Layer-by-Layer Assembly of Bioengineered Flagella Protein Nanotubes

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Flagella nanotubes present on the surface of *E. coli* bacteria were bioengineered to display arginine–lysine and glutamic acid–aspartic acid peptide loops. These protein bionanotubes were demonstrated to self-assemble, layer-by-layer, by atomic force microscopy (AFM) on gold-coated mica and quartz surfaces. Flagella with arginine–lysine loops were assembled in a bottom-up manner on a gold-coated mica surface by employing the molecular complementarity of the biotin–streptavidin interaction. Self-assembled monolayers of alkylamines on the gold surface were derivatized with biotin, followed by binding of streptavidin to the biotinylated surface. The amine groups of the flagella peptide loops were chemically attached to biotin through a polyethyleneoxide spacer and paired with streptavidin on the gold surface. This process could be repeated to generate multiple layers of flagella. Flagella with glutamic acid–aspartic acid peptide loops were self-assembled on quartz surfaces by electrostatic attraction to protonated amine groups. The quartz surface was silanized to obtain amine groups, which were used to assemble the first layer of glutamic acid–aspartic acid peptide loop flagella nanotubes. This layer was covered with polyethyleneimine through electrostatic attraction and employed to assemble a second layer of flagella. The self-assembled glutamic acid–aspartic acid flagella were also used to demonstrate the biomineralization of $CaCO_3$. The layer-by-layer self-assembly employing electrostatic attraction yielded a more uniform layer of flagella than the one obtained with the molecular complementarity of the biotin–streptavidin pair.

Introduction

Applications of layer-by-layer assembly as a versatile technique for the fabrication of thin layers and coatings have increased dramatically over the last two decades; layer-by-layer assembly of polycations and polyanions was first proposed by Decher in the early 1990s.[1,2] Electrostatic interaction, hydrogen bonding, van der Waals forces, and covalent bond formation between layers were some of the methods used to hold multiple layers together.[3] As recently reviewed by Tang et al.,[4] this newer approach to the design and construction of thin solid films has potential utility in various biomedical applications and represents an alternative method to more traditional approaches relying on self-assembled monolayers (SAMs) and Langmuir–Blodgett deposition. Many different types of synthetic and natural materials have been employed in layer-by-layer assembly. Organic polymers,[5] nanoparticles,[6] and biological molecules, such as enzymes,[7] DNA,[8] peptides,[9,10] proteins,[11] chitosan,[12] and viruses,[13] have been demonstrated as building blocks in multilayer construction and employed as biosensors, as supports for cell adhesion, as templates for biomineralization, in the controlled release of drugs, and as antibacterial coatings. Layer-by-layer assembly of a viral protein cage architecture was reported by Young and co-workers,[14] where the strong, highly specific, noncovalent interaction between streptavidin and biotin was used. Biological molecules represent genetically encoded natural building blocks, which can be used in the "bottom-up" construction of nano to microscale structures and can overcome the inherent limitations of traditional "top-down" assembly approaches such as photolithography.

In this paper, we discuss the layer-by-layer assembly of an engineered fusion protein of bacterial flagellin and thioredoxin, termed FliTrx, as a functionalized bionanotube. The functionalization was achieved by display of peptide loops on the surface of flagella and chemical modification of the peptide side chains on the flagellin proteins. The bacterial flagella is a whip-like structure that extends from the surface-bound motor complex, followed by a universal joint hook structure and stem, and an elongated fiber, which is composed of the protein flagellin (FliC). The flagellin protein self-assembles to form the helical flagella nanotube, consisting of 11 subunits of flagellin per complete turn; furthermore, the distal end of the fiber is capped with a lid complex formed from a protein pentamer called FliD or Hap2.[15,16] Extensive details of bacterial flagella structure and assembly have been reviewed elsewhere.[17–23] Our research program involves the development of engineered mesophilic and hyperthermophilic bacterial flagella nanotubes for the fabrication of hybrid nanomaterials and bionanotube systems. Recently, we demonstrated a computational model for a thermophilic bacterial flagellin protein[24] and followed this by functional characterization of a D3 domain deletion library of *Salmonella* flagellin.[25] Furthermore, the FliTrx *E. coli* flagellin system[26–29] was used to display rationally designed polypeptide loops in a highly ordered manner on the flagella surface with various functional side chains such as cysteine, arginine, lysine, histidine, aspartic acid, glutamic acid, and tyrosine. These functionalized flagella were used to make covalently bonded

