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The Nano Revolution: Bottom-up Manufacturing with Biomolecules

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ABSTRACT

As the nano-scale becomes a focus for engineering electronic, photonic, medical, and other important devices, an unprecedented role for biomolecules is emerging to address one of the most formidable problems in nano-manufacturing: precise manipulation and organization of matter on the nano-scale. Biomolecules are a solution to this problem because they themselves are nanoscale particles with intrinsic properties that allow them to precisely self-assemble and self-organize into the amazing diversity of structures observed in nature. Indeed, there is ample evidence that the combination of molecular recognition and self-assembly combined with mutation, selection, and replication have the potential to create structures that could truly revolutionize manufacturing processes in many sectors of industry. Genetically engineered biomolecules are already being used to make the next generation of nano-scale templates, nano-detailed masks, and molecular scaffolds for the future manufacturing of electronic devices, medical diagnostic tools, and chemical engineering interfaces. Here we present an example of this type of technology by showing how a protein can be genetically modified to form a new structure and coated with metal to lead the way to producing "nano-wires," which may ultimately become the basis for self-assembled circuitry.

Keywords: Nanotechnology, biomolecule, chaperonin, self-assembly, nanowire

1. INTRODUCTION

The controlled organization of materials on the nanoscale is the ultimate goal of the bottom-up manufacturing pursued by nanotechnology. At this scale, material packing densities and manipulations present technical challenges for current patterning manufacturing technologies, such as dip-pen and electron and ion beams lithography. While this scale exceeds the limits of most lithographic patterning processes, packing densities and quantum effects (e.g., single electron tunneling quantum confinement) are strong incentives to pursue this miniaturization process to nanometer size scales. The alternative approach that is widely being pursued involves self-assembly and self-organization.

Self-assembled inorganic and organic molecules that naturally form one-, two- and three- dimensional structures are a major focus of research in nanotechnology. One- and two-dimensional nano-structured materials are being investigated for their use as templates, scaffolds, or guides for fabricating prototype devices, such as quantum-dot lasers (1), single-electron transistors (2), memory units (3), sensors (4), optical detectors (5), and light-emitting diodes (LEDs) (6). There is currently also a growing interest in fabricating one-dimensional (1D) nanostructures from metal or semi-conducting materials, which can be used as both interconnects and functional units in electronic, electrochemical, and electromechanical nano-devices (7). Efforts to fabricate such nano-wires and nano-tubes include using inorganic templates, which take advantage of step edges of solid substrates, and organic templates, which take advantage of self-assembling polymers, including synthetic polymers and biological macromolecules, including DNA and proteins.

Biomolecules in general and proteins in particular, are not only capable of self-assembling into intricate patterns with nanoscale architecture, they can be manipulated and functionalized using methods developed for biotechnology. The astonishing diversity of structures formed by proteins is apparent in nature. Because their synthesis is genetically directed, both their structure and their function can be effectively manipulated. DNA and various proteins have already been used as templates for nanowires and nanotubes that have been incorporated into nano-structured materials and devices (8-14). We are exploring potential nanotechnology applications for a class of 60 kDa proteins, known as Hsp60s.

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1.1 Obtaining the protein building blocks

The HSP60s are proteins that in the presence of ATP/Mg self-assemble into regular double-ring structures known as "chaperonins." In nature, chaperonins are ubiquitous and essential biological structures comprised of 14-, 16-, or 18-HSP60 subunits arranged as two stacked rings forming supramolecular structures 16 to 18 nm high and 15 to 17 nm wide, depending on their species of origin. The HSP60-subunits consist of three structural domains named in accordance with their position in the double ring. The equatorial domain is the interface between the two rings and includes an ATP binding site that affects changes in the overall conformation of the double ring. ATP binding causes shift in the apical domain by a shift in the intermediate domain, hence the name "hinge domain".

