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REVIEW

Development of Protein Nanotubes from a Multi-Purpose Biological Structure

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One approach to develop nanosystems that incorporate biological concepts involves the addition of biotic moieties (carbohydrates, DNA, protein) to abiotic scaffolds such as carbon nanotubes. These hybrids have interesting properties but incorporation of specific, site-directed functionalization is challenging and the resulting material is best described in terms of its bulk properties. An alternative approach to the development of bionanosystems is to adapt an existing biological system. This method has several advantages, including access to the powerful tools of protein engineering and ready biological acceptance as these structures themselves are biotic in origin. We have chosen the type IV pilus, a fiber-like structure from the bacteria *Pseudomonas aeruginosa*, as our model system for the development of a protein-based nanotube. This review highlights the biological characteristics of our model system, presents the novel features of our pilin-derived protein nanotubes, and discusses how these protein nanotubes may contribute to bionanotechnology.

Keywords: Protein Nanotubes, Type IV Pilin, Pili, Nanomaterials.

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1. INTRODUCTION

In our desire to create nanodevices with defined functions, we need to discover new materials that have desirable properties at the nanoscale and new methods to assemble these materials into functional units. Carbon nanotubes (CNTs) are a prime example of such a new material. Their excellent tensile strength-to-weight ratio makes them ideal components for the development of novel fibers and structures.[1] Also, their unique electronic and electrochemical properties make them suitable for integration into nanoelectronics.[2] However, while nanotubes have highly attractive intrinsic properties our ability to alter those properties or control their structure in precise ways is limited. Moreover, CNTs may be less attractive for use in biological environments as biosensors or nanomachines due to toxicity issues.[3–5] There are also environmental concerns during CNT production, especially as CNT generation moves from the laboratory to the factory.[6] To this end, it has been recently proposed that multiple independent tests be employed when assessing CNT-based nanomaterials in cellular viability assays.[7]

As an alternative approach to nanotube development, research has begun to look for examples from the natural world. One approach is to functionalize CNTs for biocompatibility; in other words, the CNT is employed as an immobilization substrate for a functional biochemical moiety. Specific biological properties have been imparted to CNTs through covalent and non-covalent attachment of functional groups including carbohydrates,[8] biotin,[9] DNA,[10–12] and proteins.[13,14] Interestingly, Chen and colleagues[15] have reported interfacing CNTs with Chinese Hamster Ovary cells through functionalizing CNTs with polymers designed to mimic surface glycoproteins. In another study, Kam et al.[16] reported functionalization

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of CNTs with cleavable disulfides for the delivery of short interfering RNA molecules into cells for gene silencing. This merging of abiotic and biotic materials is likely to generate many interesting applications, but it still considers nanomaterials in terms of bulk properties.

A separate approach to the development of bionanosystems is to learn from the wide variety of nanostructures that perform critical functions in all living organisms. Indeed, even the simplest cells, bacteria, produce a host of specialized nanomachines whose functions include intra and inter-cellular signaling, energy production and utilization, self-sustained growth, and motility. Perhaps the best examples of abiotic nanomachines that use biological materials are bacteriophages (bacteria-specific viruses). For instance, bacteriophage lambda is considered a lifeless collection of protein and DNA, yet it can specifically bind to bacteria, use an elegant apparatus to deliver its DNA genome into the cell,[17, 18] and direct the bacteria to reproduce and package new viral particles. Evolution has not just selected a number of materials with interesting properties, it has also created the machinery to assemble these macromolecules with great precision from small building blocks, amino acids for proteins and deoxyribonucleotides for DNA. Learning nature's engineering principles is going to be a major challenge but a potentially very rewarding one. An additional advantage of this approach to nanotechnological development is that the building blocks of the chosen system are ready-made for biological integration. Here we will focus on the type IV pilus from the bacterium *Pseudomonas aeruginosa* as our template for the study and development of protein-based nanotubes.[19] The present work presents the biological origins of our model system, details its unique features, and looks forward to the development of protein nanotubes (PNTs) for applications in bionanotechnology.