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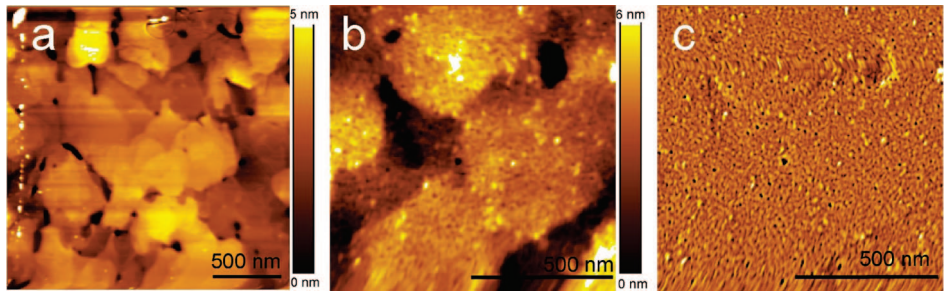

Figure 1. AFM images of original gold-SAM surface and streptavidin monolayer assembled on this surface: (a) topographic AFM image of gold surface with biotinylated amine-terminated mixed SAM on gold-coated mica surface; (b) topographic AFM image of streptavidin monolayer assembled on (a); (c) phase AFM image of (b).

flagella bundles[21] for the complexation and reduction of metal ions on functional groups of the flagella surface to form uniform metal nanotube and nanoparticles,[23] for the directed assembly and polymerization of aniline to form conductive nanotubes,[22] for biomineralization resulting in the formation of SiO_2 and TiO_2 nanotubes and hydroxyapatite crystals,[22] and for the assembly and synthesis of semiconductor quantum dots in an ordered array, where exciton energy transfer between small and large size quantum dots was demonstrated.[30] The work reported here is a continuation of this flagella bionanotube research; it demonstrates the possible application of engineered flagella in surface fabrication using layer-by-layer assembly, aided by electrostatic forces, and recognition through molecular complementarity as the primary interactions. Finally, we discuss potential applications of a surface-assembled bionanotube system as a template for the biomineralization of calcium carbonate.

Experimental Section

Materials. Polyethylene glycol (PEG) 750 (MW 750), polyethylenimine (MW 2000), streptavidin, and 3-[2-(2-aminoethylamino)ethylamino]propyl-trimethoxysilane (AEPTS) were obtained from Sigma-Aldrich (St. Louis, MO). The 11-amino-undecanethiol was obtained from Dojindo Molecular Technologies, Inc. (Gaithersburg, MD). NanoThinks ethanolic solutions of *n*-octane thiol (5 mM) were obtained from Sigma-Aldrich. The *N*-hydroxysulfosuccinimide-polyethyleneoxide-biotin (NHS-PEO_4-biotin) was obtained from Pierce Biotechnology, Inc. (Rockford, IL). Gold-plated mica was obtained from Molecular Probes (Eugene, OR). Cellulose dialysis membrane with a 50 kDa molecular weight cutoff was obtained from Membrane Filtration Products Inc. (Segiun, Texas). Atomic force microscopy of samples was performed using an Agilent 5500 AFM/SPM Microscope (Agilent Technologies, Tempe, AZ) with model FESP AFM tips (Veeco, Santa Barbara, CA), using the "tapping" mode with force feedback control for imaging biological samples.

Preparation of Amine-Terminated Mixed Self-Assembled Monolayers on Au(111) Surface. A calculated weight of 11-amino-undecanethiol was mixed with an ethanolic solution of *n*-octane thiol (5 mM, Nanothinks 8, Sigma) to form a 2 mM solution with an amine thiol/alkyl thiol ratio of 2:5. Self-assembled monolayers (SAMs) were prepared by incubating gold-plated mica plates in the thiol solution for 16 h and cleaned by thoroughly washing with ethanol, followed by rinsing with deionized (DI) water with a resistivity of 18 Mohm.

Biotinylation of Amine-Terminated Mixed Self-Assembled Monolayers. Amine-terminated gold-plated mica plates were placed in a 1 mM solution of NHS–PEO_4–biotin in 100 mM phosphate buffer (pH 7.2) for 2 h. Biotinylated plates were cleaned by rinsing with DI water.