We used the chaperonin with 18 subunits produced by *Sulfolobus shibatae,* an organism which lives in geothermal hot-springs and grows optimally at temperatures of 83°C and at pH 2.0 (15). This organism makes three related Hsp60 subunits and we chose the subunit called "beta." Sequence and structural information are available for beta and we have previously established that it forms octadecameric chaperonins (9-subunits/ring) that can be induced to assemble into filaments (16). Expressing the thermostable beta subunit in *Escherichia coli* allowed us to eliminate most *E. coli* proteins, which are thermolabile, simply by heating total protein extracts (17). A structure for the wild-type beta-chaperonin was constructed by homology modeling using the X-ray structure of the isomorphic chaperonins (18). The structure was used as a guide for mutagenesis to modifying beta to produce chaperonins that can be used for patterning (19). In previous experiments, we have demonstrated that the beta subunits retain their ability to form chaperonin double rings even after their ends are moved to a variety of new locations in the protein (20). This process called, circular permutation, allowed us to explore the effects of truncating the beta subunit.

2. MATERIALS AND METHODS

2.1 Cloning and expression of the dwarf protein

Gene construct and cloning of the dwarf protein are based on the procedures of circular permutation of the chaperonin protein Beta (20). Fragments of DNA before and after the permutation site are amplified separately using the PCR method with the flexible linker with the sequence GGSGGT added to the beginning and end of the genes. The two fragments are annealed together at the flexible linker and the resulting template DNA was cloned into a standard *E. coli* expression plasmid (pET19b, Novagen) (21). The protein was expressed from this plasmid in *E. coli* BL21DE3 and purified by heat treatment and ion exchange chromatography using Mono-Q column.

2.2 Assembly of the dwarf protein into rings, filaments and 2D arrays

Subunits of the dwarf protein in HEPES buffer were mixed with NaCl, $MgCl_2$, and ATP and the final concentrations are 1-5 mg/ml, 0.1 M, 25 mM, and 1mM, respectively. The mixtures were incubated at 4 °C - 90 °C for 1 hour; rings or filaments or 2D arrays were formed depending on temperatures.

2.3 Nickel deposition of dwarf filaments

The procedure of electroless metal plating (22) was followed with a minor modification. Dwarf filaments, 100 μL, in 25 mM of HEPES buffer pH 7.5 and $Pd(CH_3COO)_2$ 11 μL, 2mM were mixed and incubated at room temperature for 1 hour. The mixtures were dialyzed against MES buffer pH 5.26 at 4 °C overnight first, then against distilled water. The Pd-filaments solution was added to the metallization bath containing 4 g/l of DMAB and 200 mM of NiSO4 for 2 min to 1 hour.

2.4 Electron microscopy

Protein samples were attached to lacy carbon grids with ultrathin formvar (Ladd Research Industries), stained with 0.22 μm filtered 12% uranyl acetated for 3 min, rinsed with water, and air dried at room temperature. Nickel-coated protein samples were not stained with uranyl acetate. The grids were viewed in a LEO 912 AB with tungsten filament at 100 kV. Images were recorded with a MegaView digital camera using ANALYSIS 3.5 software.

2.5 Conductivity measurement of the protein nanowires

The conductivity of protein nanowires and nickel coated protein nanowires were measured using a HP semiconductor parameter analyzer 4155 B. The nanowires were laid on an interdigitated electrode structure (IDE) across two electrode leads by casting a droplet of aqueous solution of protein samples (23). The IDE with many pair of fingers provides a large contact area of electrode to the nanowires, which ensured that the contact between the metal electrode and protein nanowires are reliable. The measurement was done by sweeping the voltage with scan rate of 2mV/sec and recorded the current passing through these nanowires.

3. RESULTS

3.1 Truncating beta for new functions

Although a detailed 3D structure of beta is not yet known, X-ray structures for homologous chaperonin subunits are known (18, 23-26) and detailed transmission electron microscopic (TEM) and cryo-electron microscopic analyses of *Sulfolobus shibatae* chaperonin have been reported (27, 28). Using X-ray structures of homologous subunits and TEM analysis of *Sulfolobus* chaperonins, we produced a hypothetical 3D model for the beta.

To truncate the Hsp60 subunits, we began with the circular permutant of beta in which the native amino and carboxyl termini were shortened by 45 amino acids, joined by a six amino acid linker, and new termini were created at amino acid position 267 (20). The new termini of this permutant, referred to as beta-267, are in the loop region of the apical domain.

