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Cell-Free Co-synthesis of Protein Nanoassemblies: Tubes, Rings, and Doughnuts

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ABSTRACT

We used a cell-free transcription/translation system to synthesize structural proteins of the T4 bacteriophage. We focused on two proteins that participate in the formation of the virus tail tube assembly. Synthesized separately, the proteins assembled into their in vivo forms, namely one polymerized into rigid hollow nanotubes ~20 nm thick and hundreds of nanometers long, the other assembled into 10 nm tube-capping hexameric rings. Co-synthesis of the two proteins, however, revealed a novel structure of a nanodoughnut with an outer diameter of ~50 nm and thickness of ~20 nm. Cell-free co-synthesis and assembly of T4 structural proteins can be extended in a combinatorial fashion. The addition of other structural genes offers control of native nanoassemblies and may reveal ones not observable by mixing purified components.

Biological molecular machines that fulfill both a structural role and perform mechanical work, such as viruses, flagella motors, and cytoskeleton motility elements, are an inspiration for artificial nanoscale systems (reviewed in ref 1). DNA and proteins are routinely used in combination with a variety of inorganic materials to construct hybrid nanodevices. A common modular approach for utilizing DNA and proteins for nanodevices is to assemble hybrid structures by fusing together biomolecules with distinct features. The molecules are taken out of their biological context to create a desired architecture using molecular biology techniques in combination with organic synthesis. The structural modules are interconnected by specific high-affinity molecular pairs, e.g., biotin/avidin, 6xHistidine tag/Ni^{2+}, etc.[2] In this approach, the repertoire of envisioned structures is limited to the conformations of the initial building blocks.[2−5]

DNA can be programmed into artificial higher-order self-assemblies due to its unique base pairing capability, which facilitates sequence-dependent coding of novel structures (reviewed in ref 6). Peptides have also been used to self-assemble into nanowires, nanotubes, and nanopaticles depending on their primary amino acid sequence (reviewed in ref 7). A more challenging task is to employ in such construction schemes larger polypeptides with biological recognition and activity capabilities. However, it is hard to program proteins into self-assembling structures other than those allowed by their in vivo native conformations. In an attempt to expand the repertoire of structural modules and to create a new protein toolbox of nanoscale building blocks, we propose an approach in which a complex biological construction process such as virus assembly is taken apart into its elementary assembly steps and is then put together in various combinations. In this combinatorial approach, the viral proteins are biosynthesized in a cell-free transcription/translation system. The newly synthesized proteins self-assemble as their concentrations gradually build up, a process that is inherently different from mixing together purified proteins. While the onset for self-assembly may occur at a different concentration for each of the proteins synthesized, their co-synthesis may lead to co-assembly at a unique onset. In a cell-free reaction, one can tune the protein concentrations by their respective genes. Biochemical circuit elements could then be added to control biosynthesis.[8] Outside of their cellular environment and without the intricate network of cellular regulation, the viral building blocks are allowed to manifest their entire spectrum of putative interactions, some of which may be down-regulated in vivo because they lead to misassembled viruses. Therefore, an ensemble of structural modules may arise, some resembling their in vivo forms, some presenting novel structural motifs. As an example for the general approach of in vitro co-synthesis and co-assembly, we present the reconstruction of two native nanostructures, tubes and rings, as well the discovery of novel doughnuts, all with two structural viral proteins.

