Decorating the Outer Surface of Microbially Produced Protein Nanowires with Peptides

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Supporting Information

ABSTRACT: The potential applications of electrically conductive protein nanowires (e-PNs) harvested from Geobacter sulfurreducens might be greatly expanded if the outer surface of the wires could be modified to confer novel sensing capabilities or to enhance binding to other materials. We developed a simple strategy for functionalizing e-PNs with surface-exposed peptides. The G. sulfurreducens gene for the monomer that assembles into e-PNs was modified to add peptide tags at the carboxyl terminus of the monomer. Strains of G. sulfurreducens were constructed that fabricated synthetic e-PNs with a six-histidine “His-tag” or both the His-tag and a nine-peptide “HA-tag” exposed on the outer surface. Addition of the peptide tags did not diminish e-PN conductivity. The abundance of HA-tag in e-PNs was controlled by placing expression of the gene for the synthetic monomer with the HA-tag under transcriptional regulation. These studies suggest broad possibilities for tailoring e-PN properties for diverse applications.

KEYWORDS: Bioelectronic materials, e-biologics, pili, Geobacter, sustainable electronics

Microbially produced, electrically conductive protein nanowires (e-PNs) possess properties and possibilities for functionalization not found in other electronic nanowire materials.1−5 For example, microorganisms can produce e-PNs from renewable feedstocks with low energy inputs. No toxic chemicals are required for microbial e-PN synthesis, and the final product is robust yet also biodegradable. Unlike silicon nanowires,6 e-PNs do not dissolve in water or bodily fluids, a distinct advantage for wearable and environmental electronic sensor applications as well as implantable electronics. A dramatic change in conductivity in response to pH observed in some e-PNs7−10 suggests that they may be readily adapted for diverse sensor functions.7 Some e-PNs have evolved for making cell-to-cell electrical connections,1,9 which may facilitate biocompatibility. Microwires offer the possibility of facile taping of nanowire properties with the design of synthetic genes that modify microbial e-PN structure.7 Diverse microorganisms in both the Bacteria10−14 and Archaea15 naturally produce e-PNs.

The most intensively studied microbially produced e-PNs are the electrically conductive pilus (e-pili) of Geobacter sulfurreducens.7,8,10−11,16−23 Functional analysis of G. sulfurreducens e-pili has demonstrated that, when attached to cells, they serve as conduits for long-range electron exchange with other cells or minerals.1,9 Filaments comprised of the multiheme c-type cytochrome OmcS can also be recovered from preparations of outer-surface proteins of some strains of G. sulfurreducens.14,23 However, the in vivo relevance of OmcS filaments is not clear, and little is known about their electronic properties.24 G. sulfurreducens strain KN400, which produces the e-pilin monomer PilA in abundance but very little OmcS, provides the opportunity to grow cells that express copious e-pili but apparently not OmcS filaments.10,26−28

The available data indicate that G. sulfurreducens assembles its thin (3 nm), long (ca. 10 μm) e-pili from multiple copies of a short (61 amino acids) pilin monomer peptide.10,16,29 The simplicity of e-pili composition offers the potential for broad possibilities in the design of novel wires through genetic manipulation of the monomer peptide gene. For example, the conductivity of e-PNs produced with G. sulfurreducens has been tuned over 6 orders of magnitude (ca. 4 × 10−5 to 3 × 102 S/cm at pH 7)8,11 by genetically manipulating the abundance of aromatic amino acids in the monomer.

Modifying the surface properties of e-PNs with the addition of short, surface-exposed peptides could expand possibilities for attaching wires to substrates, enhance binding of desired analytes for sensor functions, or enable covalent bonding of e-PNs with materials.1,9,11,23
PNs to polymeric materials to produce conductive composites. Inspiration for this possibility comes from previous studies that successfully expressed E. coli curli fibers modified with peptides to confer novel functions such as improved attachment to surfaces as well as metal, mineral, and protein binding. However, curli fibers have a significantly different structure than e-pili and self-assemble outside the cell, whereas pili are assembled from pilin monomers through a complex intracellular process.