2. TYPE IV PILUS FUNCTION AND ASSEMBLY

The type IV pilus (T4P) is a filamentous protein structure produced at the poles of a range of both pathogenic and environmental gram-negative bacteria (Fig. 1).[20–22] The T4P are involved in a wide array of functions including host-cell adhesion,[21, 23] motility,[20] biofilm formation,[24, 25] cell signaling,[26] DNA uptake,[27] bacteriophage infection,[28] and attachment to abiotic surfaces such as stainless steel.[29, 30] Bacterial locomotion is achieved by extension of the pilus, followed by binding of the tip to a solid support and subsequent retraction of the T4P. This motion, known at twitching motility, is an important biological mechanism for the development of stable microcolonies known as biofilms (reviewed in Ref. [20]). Assembly and disassembly of T4P is an energy dependant process involving a large number of cellular proteins related to a type II secretion system.[23, 31–33] It has been estimated that *P. aeruginosa* can retract its T4P at rates between 0.5–1 μm s^{-1} ($\sim$1500 subunits s^{-1}), enabling the bacteria to move efficiently across a surface.[22, 34, 35] Using laser tweezers, Maier et al.[36] demonstrated that the forces associated with the retraction of a single T4P exceeded 100 pN. The source of energy for both extension and retraction comes from the hydrolysis of ATP, which is used to either assemble or disassemble pilin monomers at the base of the pilus.[23, 31] However, little is known about the mechanism of (dis)assembly or the signal to switch between extension and retraction.

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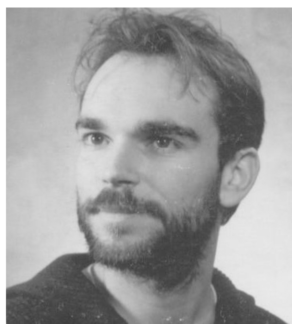

Dr. Bart Hazes received his undergraduate and graduate training at the University of Groningen (The Netherlands) where he completed his Ph.D. studies in 1993 under guidance of Professor Hol. Following post-doctoral studies at both the University of Alberta (Canada) and Cambridge University (UK) he returned to the University of Alberta where he became an Assistant Professor in 2000. His main focus is to use protein crystallography to study the structural basis of protein function, with a focus on medically important proteins. In a separate line of research, Dr. Hazes is developing nanovolume dispensing techniques to create the complex chemical mixtures needed to grow protein crystals.

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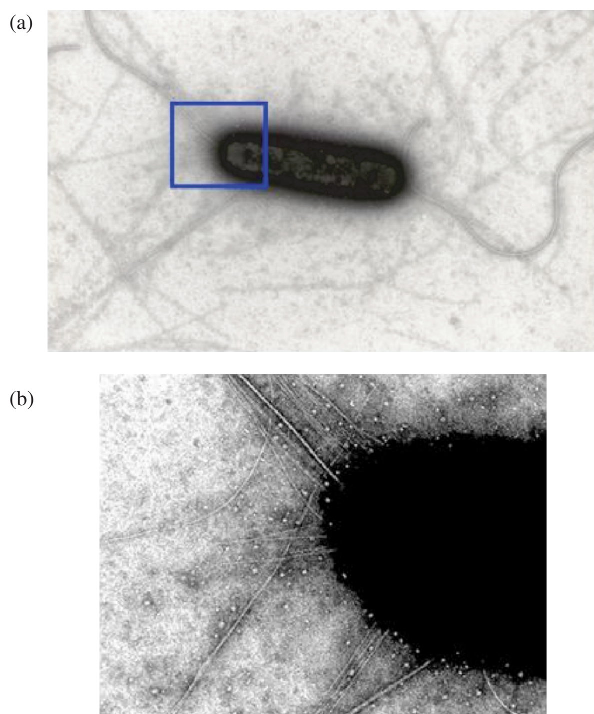

Fig. 1. Type IV pili from the bacterium *Pseudomonas aeruginosa*. (a) Negatively stained transmission electron micrograph of the bacterium. The type IV pili (T4P) are the thin fibers that extend from the poles of the bacteria; the thick fibers are flagella. (b) Magnified view of the bacterial pole (blue square in (a)) highlighting the T4P. Images courtesy of Dr. R. T. Irvin, University of Alberta.

