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Initial Studies of Protein Nanotube Oligomerization from a Modified Gold Surface

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The type IV pilus of *Pseudomonas aeruginosa* is a nanofibre composed of multiple copies of a single protein subunit, the type IV pilin. In the presence of a hydrophobic surface or solution, engineered pilin monomers oligomerize into soluble, high molecular weight structures—protein nanotubes (PNTs). *P. aeruginosa* pilins, pili and pilin-derived PNTs have been shown to bind both biotic surfaces (cells) as well as abiotic surfaces such as stainless steel. Specific binding of PNTs to abiotic surfaces has the potential for the development of novel biometallic interfaces, leading to possible applications such as biomolecular probes and nanoelectronics. In the current report, we discuss our initial findings on growth of PNTs directly from a decanethiol functionalized gold surface. Utilizing atomic force microscopy, PNTs oligomerized from surface constrained alkylthiols were observed to be several micrometers in length with an average diameter of 36±3 nm. In comparison with reported values for the diameter of native type IV pili and PNTs in solution (~6 nm), the average observed diameter of surface oligomerized PNTs suggests a multiple PNT clustered filament on the gold surface.

Keywords: Protein Nanotubes, Type IV Pilin, Nanomaterials.

Development of biologically relevant nanosystems such as biomolecular probes, sensors and nanoelectronics requires the development of novel systems that effectively interface a specific biochemical environment with abiotic architectures such as computing or electronics. Research focusing on the functionalization of carbon nanotubes (CNTs) for incorporation into more biologically relevant settings leverages the excellent tensile strength to weight ratio[1] and favourable electronic, thermal and mechanical properties[2] of CNTs. These approaches include the addition of functional groups or biochemical moieties such as carbohydrates,[3] proteins[4,5] and DNA[6] to the CNTs through covalent and non-covalent interactions. However, CNT cytotoxicity[7] and significant environmental and health hazards associated with their large-scale production[8] limit their wide-scale application. Alternatively, approaches that harness and engineer an existing biological system have an advantage over functionalizing CNTs in that the building blocks of the system are ready-made for biological integration, resulting in fewer overt toxicity issues. One such system is the type IV pilus from bacterium *Pseudomonas aeruginosa*.

An opportunistic pathogen, *P. aeruginosa* is a significant causative agent of morbidity and mortality in clinical settings, particularly amongst immuno-compromised individuals.[9–11] *P. aeruginosa* infection is initiated by the Type IV pilus (T4P), a long fibrous structure that extends from the poles of the bacterium and attaches to specific receptors on the host cell.[12,13] T4P are also involved in a wide array of functions including motility,[13,14] DNA uptake,[15] and attachment to abiotic surfaces.[16,17] Structurally, T4P are very robust; it has been estimated that *P. aeruginosa* can retract its T4P at rates between 0.5–1 μm s^{-1} (~1500 subunits s^{-1}),[14] and the forces associated with the retraction of a single T4P exceed 100 pN.[18] T4P are polymers of a single monomeric subunit, the type IV pilin. Structural studies of several bacterial pilins have revealed that the pilin protein consists of a β-sheet wrapped around one end of a long α-helix (Fig. 1(a)).[19–25] The N-terminal portion of the α-helix is highly conserved in the type IV pilins, whereas the remainder of the sequence displays little to moderate sequence conservation.[19–25] The C-terminal domain of the pilin is a disulfide-bonded loop, which has been identified as the binding domain for glycolipid receptors on host cells[26–31] and has also been shown to mediate interactions with abiotic surfaces.[16,17]