The chaperonin double rings formed by beta-267 are indistinguishable from wild-type beta rings by TEM and have nearly identical thermodynamic stability based on differential scanning calorimetry (20). Guided by structural information, we truncated beta-267 by deleting 101 amino acids from the amino-terminus of beta-267, which deleted half the apical domain, creating a mutant of 45.7 kDa, the dwarf protein (Figure 1, 2).

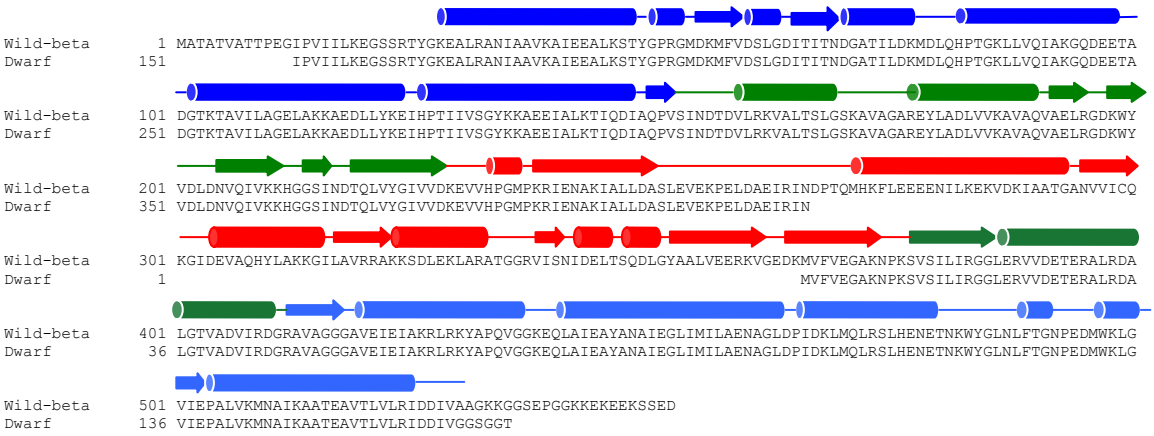

Figure 1: The amino-acid sequence alignments and secondary structures of the wild type beta and the dwarf mutant. The equatorial domain region is colored in blue, the intermediate domain in green, and the apical domain in red.

This dwarf subunit self-assembles into double-rings with 9 subunits per ring, like the wild-type beta from which it was derived. To produce a model of the dwarf double-ring, we used nine-fold rotational operations with a proportionally expanded diameter relative to the structure of the eight-fold rings of the thermosome. To generate the lower ring, a 2-fold rotational operation was applied to the upper ring with the rotational axis running along the center of the upper ring and perpendicular to the 9-fold rotational axis. The overall shape of the complex is an ellipsoid with a height of 12.5 nm

along the pseudo 9-fold axis and a diameter of 17 nm along the 2-fold axes (see Figure 3). The diameter of the filaments shown in the image under Scanning Electron Microscope (SEM) in Figure 3 is 17 nm and the height of the double-ring is 12.5 nm, as predicted by the models.

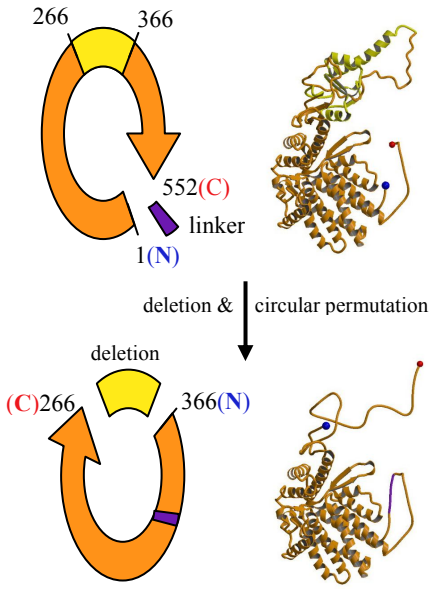

Figure 2: Design of the dwarf protein based on a circular permutant called beta-267 (see text for description). There are 99 amino acids (color in yellow) deleted from the apical domain of beta (top); a flexible linker consisting of the sequence GGSGGT (colored in purple) are connected to the original N- and C-termini; the new ends of the protein are now located at the cuts in the apical domain shown as blue and red balls in the dwarf protein model (bottom).