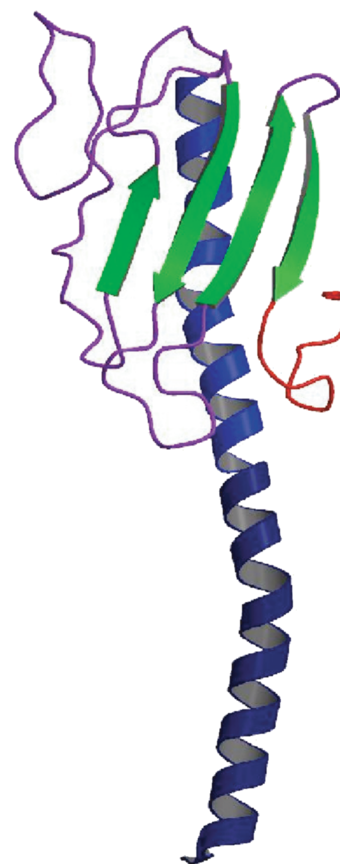

Fig. 2. Schematic representation of the PAK pilin from *P. aeruginosa* (PDB ID 1OQW).[38] The main structural features of the pilin are the N-terminal α-helix (blue), the four-stranded antiparallel β-sheet (green), connecting loop regions (purple), and C-terminal disulfide bonded D-region (red). The N-terminal helix of the pilin is sub-divided into the conserved and hydrophobic α1-N region (dark blue), and the less conserved α1-C region (light blue). Figures 2 and 3 were produced using Molscript[76] and Raster3D.[77]

3. THE ARCHITECTURE OF THE TYPE IV PILUS

T4P are polymers of a single monomeric subunit, known as the type IV pilin, that form a flexible rope-like structure with a diameter of about 6 nm and a length of several micrometers. Structural biology has revealed the atomic models for pilins from several bacteria: *Neisseria gonorrhoeae* strain MS11,[37] *Vibrio cholerae* toxin coregulated pilin (TcpA),[38] *P. aeruginosa* strains K (PAK)[39] and K122-4,[40,41] and the pilin from *Salmonella typhi*.[42] The *V. cholerae* and *S. typhi* pilins are of a separate sub-type (IVb) versus the *Pseudomonas* and *Neisseria* pilins (IVa), based upon size and sequence differences.[21] We will focus our discussion on the type IVa pilins for clarity as our PNTs are generated from the *Pseudomonas* pilins. The reader is directed to the review of Craig and colleagues[21] for a discussion of the major differences between the pilin sub-types.

Figure 2 shows a schematic representation of the three-dimensional structure for PAK pilin. The protein consists of a long α-helix (blue) with a β-sheet (green) wrapped around one end. The amino acid residues that form the N-terminal α-helix (α1-N, dark blue) are very similar in pilins from all bacteria. The T4P assembly machinery also includes proteins with a closely related amino acid sequence pattern.[31] This sequence similarity, combined with the fact that pilins from one species can be polymerized by the machinery of another species,[43] suggests that the conserved N-terminal region of the helix plays a role in pilus assembly. The C-terminal half of the α-helix (α1-C, light blue) exhibits only moderate sequence conservation and the amino acid sequence of the β-sheet and connecting loops (purple) is extremely variable. This variability has been proposed to help bacterial pathogens avoid recognition by antibodies of the immune system.[44] At the C-terminal end of the pilin is a disulfide-bonded loop (D-region, red). This 14–19 residue region has been identified as the binding domain for glycolipid receptors on host cells.[45] Moreover, the D-region of the *Pseudomonas* pilins has been shown to mediate interactions with abiotic surfaces, in particular stainless steel.[30]

Although structures of several T4P monomers have now been determined, it has turned out to be much more difficult to establish how they are arranged inside the T4P filament. Fiber diffraction studies indicate that the T4P is a helical structure with an outer diameter of