Modeling of the G. sulfurreducens e-pili structure predicted that amino acids at the carboxyl terminal of the pilin monomer are likely to be exposed on the outer surface of the e-pili. This suggested that peptides added at the carboxyl terminus might have minimal interference on e-pili structure and thus not destroy e-pili conductive properties, which are hypothesized to rely on a core of closely packed aromatic amino acids. Therefore, we examined whether G. sulfurreducens would express e-pili containing monomers in which peptides were added to the carboxyl terminus, whether the peptides introduced would be accessible on the outer surface of the e-pili, and the potential impact of the added peptides on e-pili conductivity. The results suggest that e-PNs decorated with outer surface peptides to introduce new binding properties can readily be produced with G. sulfurreducens.

RESULTS AND DISCUSSION

Wires Decorated with a Six-Histidine Peptide (His-tag). To evaluate the possibility of displaying peptides on the outer surface of e-PNs, the wild-type G. sulfurreducens gene for the pilin monomer (PilA) was modified (Supplementary Figure 1) to encode six histidines (i.e., a “His-tag”) at the carboxyl end (Figure 1a). The His-tag was chosen for these proof-of-concept studies because highly selective reagents to detect its localization are commercially available. This synthetic gene was inserted into the chromosome of the KN 400 strain that was used for constructing strain PilA-WT/PilA-6His. The resultant strain, which contained genes for the wild-type PilA as well as the histidine-modified PilA pilin monomer (PilA-6His), was designated strain PilA-WT/PilA-6His. Western blot analysis with anti-6His antibody detected a single band in lysates of strain PilA-WT/PilA-6His (Figure 1c). The additional band in the strain PilA-WT/PilA-6His lysate was positioned at the higher molecular weight expected for the PilA-6His monomer (Figure 1c). There was no corresponding band in lysates of wild-type cells.

When strain PilA-WT/PilA-6His cells were treated with a Ni2+-NTA-gold reagent designed to label His-tags, the gold nanoparticles were specifically localized along the wires (Figures 2c–e). Wild-type cells were not labeled (Supplemental Figure 1b) along with the gene for the protein Spc that is required for pilin monomer stability. The resultant strain, which contained genes for the wild-type PilA as well as the histidine-modified PilA pilin monomer (PilA-6His), was designated strain PilA-WT/PilA-6His. Western blot analysis with anti-6His antibody of cell lysates of strain PilA-WT/PilA-6His separated with SDS-PAGE revealed a single protein band at the molecular weight expected for the PilA-6His monomer (Figure 1c). There was no corresponding band in lysates of wild-type cells. Western blot analysis with antibody that detected wild-type PilA detected a single band in wild-type cell lysates and two bands in lysates of strain PilA-WT/PilA-6His (Figure 1c). The additional band in the strain PilA-WT/PilA-6His lysate was positioned at the higher molecular weight position detected with the anti-6His antibody.

Transmission electron microscopy of cells labeled with the anti-6His antibody and a secondary antibody conjugated with gold revealed abundant His-tag loci along the wires emanating from the cells, demonstrating that the added His-tag was accessible to the antibody (Figure 2a, b). There was no immunogold labeling of wild-type cells (Supplemental Figure 2). No unlabeled filaments were observed emanating from cells of strain PilA-WT/PilA-6His, indicating that no other wires, such as those comprised of the cytochrome OmcS, were expressed. This finding is consistent with previous studies that have demonstrated that the KN 400 strain that was used for constructing strain PilA-WT/PilA-6His produces little OmcS and that the filaments emanating from the cells are e-pili.

When strain PilA-WT/PilA-6His cells were treated with a Ni2+-NTA-gold reagent designed to label His-tags, the gold nanoparticles were specifically localized along the wires (Figures 2c–e). Wild-type cells were not labeled (Supplemental Figure 1b).
G. sulfurreducens strain PilA-WT/PilA-6His produced maximum currents comparable to the wild-type strain (Figure 3), indicating the pili retained the capacity to promote long-range electron transport. There was a slightly longer lag period in the initiation of current production in strain PilA-WT/PilA-6His (Figure 3), but this is a period of initial growth in which attachment to the anode and short-range cell-to-anode electron transport are important and thus not indicative of a change in the capacity of the cells for long-range electron transport via e-pili.