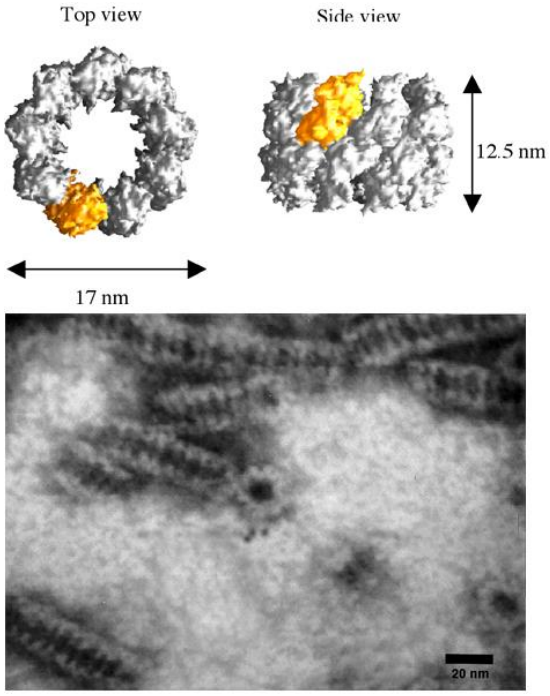

Figure 3: Surface representations of dwarf double-rings and SEM image of the chaperonin and the filaments it forms. The diameter of the ring is 17 nm, similar to native BETA(β); the height of the double-rings is shortened from 15.5 nm of wild type chaperonins to 12.5 nm. The SEM image shows how the rings stack through their apical domains to form filaments. Images taken at 30 kV on a Hitachi S4800 SEM courtesy of Konrad Jarausch at Hitachi High Technologies America.

3.2 The dwarf protein self-assemble into individual filaments or 2-dimensional arrays

We observed the double rings derived from dwarf subunits assembled into filaments, filament bundles, or 2-dimensional (2D) arrays depending on conditions. Under most circumstances, assembly required magnesium chloride ($MgCl_2$) and ATP and was influenced by temperature. The assembly rate and length of filaments was increased at higher temperatures. For example, within 30 min at 75°C most dwarf rings assembled into long individual filaments, while at room temperature they assembled into short filaments, and at 4°C they mostly remain as double-rings. Once long filaments formed, they remain intact for at least 7 days when stored at 4 °C, although were continuously released at a low rate. When incubated overnight with $MgCl_2$ and ATP, at 4° C or at room temperature there were mixtures of short filaments and 2D arrays of double rings, and a few filamentous aggregations also appeared. After overnight incubation at 75°C, we observed some of the filaments were associated with denatured proteins. Figure 4 shows images of the dwarf protein incubated at different temperatures for one hour or over night visualized by transmission electron microscopy (TEM).

In general, at temperatures between 25°C and 75°C and at concentrations above 2 mg/ml long filaments formed, ranging from 0.1 to 3 μm. The upper size limit was presumably set by mechanical forces during transferring. At temperatures >75 °C the dwarf rings and filaments denature slowly and at 90 °C the protein solution turns turbid after one hour, presumably due to protein denaturation.