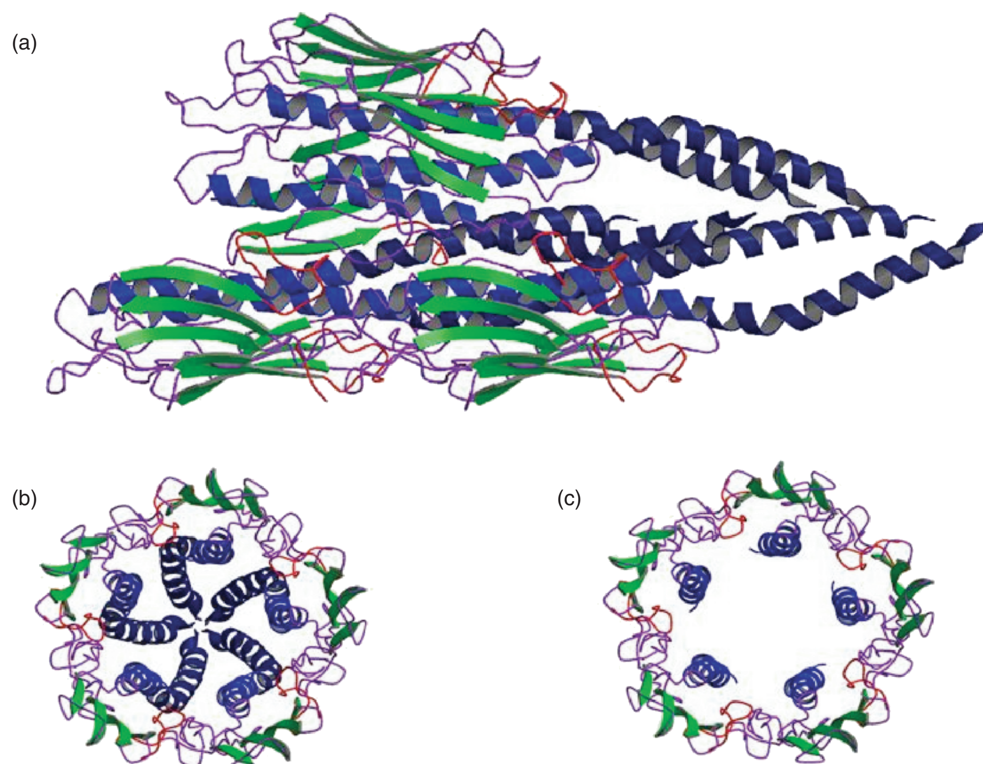

Fig. 3. T4P and protein nanotube models. (a) Schematic representation of the 1-start right-handed helical model[37,48] of T4P based on the PAK pilin[38] monomer. In this model there are 5 pilin monomers per helical turn, with a helical pitch of 42 Å and an outer diameter of ∼52 Å. Colors for the structural features of the pilin monomer are as in Figure 2. (b) Axial view of the T4P from (a). In all current models of T4P, the hydrophobic N-terminal α-helices are sequestered from aqueous milieu with the β-sheet of the pilins forming the outer surface of the structure. (c) Axial view of the pilin-derived PNTs, using the truncated PAK pilin[39] as the monomeric subunit. In this model, the outer diameter of the PNTs (∼60 Å) are similar to those of T4P, however there is a central channel, ∼20 Å in diameter.

approximately 52 Å, a helical pitch of 42 Å, and 5 pilin monomers per helical turn.[46,47] Based on these parameters, as well as electron microscopy images and crystal packing interactions,[21] several models of pilin interactions within the T4P architecture have been proposed. Figure 3 shows the T4P model for PAK pilin based on the original 1-start right-handed helical assembly parameters determined for MS11 pilin.[37,48] Left-handed 1-start[40] and 3-start[38] helix models have also been proposed, as well as a stacked pentamer assembly model based on the 5-helix bundle of Shiga-like toxins (B. Hazes, unpublished data). Although more experimental data is obviously required to determine the true T4P structure, all T4P models sequester the hydrophobic conserved N-terminal α-helix in the interior of the pilus with the variable β-sheet exposed on the outer surface. This architecture hides the N-terminal α-helix from the immune system, which allows this region to act as a conserved oligomerization domain that enables the remainder of the protein to accommodate very different sequences without compromising its ability to be assembled into T4P.