Conductivity of individual wires was more directly evaluated with conductive atomic force microscopy (c-AFM), as previously described. The wires were readily identified in topographic imaging in contact mode (Figure 4a), and height measurements (Figure 4c, e) indicated a diameter of 3.1 ± 0.3 nm (mean ± standard deviation; n = 18, 6 points on 3 wires). This corresponds with the 3 nm diameter previously reported for individual e-pili from wild-type G. sulfurreducens and the diameter of individual e-pili when the G. sulfurreducens pilin monomer gene is heterologously expressed in Pseudomonas aeruginosa. The conductive tip was translated to the top of the wire, and the point-mode current—voltage response (I−V) spectroscopy revealed a conductance of 7.2 ± 1.5 nS (mean ± standard deviation; n = 9) under a load force set point (1 nN) (Figure 4g, Supplemental Figure 4). This is slightly higher than the previously observed conductance of 4.5 ± 0.3 nS for e-PNs comprised solely of the wild-type monomer and much higher than the previously reported conductance of the e-PNs from strain G. sulfurreducens strain Aro-S, which lacks key aromatic amino acids required for high conductivity.

**Wires Decorated with Two Different Peptides.** To determine whether two peptides with different functions could be displayed on one e-PN, a gene (Supplemental Figure 1) encoding the previously described nine-peptide “HA-tag” (YPYDVPDYA) at the carboxyl end of the wild-type PilA pilin monomer (Figure 5a) was incorporated into the chromosome along with the PilA-6His and wild-type (WT) genes (Figure 5b). Like the His-tag, the HA-tag was convenient for proof-of-concept studies because a HA-tag antibody is commercially available. The gene for the PilA with the HA-tag (PilA-HA) was located downstream of the IPTG-inducible lac promoter/operator to provide the option of controlling the stoichiometry of incorporation of the PilA-HA monomer in the e-PNs (Figure 5b). This strain was designated G. sulfurreducens strain PilA-WT/PilA-6His/PilA-HA. Western blot analysis demonstrated that, in the presence of 1 mM IPTG, monomers of WT-PilA, PilA-6His, and PilA-HA were expressed in this strain (Figure 5c).

Immunogold labeling for just the His-tag (Figure 6a, b) or the HA-tag (Figure 6c, d) demonstrated that both tags were abundant in the e-PNs from strain PilA-WT/PilA-6His/PilA-HA grown in the presence of 1 mM IPTG. Dual labeling with secondary antibodies with different size gold particles demonstrated that both tags were present in the same e-PNs (Figure 6e, f). e-PNs of strain PilA-WT/PilA-6His cells were not immunogold labeled with the anti-HA antibody (Supplementary Figure 5). The current production of strain PilA-WT/PilA-6His/PilA-HA was similar to that of strain PilA-WT/PilA-6His, indicating the addition of the peptide tag did not significantly diminish pili conductivity (Figure 3).

AFM analysis of the e-PNs (Figure 4b) revealed that incorporation of the HA-tag increased the diameter (height) to 4 nm (Figure 4d, f), suggesting an influence on the pilus assembly. Previous studies have demonstrated that making small changes in amino acid content can dramatically influence pilus diameter, but attempts to determine e-pili structure with cryo-electron microscope approaches have as yet been unsuccessful, making it difficult to interpret this phenomenon. Individual e-PNs of strain PilA-WT/PilA-6His/PilA-HA (Figure 4g, Supplemental Figure 6) yielded higher currents at...
equivalent applied voltages than observed with the e-PNs with just the His-tag with an estimated conductance of 27.2 ± 1.0 nS (n = 9). The mechanisms for electron transport within e-pili are still a matter of conjecture, but aromatic amino acids play a key role.1 Thus, a potential explanation is that the multiple aromatic amino acids in the HA-tag improve electron transport between the conductive AFM tip and e-pili.

Some PilA-HA was expressed in strain PilA-WT/PilA-6His/PilA-HA even in the absence of the IPTG inducer (Figure 7a). However, the concentration of PilA-HA monomer in the cells was greater with added IPTG (Figure 7a). Increased pools of PilA-HA were associated with e-PNs that labeled more heavily with immunogold labeling for the HA-tag (Figure 7b–d). These results demonstrate that it is possible to control the abundance of a specific peptide displayed on e-PNs with transcriptional control of the expression of the monomer modified with that peptide. It is likely that stoichiometric control of peptide display could be improved by fine-tuning the transcriptional regulation, possibly by increasing expression of LacI repressor and adding more LacI binding sites. Furthermore, multiple additional systems for inducing or repressing gene expression in G. sulfurreducens are available which provide other options for controlling the stoichiometry of expression of synthetic pilins decorated with peptide tags.41

