (PN) (Figure 3). H1-sfGFP-PN, in which PN is fused to the C-terminus of H1-sfGFP, exhibits a 55-fold increase in encapsulation of sfGFP relative to H1-sfGFP with the improvement provided by the cooperative effects of the H1-helix, PN, and the His-tag at the C-terminus of PN. This method is expected to provide valuable materials based on the encapsulation of various functional proteins.