From a nanotechnology perspective, T4P form soluble, robust nanofibres that can bind biotic and abiotic surfaces via their tips. Moreover, tolerance to amino acid sequence variation on the fiber surface makes it possible to use mutagenesis to engineer fibers with altered surface properties.

4. *IN VITRO* CREATION OF PROTEIN NANOTUBES

The assembly of pilin monomers into fibers is not normally a spontaneous process and researchers have not been able to recreate natural T4P or other bacterial filaments from their monomeric precursors in a test tube. The prevention of self-assembly may be a common theme allowing the cell to use the assembly machinery to control the number and length of pili. Structural studies on P-pili have provided insight into the molecular mechanism for this behaviour in that class of filaments.[49,50] In the case of the P-pili, the pilin monomer (PapK) forms a β-barrel with one β-strand missing. The missing β-strand is present in PapK but its structural organization prevents it from completing its own β-barrel. To stabilize the molecule, PapK interacts with a chaperone (PapD) that provides the missing β-strand. During filament polymerization, PapD is proposed to join PapK to the growing filament by making the β-strand of one PapK complete the β-barrel of another PapK in the filament.[49] For T4P the method of pili polymerization is unclear. However, the fact that the assembly

machinery can create normal T4P filaments out of type IV pilins from multiple species[43] suggests that the hydrophobic α1-N region of the N-terminal α-helix, the only part that is conserved between species, plays an important role.

As part of our structural studies of T4P, we have recently deleted the α1-N region of the N-terminal α-helix from *P. aeruginosa* strain K122-4 to increase protein solubility.[41] Although the crystal structure was obtained, we noted that the protein aggregated on Superdex size exclusion chromatography resins, but not on the more hydrophilic Sephadex resins. To our surprise, we found that the highly soluble engineered pilin protein oligomerizes into soluble high molecular weight structures in the presence of hydrophobic surfaces or compounds. Inspection by electron microscopy found that the aggregates formed protein nanotubes (PNTs; Fig. 3).[19]

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5. K122-4 PNTs AND T4P SHARE SIMILAR STRUCTURAL AND FUNCTIONAL PROPERTIES

In EM images, the PNTs and T4P share a very similar morphology and diameter (~5–6 nm). However, the pilin-derived PNTs can reach several hundred micrometers in length,[19] significantly longer than native pili that tend to be around 10 μm long,[21] and may be due to the lack of assembly machinery *in vitro* versus *in vivo*. A second difference is that the pilin-derived PNTs appear to coalesce from nanofibrils.[19] It is unclear at this moment if the observed nanofibrils represent a T4P assembly precursor or if they are an artifact of PNT binding to the EM substrate. Further research is required to clarify these differences between T4P and PNTs.

In order to investigate if the PNTs are structurally related to the native T4P, we tested if they displayed properties exhibited by T4P filaments but not by the monomeric pilin protein or the more common β-amyloid protein fibers.[51] PNTs do not bind Congo-red,[19] suggesting that the pilin subunits do not adopt a β-amyloid structure. Antibodies that bind specifically to the side[52] and tip[53] of the T4P also bind to PNTs,[19] indicating that they share similar surface epitopes. In addition, *Pseudomonas* T4P filaments, but not the monomers, have recently been reported to bind DNA.[54] Again, this property is mimicked by the PNT (discussed below).[19] Lastly, PNTs have been demonstrated to bind to both biotic and abiotic surfaces similar to T4P.[30] Taken together, these data indicate that PNTs retain a conformation similar to T4P, and that they retain biochemical functionalities observed in T4P.

6. CONTROLLED BINDING OF T4P AND PNTs TO ABIOTIC SURFACES

In order to develop PNTs for use in nanoelectronics, adherence of the PNTs to an abiotic surface is required. One method to achieve this would be to grow the PNTs on the abiotic surface itself. We have observed that PNT generation can be triggered by hydrophobic surfaces.[19] While that study employed a non-specific coating of hydrophobe onto the abiotic surface, it may be possible to direct PNT growth to specific positions on a surface through localized application of the hydrophobe on the surface substrate. We are currently investigating this possibility for directed PNT growth from an abiotic surface.