We chose to work on T4, an *E. coli*-infecting virus, which is a magnificent nanomachine assembly and an inspiration to nanoengineering. Recently, the self-assembly domains of

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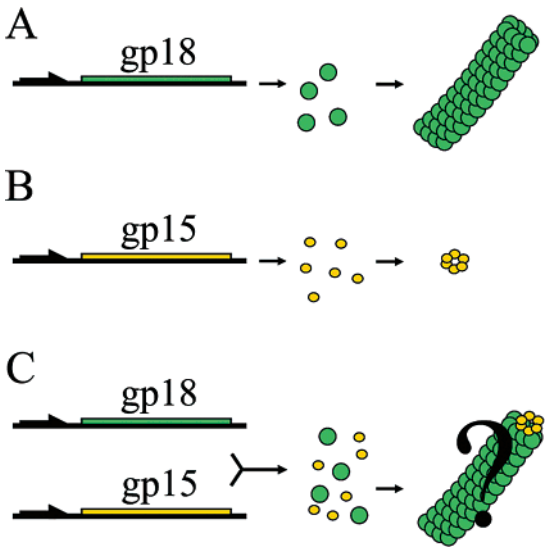

Figure 1. Schemes of T4 tail tube co-synthesis and sub-assembly. T4 respective genes transcribed by T7 RNA polymerase (T7 promoter denoted by arrow in front of genes) and coupled translation in *E. Coli* extracts. Synthesis and assembly of gp18 tubes (A), gp15 hexameric rings (B), and co-synthesis of gp18 and gp15 assembled into unknown structures (C).

the fiber proteins of the T4 tail were used as elements in the construction of novel structures by rearranging their genes.[9] Because T4 is composed of dozens of different proteins, we chose to focus on a small subset of proteins that compose its tail, a protein nanotube.[10,11] One of the main proteins that constitute the tail tube is gp18. It forms the tail tube sheath, covering an inner tube made of gp19 protein, through which viral DNA is injected upon infection[12]. Gp18 protein has been shown to maintain its polymerization ability in vitro in making aberrantly long tubes when purified and reassembled[13] or when overexpressed from a plasmid in *E. coli* cells.[14] Gp18 can therefore serve as a cornerstone structural element that can be modified by other viral proteins. A natural choice of a modifying protein is gp15,[10] another T4 protein involved in tail tube assembly. Gp15 is the presumed capping protein of the tail tube and, based on recent reconstitution analysis, is found within the tail structure in direct contact with gp18 sheath protein.[15] To study the assembly mechanism of gp18 and try to control the architecture of this nanotube by gp15, we have expressed gp18 and gp15 in a cell-free system both separately (Figure 1A,B) and simultaneously (Figure 1C). Coexpression of gp18 and gp15 may result in assembly of gp18 into tubes and gp15 into hexameric rings, independently. Alternatively, if gp15 hexameric rings interact with gp18 tubes, one can expect formation of shorter capped tubes. A third possibility is that co-synthesis of gp18 and gp15 would lead to novel assembled structures (Figure 1C).

As mentioned above, cell-free systems allow the flexibility of coexpressing several proteins at once with direct control over their relative amounts and time of expression.[16] Coexpression of two or more genes is a difficult task to fulfill in bacteria due to the limited ability to control expression from several promoters at once in a quantitative fashion. We have chosen to express gp15 in the extract by using the overlap PCR technique (OL-PCR). This technique adds regulatory sequences essential for gene expression in linear templates without the need to go through tedious cloning procedures into plasmids. The whole construct is then amplified by PCR rather than plasmid propagation in *E. coli* cells. This technique is essential for our future plans to screen a large number of viral proteins in a combinatorial fashion within cell-free extracts. Gp15 with a 6xHistidine tag at its C-terminus (gp15his), expressed in the extract and purified on Ni-NTA beads (Figure 2A, lane 1), formed its expected hexameric ring structure[17] as imaged by transmission electron microscopy (TEM) (Figure 3B,D). Gp18 with a streptavidin-binding protein tag[18] at its C−terminus (gp18sbp) was also expressed in the extract and purified on streptavidin-linked beads by eluting with buffer containing biotin (Figure 2A, lane 2).

TEM imaging of purified gp18sbp from the extract (Figure 3A,C) showed that it assembled into tubes with dimensions of hundreds of nanometers long. Apparently the sbp tag, 38 amino acids long added to the C-terminus, did not interfere with tube assembly. This result is in accord with previous observation that the C-ter of gp18 tolerates changes that do not hamper its polymerization ability.[14] In addition, the C-ter of gp18 contributes to the flexibility of the tube diameter in that a C-ter truncation led to a narrower tube.[14] This width flexibility manifested by the gp18 tube sheath suggests its futuristic utilization as a mechanical nanotube.