x

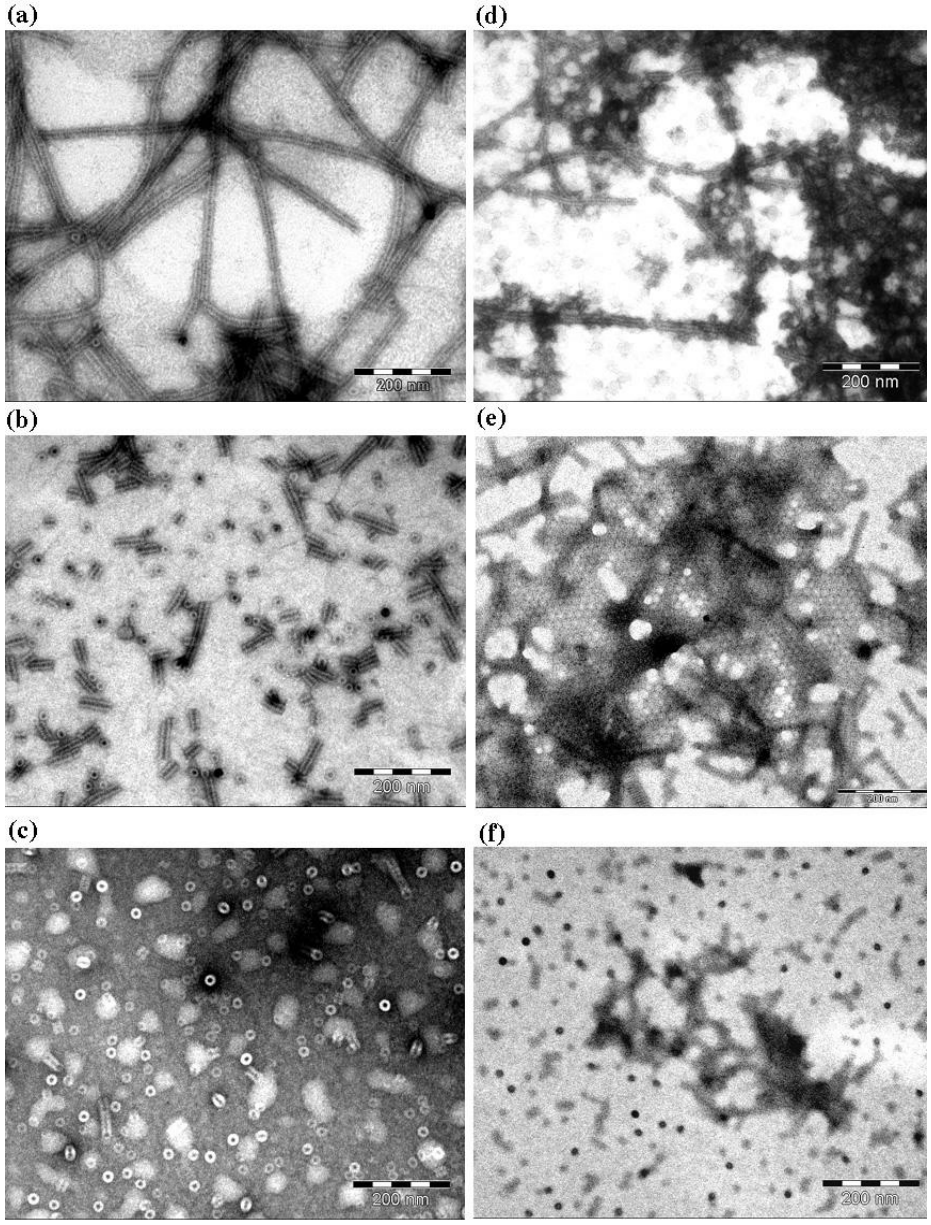

Figure 4: TEM images of the dwarf protein incubated with NaCl, $MgCl_2$, and ATP at different temperatures for one hour or over night. After one-hour incubation (a) at 75 °C most of the rings are incorporated into long single filaments; (b) at room temperature short filaments are assembled; (c) at 4 °C most of the proteins remain as double-rings. When incubated over night (d) at 75 °C some denatured proteins precipitate on filaments; (e) at room temperature 2D arrays are formed; (f) at 4 °C dwarf protein filamentous aggregations appear.

3.3 Other factors affecting filament formation

The dwarf subunit concentrations and ATP/Mg were important for both the assembly of rings and ring association into filaments. Without ATP/Mg at 4 °C or room temperature, we observed few filaments and bundles by TEM. At 75 °C after one hour, we observed mostly aggregates. At higher concentrations of dwarf subunits (> 6 mg/m), a few filaments assembled without ATP/Mg.