**CONCLUSIONS**

These results demonstrate that e-PNs produced with G. sulfurreducens can be decorated with one or more peptides while maintaining, or possibly increasing, their conductivity. The stoichiometry of peptide display can be controlled with transcriptional regulation. These capabilities greatly expand the potential applications of e-PNs in electronic devices and for the fabrication of electrically conductive composite materials.
For example, sensors developed from other nanowire materials show promise for providing highly sensitive and specific, real-time electrical response for detection of diverse chemicals and biologics. Analytes of interest are detected as a change in nanowire conductivity that results from changes in pH associated with the activity of enzymes incorporated into the sensors or binding of analytes to nanowires functionalized with antibodies, peptides, or other ligands. The conductivity of G. sulfurreducens has already been shown to be highly responsive to pH. Short peptides for binding enzymes and antibodies displayed on the outer surface of e-PNs could be an effective method for functionalizing e-PN-based sensors. Both peptides investigated in this study were effective in promoting antibody binding to e-pili. Furthermore, peptides can be designed to function as ligands for a wide diversity of chemical and biological analytes or to enhance attachment to cells. The His-tag demonstrated the potential for a peptide tag to serve as a metal ligand. Thus, the simplicity of modifying the peptides displayed on e-PNs, and controlling the abundance of peptide display, could provide unprecedented flexibility in nanowire sensor design not readily achieved with other materials such as silicon nanowires or carbon nanotubes. In a similar manner, modifying the surface chemistry of e-PNs with short peptides may enable chemical linkages with polymers or enhance binding to materials to aid in e-PN alignment in electronic devices.

The studies described here demonstrate that peptides of up to 9 amino acids can be added to the 61 amino acid monomer backbone of G. sulfurreducens e-PNs. However, it may be possible to decorate e-PNs with much larger peptides because the monomers of other e-pili have an N-terminal region homologous to the G. sulfurreducens monomer but are comprised of over 100 amino acids. These broad possibilities for modifying e-PNs with peptides coupled with...
For the construction of pilA-6His-KN400_3442 by recombinant PCR, pilA-6His-KN400_3442 was cloned at NotI/Xhol sites in pCR2.1 UP-Gm’loxP downstream of Gm’loxP, resulting in pCR2.1 UP-Gm’loxP-pilA-His-3442. The primer pair dnrAvrII (CTTCAGAGGAGCAGACATTTGCGAACTG) and dxXhoI (CATCTCGAGCGGTCCGCTGCGTCTG) was used to amplify by PCR. ca. 530 bp of KN400_0787 downstream of the integration site. This PCR product was cloned at AvrII/Xhol sites in pCR2.1 UP-Gm’loxP-pilA-His-3442, resulting in pCR2.1 UP-Gm’loxP-pilA-His-3442-DN. The final plasmid was linearized with Xhol for transformation as previously described. Transformants were selected with the medium containing gentamicin (20 μg/mL) and were verified by PCR.

Construction of G. sulfurreducens PillA-WT/PilA-6His/PilA-HA Strain. G. sulfurreducens PilA-WT/PilA-6His/PilA-HA strain was constructed by introducing a gene encoding PilA monomer with the HA tag (pilA-HA) together with the gene KN400_3442 in the chromosome of the G. sulfurreducens PillA-WT/PilA-6His strain (Figure 1b). The pilA-HA gene was amplified by PCR with a primer pair TCTGGAGTCAGGAGGACACTTTATGCGTACAGAACAG/CTATTTAACATCCTAGGTATCGCTGCCGCG, resulting in pCR2.1 UP-Gm’loxP-pilA-His-3442-DN. The final plasmid was linearized with Xhol for transformation as previously described. Transformants were selected with medium containing gentamicin (20 μg/mL) and were verified by PCR.

MATERIALS AND METHODS

Strains and Growth Conditions. G. sulfurreducens strains were grown under anaerobic conditions at 30 °C in a defined medium with acetate as the electron donor and fumarate as the electron acceptor as previously described unless otherwise described. Escherichia coli was cultivated with Luria–Bertani medium with or without antibiotics.