The relative and simultaneous expression of gp15 and gp18 proteins can be demonstrated by the use of radioactive ^{35}S-methionine, which is incorporated into newly synthesized proteins only. Figure 2B shows that, indeed, the two proteins can be coexpressed within the extract to different extents. To identify structural elements that may form when gp18 and gp15 are coexpressed, an extract expressing the two proteins at a 1:1 gene ratio was divided in two: one aliquot was combined with streptavidin-linked beads to purify gp18sbp, while the other was combined with Ni-NTA beads to purify gp15his proteins. The rationale of this approach is that high-affinity protein complexes would coelute from an affinity column despite the fact that only one of the proteins contains a specific tag matching the column. TEM analysis of the eluant from the Ni beads revealed amazing structures of tubes interconnected with doughnutlike structures with an outer diameter of ∼50 nm (Figure 4A). We did not observe these doughnut structures in either gp18sbp or gp15his only samples. In addition, no doughnuts were observed by mixing independently expressed and purified gp15his and gp18sbp. Instead, we could detect only gp18sbp tubes and gp15his hexamer coexisting on the TEM grids (Figure 4C). The fact that the doughnut structures were eluted from a Ni-NTA affinity column strongly suggests that they were partly composed of gp15his proteins and that the His tag was exposed in the structure to allow binding to the Ni-NTA resin. The thickness of the doughnut is similar to the thickness of the gp18sbp tubes (20 ± 5 nm). This similarity strongly suggests that the doughnuts observed are composed mainly of gp18sbp tubes that were somehow modified by gp15 protein, most likely by its direct integration into the