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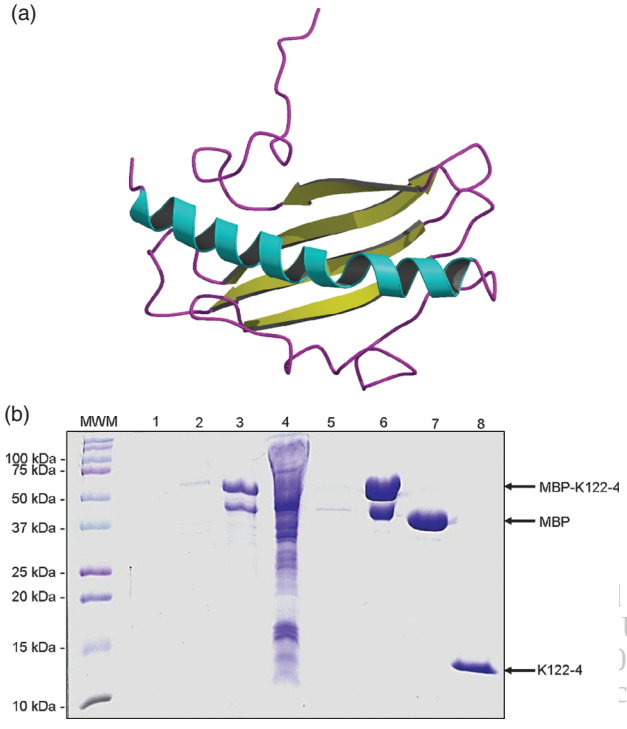

Fig. 1. (a) The structure of the monomeric K122-4 pilin (PDB ID 1QVE),[24] from which PNTs oligomerize. The N-terminal α-helix is in cyan, the β-sheet is in yellow, and coil regions in magenta. The image was produced using Molscript[40] and Raster3D.[41] (b) SDS-PAGE analysis of the purification of monomeric truncated K122-4 pilin. Lanes 1 through 8 are as follows: 1—extracellular solution from expression culture; 2—pre-osmotic shock solution; 3—periplasmic solution (post-osmotic shock); 4—cytosolic contents after osmotic shock; 5—flow through of amylose column; 6—amylose column peak (containing MBP-K122-4 and MBP); 7—flow through of the cation exchange column after trypsin digestion; 8—cation exchange peak containing isolated monomeric K122-4 pilin. The positions of MBP-K122-4 (55.2 kDa), MBP (42.4 kDa) and K122-4 (12.8 kDa) are shown on the right and molecular weight markers (MWM) are in the left most lane.

The N-terminal portion of the native pilin is highly hydrophobic, leading to poor solubility of the intact pilin monomer. Truncation of the 28 N-terminal amino acids of this α-helix results in a highly soluble pilin monomer (Fig. 1) that retains the receptor-binding characteristics of the full-length pilin.[21–25] These truncated pilin monomers were found to aggregate on agarose crosslinked size exclusion chromatography resins, but not on the more hydrophilic, un-crosslinked dextran resins, which led to the discovery that these engineered pilin monomers oligomerized into soluble, high molecular weight structures in the presence of hydrophobic surfaces and solutions.[31] Analysis of these aggregates by electron microscopy identified that they were similar in morphology to native T4P.[31] Noting that the monomeric pilin within the aggregates was a truncated monomer lacking half the N-terminal α-helix, which forms the core of the native T4P fiber,[12, 37–39] the resultant structure was in effect a protein nanotube (PNT).[31] While the T4P and PNTs share a similar morphology and diameter (~6 nm), pilin-derived PNTs can reach lengths of several hundred micrometers, significantly longer than the native pili that tend to be approximately 10 μm long.[12]

While it has been previously determined that PNT oligomerization could be triggered by the addition of a hydrophobe in solution,[31] the development of PNTs for use in nanoelectronics requires binding them to abiotic surfaces or the growth of the PNTs directly from the surface itself. The focus of the current study was to investigate the growth of pilin-derived protein nanotubes from an alkylthiol constrained to a gold surface. The gold-thiol interaction is well characterized, and the assembly of thiol-containing synthetic DNA oligomers on gold particles has been reported recently,[32, 33] suggesting that growth of PNTs from a gold surface constrained alkylthiol is feasible. We report below our initial studies of PNT oligomerization on an alkylthiol-constrained gold surface using atomic force microscopy (AFM).