We also observed that dwarf subunit assembly depended on the order of addition of reagents. That is, assembly into individual filaments required that ATP/Mg was added to a mixture of protein in buffer. If the protein was added to a solution of ATP/Mg and buffer, bundles formed rather than individual filament.

We suspect that the conformation of the dwarf subunits influenced their assembly into single filaments or bundles. Studies of wild-type chaperonins have revealed two conformations referred to as "open" and "closed" (27, 29-31). In the TEM, we observed what appeared to be different conformations of the dwarf chaperonins in single filaments and bundles (Figure 5). In side views of the rings in single filaments, they appear rounded and resemble the closed conformation of the wild-type chaperonin ring, while in side views of the rings in bundles, they appear more rectangular and resemble the open conformation of the wild-type chaperonin ring. We therefore suspect that assembly into single filaments or bundles depends on the conformations of the rings: the closed rings form single filaments and the open rings form bundles. We suggest this difference in conformation influences the side-to-side interactions between rings and thereby impacts bundling.

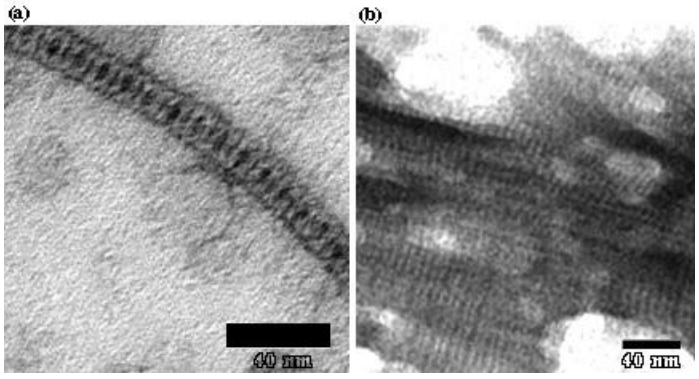

Figure 5: TEM images show (a) the side view of the rings in single filaments is rounded and resembles the closed conformation of the beta ring; (b) the side view of the rings in bundles is rectangle and resembles the open conformation of the beta ring.

The impact of temperature, protein concentration, ATP/Mg and subunit conformation on the formation of rings and the nature of the filaments formed by rings, require further investigation.

3.4 Transforming dwarf filaments into nanowires

To use chaperonin filaments as templates for creating nanowires, we used established electroless metal plating methods to deposit a thin metal film onto dwarf surfaces (22, 32). Electroless deposition occurs by a redox process in which the cation of the metal is chemically reduced on an appropriate catalytic surface. Prior to metal plating the insulating surface of the biomolecular template was activated by attaching catalytic particles. Dwarf filament surface catalysis was accomplished by adsorption of colloidal palladium salts $Pd(CH_3COO)_2$ (Figure 6). The palladium catalyst particles increased the average diameter of the dwarf filaments to approx. 24 nm (Figure 6a). Rinsed filaments with palladium nucleation sites were soaked in a solution of nickel sulfate ($NiSO_4$), with the reducing agent dimethylamine borane (DMAB). The Ni nanoparticles coalesced into a continuous metallic coating covering the dwarf filaments and increased in thickness with time. After 10 min, the average diameter of filaments was 36 nm (Figure 6b). After 15 min, the average diameter of Ni-coated filaments was 63 nm (Figure 6c). After 1 hour, the nickel particles reached a diameter of 200 to 300 nm (Figure 6d). The preferential and very regular deposition of nanoparticles observed in the presence of the dwarf filaments suggested that defined interactions between the functional groups of the protein surface and the palladium in solution were important during particle nucleation. The metallized nanowires appear aggregated and slightly bent, which is also observed on Ni-coated microtubules. The aggregation of dwarf filaments during metallization may be a result of their magnetic properties, causing attraction of the individual tubes.