Construction of G. sulfurreducens PillA-WT/PilA-6His Strain. G. sulfurreducens PillA-WT/PilA-6His strain was constructed with G. sulfurreducens KN400. A gene for PilA 6His, the gene KN400_3442, which is located downstream of the pilA gene (KN400_1523) on the chromosome, and the putative transcription terminator were integrated at a non-coding region between KN400_0788 and KN400_0787 in the chromosome (Figure 1b). The primer pair upKpnI (CTAGGTACCCGTGTTGACCCCTTACGGGT) and spSpeI (CGAACAAGGAGTGGACCCCTGCGTGCGCC) was used to amplify by PCR ca. 550 bp of KN400_0788 upstream of the integration site with the genomic DNA as template. This PCR product was digested with KpnI/SpeI and ligated with the vector pCR2.1loxP downstream of the integration site. This PCR product was amplified by ImageJ (http://imagej.nih.gov/ij/index.html). The amount of protein was measured with the Bradford Protein Assay (Bio-Rad) as instructed by the manufacturer. Cell extracts were prepared with B-PER Complete Bacterial Protein Extraction Reagent (Thermo Fisher Scientific), and the amount of protein was measured with the Bradford Protein Assay (Bio-Rad) as instructed by the manufacturer. Cell extracts were separated on 16.5% Tris-Tricine gel (Bio-Rad). An anti-PilA antibody was obtained against protein, ESAFADDQTYPPES, corresponding to the C-terminal end of PilA (New England Peptide). Anti-His antibody (6x-His Tag Polyclonal Antibody) and an anti-HA antibody were purchased from Invitrogen. Western blot analysis was conducted as previously described. The molecular weight standard markers were Precision Plus Protein Standards, all blue prestained (Bio-Rad). The amount of PilA-HA was quantified by ImageJ (http://imagej.nih.gov/ij/index.html).
**Immunogold Labeling.** The strains were grown with acetate and fumarate at 25 °C. The PilA-WT/PilA-6His/PilA-HA strain was grown with 1 mM IPTG unless otherwise specified. Immunogold labeling was conducted as previously described with the modification that after the reaction with second gold antibody the grid was washed with PBS three times and water once before staining with 2% uranyl acetate. For immunogold labeling of just one type of peptide tag, the 6x-His Tag Polyclonal Antibody or HA Tag Polyclonal Antibody was the primary antibody and the antirabbit IgG-gold (10 nm) antibody (Sigma-Aldrich) was the secondary. Dual immunogold labeling was conducted with 6x-His Tag Monoclonal Antibody (Invitrogen) and the HA Tag Polyclonal Antibody as primary antibodies and an antimouse IgG-gold (40 nm) antibody (40 nm Goat Anti-Mouse IgG gold conjugate, Expedeon) and the antirabbit IgG-gold (10 nm) antibody as secondary antibodies. Samples were examined with transmission electron microscopy as described previously.

**Ni²⁺-Binding Assay.** The wild-type and PilA-WT/PilA-6His strains were grown with acetate and fumarate at 25 °C. Ni²⁺-binding assay was conducted with Ni-NTA-Nanogold (5 nm) (Nanoprobes). Seven microliters of the culture was placed on a copper grid and incubated for 5 min. The grid was floated upside down for 5 min in phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) and 40 mM imidazole for 15 min and in PBS containing 0.3% BSA, 40 mM imidazole, and the Ni-NTA-Nanogold for 30 min at room temperature. The grid was washed with PBS containing 40 mM imidazole three times and with water once. Samples were stained with 2% uranyl acetate and examined by transmission electron microscopy as described previously.

**Current Production.** The capacity to produce current was determined in the two-chambered H-cell system with a continuous flow of medium with acetate (10 mM) as the electron donor and graphite stick anode (65 cm²) poised at 0.6 to 0.6 V at 0.99 Hz. The voltage sweep of medium with acetate (10 mM) as the electron acceptor as described previously. Briefly, cells were initially grown in the anode chamber in a medium containing 20 mM acetate and 40 mM fumarate. When the current reached 0.5 mA, fresh medium was added to the anode chamber and actuation was continued until the distance: long-range conductivity in protein and peptide bioelectronics: reductionist design of conductive pili mimetics. Bioelectron. Med. 1, 131–137.

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