A second approach exploits the observation that T4P can bind to different biotic and abiotic surfaces. This binding is a tip-associated event that has been mapped to the D-region of the pilin.[30,55] It has been proposed that binding is prevented along the length of the pilus because the D-regions of non-tip pilins are occluded in the quaternary structure of the T4P.[19,21] We have recently observed that PNTs can bind stainless steel with similar affinity as native T4P (Giltner et al., submitted), while the binding of epithelial cells by PNTs is significantly less than that observed for native pili (Giltner et al., in preparation). The PNT-steel interaction is mediated by the D-region of the terminal pilin monomers, similar to the binding observed for T4P (Giltner et al., submitted). It has also been demonstrated that T4P can bind polyvinylchloride and polystyrene surfaces,[30] although this has not to date been demonstrated for PNTs. It is plausible that photolithographic methods could be used to create surface patterns with areas that can and cannot bind T4P and thus control T4P surface distribution on a nanoscopic scale.

When there is no need to limit binding to the tip of the T4P/PNT then protein engineering can be used to introduce specific capture functions along the length of the filament. It has been shown that T4P assembly tolerates extra amino acids added to the C-terminus of the protein[45] and it is likely that the same applies to PNTs. One example is to add a poly-histidine tag that directs binding to nickel and copper surfaces. Similarly, the dimeric coiled-coil system can be used, which has previously shown effective in biosensor development.[56,57] This system employs two helix-forming peptides with defined and complimentary heptad repeat sequences that form robust helical coiled-coils with a low dissociation constant.[58] Also, the strength of the coiled-coil interaction can be controlled by modification of the number of heptad repeats in the helical peptides.[59,60] A similar approach has been reported for CNTs where coiled-coil peptides immobilized on CNTs enabled specific capture of metallized peptides.[61] Protein engineering of the pilin monomer can enable PNT capture onto various abiotic surfaces, and research is ongoing to examine the potential of PNT capture systems (R. T. Irvin, personal communication).

The role of PNTs in nanotechnology need not be limited to interactions with abiotic surfaces. It must be remembered that PNTs, like T4P, bind biotic surfaces. In particular, both T4P and PNT have been shown to bind epithelial

cells through specific interactions between the D-region of the pilin and cellular glycolipid (Giltner et al., in preparation). This receptor-specific interaction could allow for the targeted delivery of therapeutics through specific loading of the PNTs. It may also be possible to deliver specific nucleotide sequences to cells via a PNT-nucleotide interaction (see below). Such PNT delivery systems are promising areas for further research.

7. CONTROLLED BINDING OF MOLECULES TO THE PNT SURFACE

The tolerance of T4P to accommodate widely different amino acid sequences on its outer surface creates a situation where protein engineering can be used to introduce binding sites for particular ligands. However, T4P already have the inherent ability to bind DNA. In *Neisseria* and a few other bacterial families, DNA binding by T4P forms part of a T4P-mediated DNA-import system used for natural transformation.[62] Natural transformation has not been observed for *Pseudomonas aeruginosa* but its T4P still bind DNA[54] and DNA has been found to enhance biofilm formation.[63] *In vitro* studies have also shown that T4Ps bind both single-stranded (ssDNA) and double-stranded (dsDNA) DNA with the highest affinity for pyrimidine-rich ssDNA.[54] As mentioned above, PNTs have retained the ability to bind both ssDNA and dsDNA.[19] Moreover, nanotube-bound ssDNA was able to specifically capture a complimentary ssDNA sequence, indicating that ssDNA is bound non-specifically via its phosphodeoxyribose backbone.[19] It was also noted that the DNA could be biotinylated without hindering PNT interaction. These properties are of particular interest for nanotechnology since they form the basic building blocks of an addressable binding system. In this system, oligonucleotides contain a specific sequence that forms an "address-region" followed by a pyrimidine rich "sticky region" for immobilization on the PNT surface. The ssDNA-coated PNTs could then specifically capture ssDNA molecules with the complimentary sequence. Other molecules can be targeted to the PNTs by direct conjugation to the complimentary ssDNA or, indirectly, by using biotinylated ssDNA. The latter approach uses the very high affinity biotin–streptavidin interaction to capture other biochemical targets. Finally, because PNTs can interact with epithelial cells in a receptor-specific fashion, albeit at a lower affinity than T4P (Giltner et al., in preparation), it may be possible to target the cellular delivery of such PNT-captured molecules.