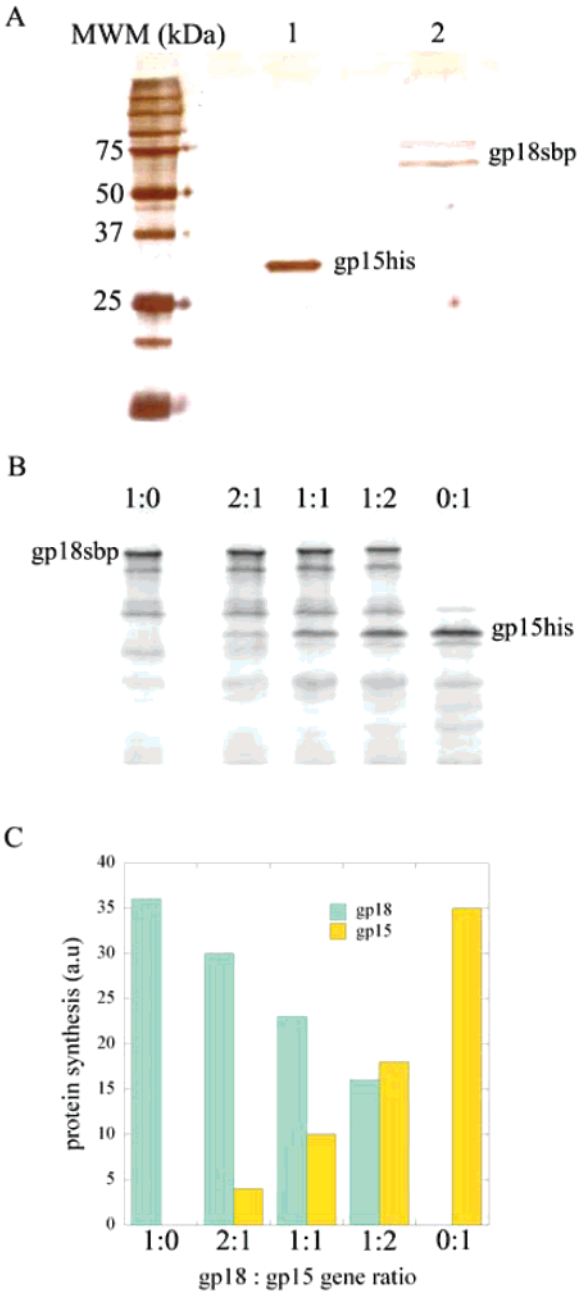

Figure 2. Analysis of T4 gp18 and gp15 protein cell-free biosynthesis. (A) gp15his (lane 1) and gp18sbp (lane 2) were expressed in an *E. coli* cell-free extract (RTS100, Roche) that had been incubated with 1 nM OL-PCR fragment or plasmid DNA, respectively, and micropurified by either Ni-NTA (Qiagen) or strepavidin affinity purification (Pierce), respectively. Analysis by 12% SDS-poly acrylamide gel electrophoresis (PAGE) stained with silver (MWM; molecular weight markers in kDa). (B) Coexpression of both gp18sbp and gp15his in the presence of ^{35}S-methionine (in vitro translation grade, ~1000 Ci/mmol, Amersham), incubated for 1 h at 30 °C, separated on a 12% SDS-PAGE, and analyzed by phosphorimaging (Fuji). Ratio of gp18sbp to gp15his genes from left to right 1:0, 2:1, 1:1, 1:2, 0:1. (C) Quantitation of gp15his (yellow) and 18sbp (green) coexpression as measured by phosphorimager analysis of the SDS-PAGE digital autoradiogram in (B).

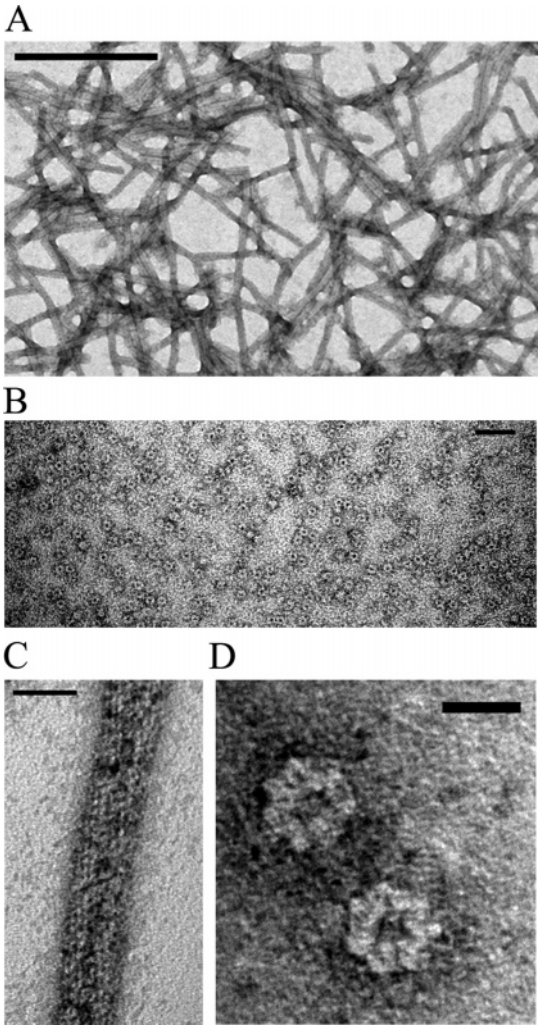

Figure 3. TEM imaging of gp18sbp and gp15his T4 proteins after cell-free biosynthesis and purification. Negative staining by 1% w/v aqueous uranyl acetate of samples deposited on carbon-coated Nickel grids. Samples were observed in a Tecnai 12 transmission electron microscope (FEI, Eindhoven, The Netherlands), operated at 120 kV. Micrographs were taken with MegaView III CCD camera (SIS, Muenster, Germany). (A, C) Gp18sbp tubes, (B, D) gp15his hexamers. Scale bars: (A) 500 nm, (B) 40 nm, (C) 20 nm, and (D) 10 nm.

helical structure, to form doughnuts. If indeed the doughnuts are formed by coexpression of gp18sbp and gp15his, doughnuts should also be purified on streptavidin-linked beads rather than by Ni-NTA beads. Because the streptavidin-purified preparation is rich with gp18sbp tubes, it was difficult to detect doughnuts in this eluant. After extensively searching the sample on TEM grids, a few cases of isolated doughnuts were found (Figure 4B), suggesting that their formation is a rare event.