The monomeric type IV pilin from *P. aeruginosa* strain K122-4 [*pilA*(Δ1-28)] was expressed and purified as previously reported.[24, 34] Briefly, K122-4 pilin was expressed in *E. coli* strain ER2507 cells as a maltose binding protein (MBP) fusion construct and exported to the periplasmic space. Protein expression was induced with the addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) to cells in mid-log phase growth (A_{600} 0.5–0.7) at 37 °C. After 3 hours expression, cells were harvested by centrifugation (6000× g, 4 °C), and the MBP-K122-4 fusion protein was released from the cell by a modified osmotic shock protocol.[35] The clarified periplasmic solution was loaded onto an Amylose resin (NEB) column equilibrated in column buffer (10 mM Tris, 50 mM NaCl, 1 mM EDTA, pH 7.4). After column washing to remove unbound protein from the column, MBP-K122-4 was eluted with 10 mM maltose in column buffer. The K122-4 pilin was released from MBP by the addition of trypsin in a 500:1 protein-to-protease ratio on ice for 15 min. Monomeric K122-4 was isolated using cation exchange chromatography with a linear gradient of 0–1 M NaCl at pH 7.4. Protein purification was monitored via SDS-PAGE; Figure 1(b) shows a representative gel of the purification of the monomeric K122-4 pilin.

The gold surfaces used in this study were 7 mm × 7 mm plates containing a 200–300 nm layer of gold over borosilicate glass (Arrandee). Plates were flame-annealed and submerged in degassed 2 mM decanethiol solution for 1 hour to thiol-link the alkane to the gold surface. The modified surface was characterized by FT-IR using a Nexus 870 Spectrometer (ThermoNicolet) and Cricket (Harrick Scientific Corporation) prior to exposure to K122-4 to confirm functionalization. The C–H bond stretching observed in the 2800–3000 cm^{-1} range of the IR spectrum confirm the $C_{10}SH$ modification of the gold surface (Fig. 2).[36] The modified gold surface was submerged in a 10 mg/ml K122-4 solution (10 mM Tris, 200 mM

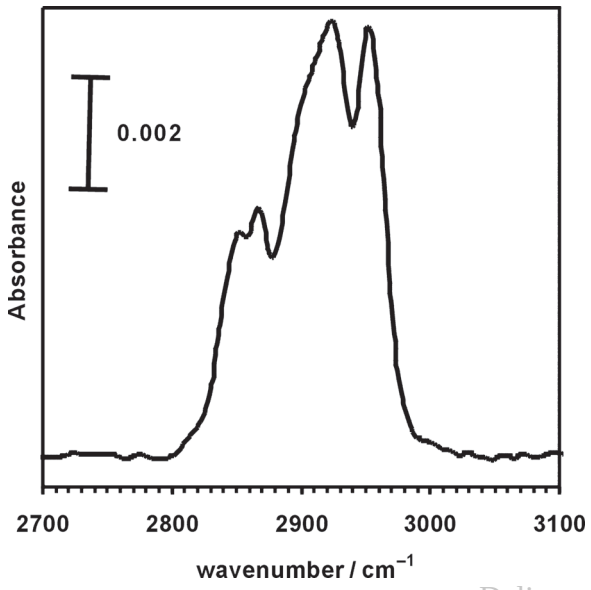

Fig. 2. FT-IR spectrum of 1-decanethiol ($C_{10}SH$) modified Au(111) surface. The spectrum of a bare gold film was used as background.

NaCl, pH 7.4) at room temperature with gentle agitation for 7 days. AFM topographic images of all samples were performed using a Dimension 3100 Nanoscope IIIa AFM instrument (Digital Instruments, USA) in tapping mode.

The AFM images in Figure 3 show the bare gold in comparison with the surface after 7 days of soaking in the K122-4 solution. Gold is not characteristically flat, therefore it was flame annealed to develop an Au(111) texture that gives rise to large flat top grains (Fig. 3(a)). All AFM images of the protein/PNTs on the surface were taken of individual plateaus to maintain a fairly flat imaging area. After 7 days, no PNTs were observed prior to washing (Fig. 3(b)), however after rinsing with ddH_2O and drying with argon, PNTs are clearly visible by AFM (Fig. 3(c)). Most likely, the washing was necessary to remove non-oligomerized pilin monomers that had adsorbed and then dried on the surface. The difficulty in seeing a start or end to the PNTs in the chosen area of the AFM images suggests lengths of several micrometers, consistent with previous observations.[31] Analysis of the PNTs using the N-Surf software package indicates an average diameter of 36 ± 3 nm. Previous studies of PNTs using negatively stained EM[31] suggested an outer diameter of ~6 nm, which is comparable to fibre diffraction[37] and cryo-EM studies of intact T4P.[12, 38, 39] The observed diameter of the PNTs suggests a coalescence of ~6 individual PNTs into thicker fibers over time when oligomerized from a surface due to spatial constraints. In solution, oligomerized PNTs likely remain separated, thereby appearing morphologically similar to native T4P.[31] We are currently investigating this possibility.