8. ENHANCING THE PHYSICAL PROPERTIES OF PNTs

Compared to CNTs, PNTs will have lower tensile strength and be more sensitive to damage by heat, pH, and chemicals. However, although CNTs will likely always remain superior in this aspect, there are ways to enhance the strength, resistance, or flexibility/rigidity of PNTs by protein engineering or chemical means. Covalent bonds can be introduced by cross-linking reagents such as glutaraldehyde that form bridges between the primary amine groups of lysine residues. This may be acceptable when the focus is on the structural aspects of PNTs, but it will likely compromise applications that depend on specific functions. For instance, lysine residues often contribute to DNA binding and if this is the case for PNTs then chemical modification of lysines must be avoided. Cross-linking between cysteine residues offers a more specific solution. Protein engineering can be used to introduce cysteine residues near protein–protein interfaces in the T4P/PNT. If a detailed structural model of the T4P/PNT filament can be obtained then cysteines can be placed in appropriate positions so that disulfide bonds can form spontaneously under oxidizing conditions.[64] Alternatively, one can use thiol-specific reagents to form intermolecular cross-links between engineered pairs of cysteine residues. By using cross-linking reagents with some inherent flexibility, pairs of cysteines at different distances can be cross-linked with reduced dependence on their relative spatial positions.

9. PNTs AS BIOLOGICAL NANOWIRES

One of the great interests in developing bionanotechnology is the use of these systems as biological nanowires and the natural world again shows examples to learn from. In particular, natural nanowire systems derived from T4P have been identified in several bacteria. The environmental bacterium *Geobacter sulfurreducens*, which is of significant interest in bioremediation,[65] has been demonstrated to transfer electrons through it's pili to insoluble Fe(III) oxides.[66] Very recently, electrically conductive pili have been identified in other species, including *Shewanella oneidensis*.[67] In both studies, functional pili are required for conductivity and a lack of the pilin protein[66] or pili assembly machinery[67] result in abolished electron transfer and metal reduction. While the structural organization of electron transfer along the pili is still under investigation, these studies suggest that T4P-based bionanowire development is a realistic goal.

Another biological nanowire that has been reported is a DNA-based system involving the coordination of divalent metal ions by DNA.[68] Aich and colleagues[69] demonstrated that normal B-DNA, at elevated pH and in the presence of divalent metals, could coordinate the metal ions through novel base pairing. The resultant DNA, denoted M-DNA, can self-assemble/disassemble by varying the pH of the system. Further investigations have shown that the coordinated divalent metal ions of M-DNA impart electrical conductivity to the structure,[69–73] enabling it to act as a molecular wire. It will be of interest to determine if M-DNA still binds to PNTs and if and M-DNA-coated PNT becomes a conductor.

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Finally, protein engineering can be considered as a tool to introduce metal binding sites into the hollow core of the PNT. Current T4P models predict that the inner wall of the PNT is formed by the α1-C helices and helical HxxxH sequences have been used in nature to chelate copper ions between the two histidine residues, for instance in hemocyanins.[74] Similarly, hemerythrin uses a Asp, Glu, and His residues in a helical arrangement to bind iron.[75] Continued research is needed to further develop the use of PNTs as molecular wires.

10. CONCLUSIONS

The discovery of a method to create T4P-like protein nanotubes *in vitro* presents a significant step forward in the development of nanosystems from biological materials. T4Ps, and the derived PNTs, already have a number of inherent properties that further increase their utility for nanotechnology. However, bionanotechnology is still in its infancy and considerable progress in our understanding of protein structure and function is needed to realize the full potential of natural nanosystems and its optimization by protein engineering. Nevertheless, given the enormous potential, learning from nature's examples can provide great benefits for bionanotechnology.

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