The persistence length of gp18 tubes is of the order of a micron, as can be deduced from their observed stiffness over hundreds of nanometers (Figure 3A). Bending such tubes to high curvature is prohibitively costly in energy unless the crystalline structure of the tube is somehow modified to relax this bending energy. We therefore hypothesize that the doughnut is formed by random incorporation of gp15 monomers into the gp18 tube, forming smaller "impurities" in the crystalline structure that facilitate local bending. The formation of the doughnut is yet a rare event. A thorough investigation of the detailed mechanism leading to the formation of doughnuts, including direct evidence for the

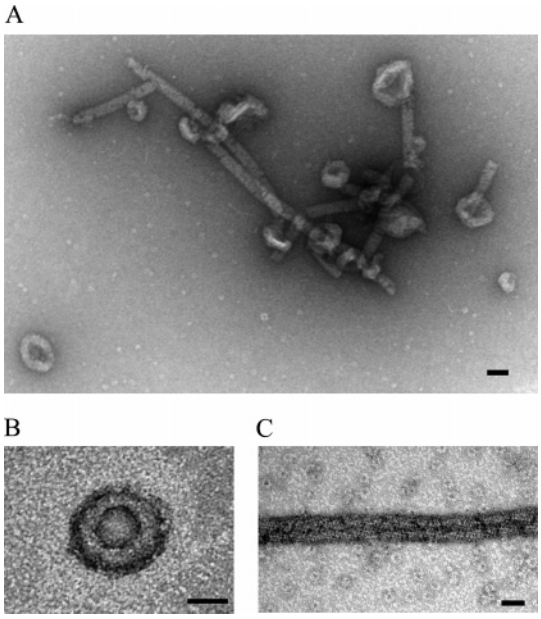

Figure 4. TEM imaging of co-synthesis and assembly of gp15his and gp18sbp. (A) Purification of gp15his, in the presence of gp18sbp, by Ni-NTA; scale bar 40 nm. (B) Purification of gp18sbp, in the presence of gp15his, by streptavidin resin. Scale bar is 20 nm. (C) Co-existence of purified gp15his and gp18sbp mixed postpurification. Scale bar is 20 nm.

incorporation of both gp18 and gp15 monomers, ratios of expressed proteins within the extract that are required for its formation, relative amounts of the two proteins within the doughnut, and parameters that influence doughnut dimensions, is crucial to our ability to utilize these assemblies. The last few decades provided clear evidence for a hierarchy of tail tube assembly steps. We find it significant that cell-free co-synthesis of a subset of only two of the ~18 proteins that comprise the T4 tail lead to a co-assembled structure that is markedly different than the in vivo wild-type structure. Is the nanodoughnut also formed in vivo but is discarded as a misassembled body part? Otherwise, is the virus tail tube assembly pathway so optimized that only a single and unique structure forms when the proteins are all present to co-assemble? It would be interesting to add more tail tube genes into the cell-free biosynthetic reaction in a combinatorial fashion to investigate whether a spectrum of unknown nanostructures is revealed. Finally, nanodoughnuts[19,20] are an addition to the repertoire of nanostructures that can be used to construct bionanodevices. Their unique shape is particularly attractive in conjunction with tubes (Figure 4A), and it would be a challenge to construct a sliding clamp nanomechanical device. Protein nanostructures could be synthesized on a bio-compatible photo-lithographic interface in order to control their localized assembly.[21]

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