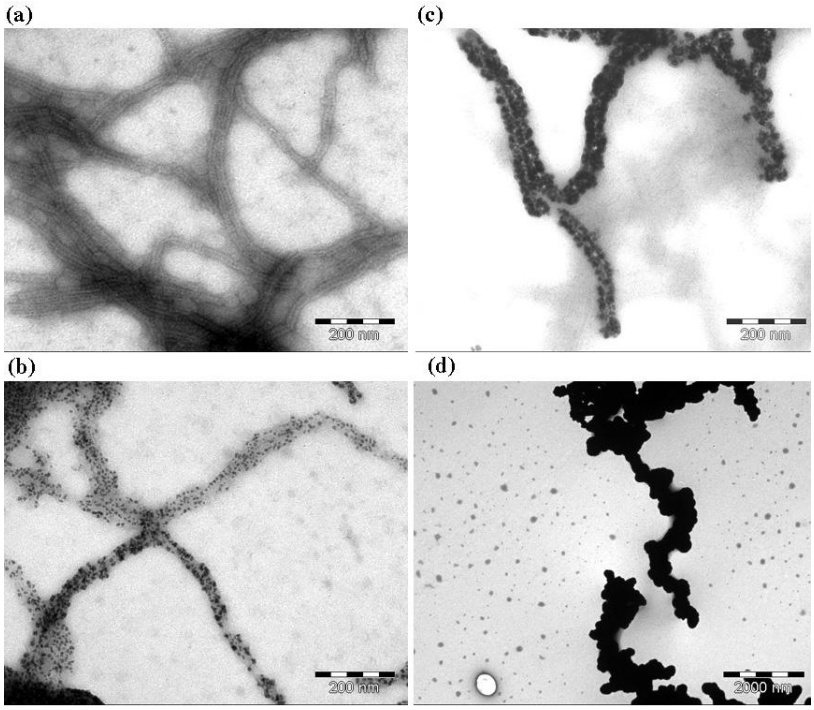

Figure 6: TEM micrographs showing the process of nickel coating on dwarf filaments. (a) Palladium catalyst particles are distributed over the dwarf filament surface negatively stained with 2% uranyl acetate. (b), (c), and (d) show nickel-metallized dwarf filaments resulting from DMAB reduction after 10 min, 15 min, and 1 hour, respectively.

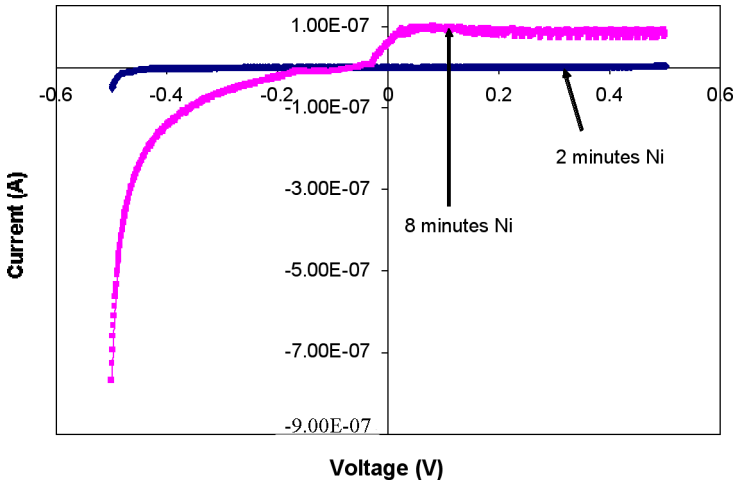

Figure 7: The conductivity of protein nanowires and nickel coated protein nanowires were measured. The nanowires were laid on an interdigitated electrode structure (IDE) across two electrode leads by casting a droplet of aqueous solution of protein samples.). Three protein nanowires were measured of their conductivity: protein itself, protein coated with Ni for 2 minutes (thinner Ni coating, blue curve) and protein coated with Ni for 8 minutes (thicker Ni coating, magenta curve). The conductivity of pure protein nanowires across the IDE electrodes cannot be measured.