PNTs were observed on the $C_{10}SH$-functionalized gold surface after 7 days incubation in the K122-4 solution; annealed gold surfaces soaked overnight in the K122-4

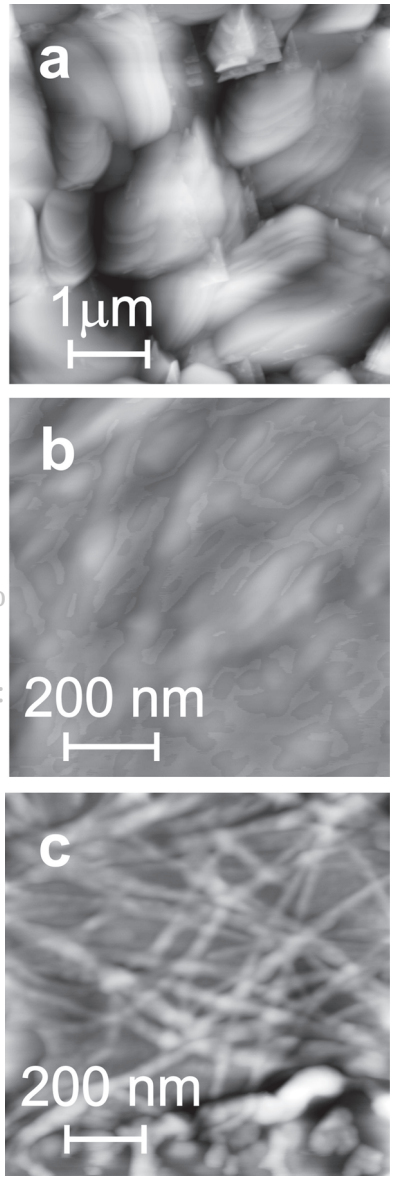

Fig. 3. AFM images (tapping mode) of (a) The unmodified Au(111) surface after annealing and prior to incubation with decanethiol. (b) The Au(111):$C_{10}SH$ surface after 7 days submerged in K122-4 solution. No PNTs are observed, likely due to residual bulk pilin monomer on the surface. (c) The same Au:$C_{10}SH$-PNT surface after rinsing with ddH_2O and dried under argon. PNT filaments are clearly observable, and are similar in morphology to those observed by negatively stained EM.[31] All images are displayed using the same height scale of 100 nm.

solution showed only slight protein aggregation and not PNT formation (data not shown). Previous studies have shown that PNTs oligomerize in ~12 hours in solution from a similar concentration of K122-4 monomer, and that PNT oligomerization from an alkylthiol covalently attached to a maleimide-activated microtitre plate was concentration dependent.[31] While the exact amount of time required for PNT growth from the $C_{10}SH$/Au surface is not clear, a possible explanation for the extended PNT oligomerization time on a surface compared to solution

may be due to the fact that pilin monomers have limited exposure to the surface-bound alkane chains due to their dense packing on the Au(111) surface. In the present study, sufficient $C_{10}SH$ was used to coat the whole Au(111) surface. We are currently engaging in oligomerization kinetics studies to identify optimal oligomerization times for PNTs from surfaces, and are examining the use of mixtures of long and short chain alkane thiols to increase the proportion of long chain hydrophobe exposed to the pilin and to elucidate the exact interactions between the pilin monomers and the surface constrained alkane.

The use of surface constrained proteins at biometallic interfaces facilitates a modular development of the systems for a wide range of applications. The protein component can be readily modified through modern protein engineering strategies and the abiotic surface can also be tailored for the desired application. We have generated a biometallic interface in which a purified protein monomer, the K122-4 pilin (Fig. 1), oligomerizes into PNTs from a $C_{10}SH$ modified Au(111) surface (Fig. 3). Surface constrained PNTs are several micrometers in length and appear to cluster into filaments of ~6 PNTs with and average diameter of 36 ± 3 nm (Fig. 3(c)). PNTs bound to a metallic surface present a unique biometallic interface, and have potential applications in nanotechnology such as molecular scaffolds, biomolecular probes and nanoelectronics. Research is on-going to understand the PNT assembly mechanism as well as the kinetics of this process. It may be possible to confine the growth of PNTs to specific areas of the surface by localized application of the hydrophobe to the surface or by using mixtures of hydrophobes with varying alkane chain lengths and properties of any chain-terminating functional groups.

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References and Notes

1. L. M. Ericson, H. Fan, H. Peng, V. A. Davis, W. Zhou, J. Sulpizio, Y. Wang, R. Booker, J. Vavro, C. Guthy, A. Nicholas, G. Parra-Vasquez, M. J. Kim, S. Ramesh, R. K. Saini, C. Kittrell, G. Lavin, H. Schmidt, W. W. Adams, W. E. Billups, M. Pasquali, W. Hwang, R. H. Hauge, J. E. Fischer, and R. E. Smalley, *Science* 305, 1447 (**2004**).
2. K. Gong, M. Zhang, L. Su, S. Xiong, and L. Mao, *Anal. Sci.* 21, 1383 (**2005**).
3. T. Hasegawa, T. Fujisawa, M. Numata, M. Umeda, T. Matsumoto, T. Kimura, S. Okumura, K. Sakurai, and S. Shinkai, *Chem. Commun.* 19, 2150 (**2004**).
4. S. S. Tatke, V. Renugopalakrishnan, and M. Prabhakaran, *Nanotechnology* 15, S684 (**2004**).
5. A. M. Kannan, V. Renugopalakrishnan, S. Filipek, P. Li, G. F. Audette, and L. Munukutla, *J. Nanosci. Nanotechnol.* 9, 1665 (**2009**).
6. C. Dwyer, M. Guthold, M. Falvo, S. Washburn, R. Superfine, and D. Erie, *Nanotechnology* 13, 601 (**2002**).
7. D. Cui, F. Tian, C. S. Ozkan, M. Wang, and H. Gao, *Toxicol. Lett.* 155, 73 (**2005**).
8. C.-W. Lam, J. T. James, R. McCluskey, S. Arepalli, and R. L. Hunter, *Crit. Rev. Toxicol.* 36, 189 (**2006**).
9. G. P. Bodey, R. Bolivar, V. Fainstein, and L. Jadeja, *Rev. Infect. Dis.* 5, 279 (**1983**).
10. J. B. Lyczack, C. L. Cannon, and G. B. Pier, *Microbes Infect.* 2, 1051 (**2000**).
11. E. Braunwald, Harrison's Principles of Internal Medicine, 15th edn., McGraw-Hill, New York (**2001**).
12. L. Craig, M. E. Pique, and J. A. Tainer, *Nat. Rev. Microbiol.* 2, 363 (**2004**).
13. L. L. Burrows, *Mol. Microbiol.* 57, 878 (**2005**).
14. J. S. Mattick, *Annu. Rev. Microbiol.* 56, 289 (**2002**).
15. D. Dubnau, *Annu. Rev. Microbiol.* 53, 217 (**1999**).