3.5 Conductivity measurement of the protein nanowires

The nanowires were laid on an interdigitated electrode structure (IDE) across two electrode leads by casting a droplet of aqueous solution of protein samples (23). Three protein nanowires were measured for their conductivity: protein itself, protein coated with Ni for 2 minutes (thinner Ni coating) and protein coated with Ni for 8 minutes (thicker Ni coating). The conductivity of pure protein nanowires across the IDE electrodes cannot be measured. There was no detectable current (instrument current limit is 10 fA) passing through when the voltage swept from 3V to -3V. The current can be detected at the level of 10^{-8} to 10^{-7}A (see the blue curve in Figure 7) by sweeping the voltage from 0.5V to -0.5V for the protein nanowires that was coated with Ni for 2 minutes. The current was measured at higher level of 10-7A (see the magenta curve in Figure 7) by seeping the voltage from 0.5V to -0.5V for the protein nanowires that coated with Ni for 8 minutes. These results show that pure protein nanowires are strong insulating material. When the Ni metal coated the outside of the protein nanowires, it introduced the conductivity to the protein. The thicker the Ni, the higher the conductivity was obtained as it can be seen in figure 7. However, I-V curves are not linear through origin, which indicates that the protein-Ni materials do not behave as metallic conducting. Further conducting mechanism will be investigated by electrical measurement as well as spectroscopic measurement.

3.6 A novel technique to make arrays of metal nanoparticles

We previously demonstrated that self-assembling native and genetically modified chaperonins that form 2D crystals could be used to organize gold nanoparticles, transition metals Pd, Ni, and Co nanoparticles, and semiconductor quantum dots into ordered arrays (19). Agarose has been used in protein crystallizations to reduce nucleation and sedimentation and grow larger protein crystals (33, 34). We discovered that the dwarf chaperonins were able to self-assemble into 2D arrays in an

agarose matix. We optimized the concentration of agarose so that its melting temperature was below 75°C, which allowed us to take advantage of the thermal stability of the chaperonins. We discovered that uranyl acetate (UA) does not stain agarose and we used UA to visualize dwarf rings, filaments, and 2D arrays by TEM in agarose gel slices.

Using Pd-activated dwarf 2D crystals in solid agarose, metallization with $NiSO_4$ and DMAB, resulted in extensive 2D arrays with Ni metal particles deposited in the centers of rings (Figure 8). By first forming Pd-activated dwarf filaments and solidifying them in agarose the nickel particles are not only coated on the filaments, but also formed nanowires with more uniform diameters (Figure 8b).

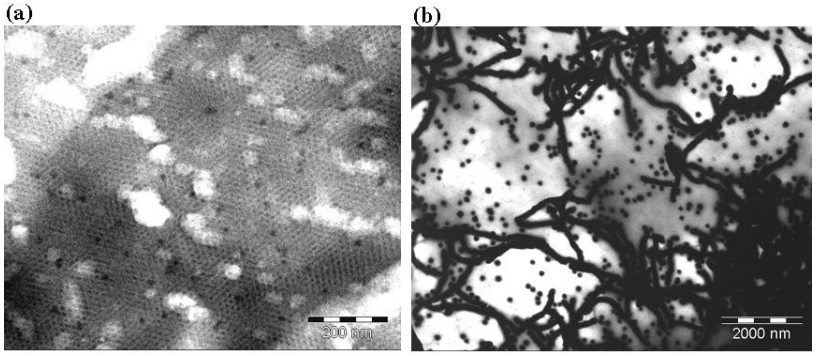

Figure 8: TEM micrographs showing (a) the nickel particles deposit on 2D arrays assembled by Pd activated the dwarf proteins in agarose gel, and (b) the nickel particles are coated on the Pd activated dwarf filaments uniformly in agarose gel.

4. CONCLUSIONS

We have demonstrated by example how a protein can be manipulated genetically to self-assemble into interesting nanostructures and how these structures can function as templates, which can be transformed by a simple chemical process (electroless deposition) from an organic to a metallic material. While our results remain crude by most manufacturing standards, we hope that our readers can see their trajectory and implications. It should be clear from our example that the intrinsic properties of biomolecules, molecular recognition and self-assembly combined with mutation, selection, and replication, have a vast potential in bottom-up manufacturing and that biomolecules will play an unequivocal role in the on-going nano-revolution.

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