16. C. Giltner, E. J. van Schaik, G. F. Audette, D. Kao, R. S. Hodges, D. J. Hassett, and R. T. Irvin, *Mol. Microbiol.* 59, 1083 (**2006**).
17. B. Yu, C. Giltner, E. J. van Schaik, D. L. Bautista, R. S. Hodges, G. F. Audette, D. Y. Li, and R. T. Irvin, *J. Bionanosci.* 1, 73 (**2007**).
18. B. Maier, L. Potter, M. So, C. D. Long, H. S. Seifert, and M. P. Sheetz, *Proc. Natl. Acad. Sci. USA* 99, 16012 (**2002**).
19. G. F. Audette and B. Hazes, *J. Nanosci. Nanotechnol.* 7, 2222 (**2007**).
20. H. E. Parge, K. T. Forest, M. J. Hickey, D. A. Christensen, E. D. Getzoff, and J. A. Tainer, *Nature* 378, 32 (**1995**).
21. B. Hazes, P. A. Sastry, K. Hayakawa, R. J. Read, and R. T. Irvin, *J. Mol. Biol.* 299, 1005 (**2000**).
22. D. W. Keizer, C. M. Slupsky, M. Kalisiak, A. P. Campbell, M. P. Crump, P. A. Sastry, B. Hazes, R. T. Irvin, and B. D. Sykes, *J. Biol. Chem.* 276, 24186 (**2001**).
23. L. Craig, R. K. Taylor, M. E. Pique, B. D. Adair, A. S. Arvai, M. Singh, S. J. Lloyd, D. S. Shin, E. D. Getzoff, M. Yeager, K. T. Forest, and J. A. Tainer, *Mol. Cell* 11, 1139 (**2003**).
24. G. F. Audette, R. T. Irvin, and B. Hazes, *Biochemistry* 43, 11427 (**2004**).
25. D. J. Kao, M. E. A. Churchill, R. T. Irvin, and R. S. Hodges, *J. Mol. Biol.* 374, 426 (**2007**).
26. M. A. Farinha, B. D. Conway, L. M. Glaiser, N. W. Ellert, R. T. Irvin, R. Sherburne, and W. Paranchych, *Infect. Immun.* 62, 4118 (**1994**).
27. K. K. Lee, H. B. Sheth, W. Y. Wong, R. Sherbourne, W. Paranchych, R. S. Hodges, C. A. Lingwood, H. Kirvan, and R. T. Irvin, *Mol. Microbiol.* 11, 705 (**1994**).
28. L. Yu, K. K. Leek, R. S. Hodges, W. Paranchych, and R. T. Irvin, *Infect. Immun.* 62, 5213 (**1994**).
29. H. B. Sheth, K. K. Lee, W. Y. Wong, G. Srivastava, O. Hindsgaul, R. S. Hodges, W. Paranchych, and R. T. Irvin, *Mol. Microbiol.* 11, 715 (**1994**).
30. H. B. Sheth, L. M. G. Glasier, N. W. Willert, P. Cachia, W. Kohn, K. K. Lee, W. Paranchych, R. S. Hodges, and R. T. Irvin, *Biomed. Pept. Proteins Nucl. Acids* 1, 141 (**1995**).
31. G. F. Audette, E. J. van Schaik, B. Hazes, and R. Irvin, *Nano Lett.* 4, 1897 (**2004**).
32. W. Zhao, Y. Gao, S. A. Kandadai, M. A. Brook, and Y. Li, *Angew. Chem. Int. Ed.* 45, 2409 (**2006**).
33. H. Yao, Y. Changqing, C.-H. Txang, J. Zhu, and M. Yang, *Nanotechnology* 18, 015102 (**2007**).
34. G. F. Audette, R. T. Irvin, and B. Hazes, *Acta Crystallog. D* 59, 1665 (**2003**).

35. H. C. Neu and L. A. Heppel, *J. Biol. Chem.* 240, 3685 **(1965)**.
36. M. D. Porter, T. B. Bright, D. L. Allara, and C. E. D. Chidsey, *J. Am. Chem. Soc.* 109, 3559 **(1987)**.
37. D. A. Marvin, K. Nadassy, L. C. Welsh, and K. T. Forest, *Fibre Diffraction Rev.* 11, 87 **(2003)**.
38. L. Craig, N. Volkmann, A. S. Arvai, M. E. Pique, M. Yeager, E. H. Egelman, and J. A. Tainer, *Mol. Cell* 23, 651 **(2006)**.
39. L. Craig and J. Li, *Curr. Opin. Struct. Biol.* 18, 267 **(2008)**.
40. J. Kraulis, *J. Appl. Cryst.* 24, 946 **(1991)**.
41. E. A. Merritt and D. J. Bacon, *Methods Enzymol.* 277, 505 **(1997)**.

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