Biotinylation of Flagella. A 5 mL solution of 1 mg/mL of previously described arginine-lysine peptide loop flagella[22] were suspended in 200 mM NaCl and 10 mM, pH 7.2 phosphate buffer. This solution was then mixed with 1 mL of a 5 mg/mL solution of NHS–PEO_4–biotin at pH 7.2 (dissolved in 10 mM phosphate buffer) and stored at 4 °C for 5 h. The *N*-hydroxysuccinimide ester groups react with the primary amine of lysine residues to generate amide bonds. In this manner, biotin molecules were attached to the flagella through the polyethyleneoxide (PEO) spacer. This was followed by addition of another 1 mL of biotin solution and storage at 4 °C for another 2 h. Excess NHS–PEO_4–biotin and other soluble reaction byproducts were removed by dialysis of the flagella sample against 200 mM NaCl, 10 mM phosphate buffer, pH 7.2, for 36 h using a 50 kDa molecular weight cutoff cellulose membrane with a buffer change performed every 12 h.

Layer-by-Layer Assembly of Streptavidin and Flagella. Biotinylated gold-plated mica plates were first placed in a solution of 0.5 mg/mL streptavidin dissolved in 50 mM phosphate (pH 7.2) buffer solution for 10 min, followed by washing three times with a solution of 0.1% (w/v) PEG 750 in 50 mM phosphate buffer (pH 7.2), followed by rinsing with phosphate buffer and DI water. The resulting streptavidin-coated plates were air-dried for 10 min and AFM images were collected using the tapping mode. For assembly of a flagella layer, the streptavidin-coated mica plates were incubated for 20 min in a 0.5 mg/mL solution of biotin-linked flagella in 200 mM NaCl, 50 mM phosphate buffer, and washed three times with a 0.1% PEG 750 solution, followed by rinsing with 50 mM phosphate buffer and DI water. AFM images were recorded after air-drying for 10 min. The same procedures were followed to prepare additional layers of streptavidin and biotinylated flagella nanotubes immobilized on the streptavidin layers.

Layer-by-Layer Assembly of Polyethylenimine and Flagella. Quartz plates with dimensions of 8 cm × 2 cm × 2 mm were cleaned with piranha solution (3:1 concentrated H_2SO_4/30% H_2O_2) and washed three times with DI water. Cleaned and dried quartz plates were placed in a 50 mM solution of AEPTS in absolute ethanol for 4 h and washed twice with absolute ethanol, followed by rinsing with DI water and pH 7.2, 50 mM phosphate buffer. The resulting amine-functionalized quartz plates were immersed in a 0.5 mg/mL solution of glutamic-acid–aspartic acid (Glu–Asp) loop peptide flagella, described previously,[22] in 100 mM NaCl at pH 7.0 (pH was adjusted with 0.01 M HCl/0.01 M NaOH) for 5 min and washed with DI water three times

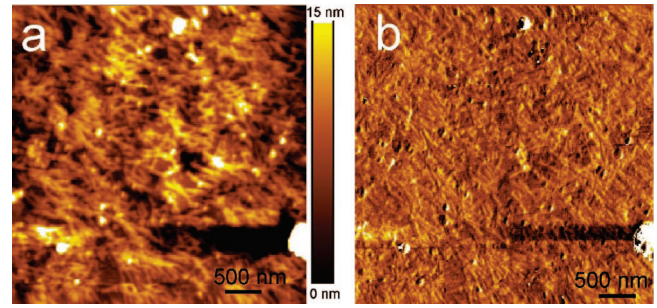

Figure 2. AFM images of the arginine–lysine peptide loop flagella assembled on streptavidin monolayer: (a) topographic AFM image and (b) phase AFM image of (a).

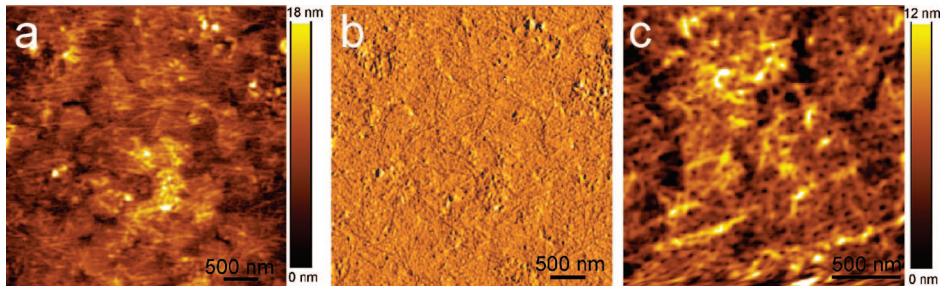

Figure 3. AFM images of streptavidin and flagella monolayers prepared by layer-on-layer assembly: (a) topographic AFM image of streptavidin monolayer assembled on flagella in Figure 2; (b) phase AFM image of (a); and (c) topographic AFM image of the second layer of the arginine–lysine peptide loop flagella assembled on (a).

by dipping. The flagella nanotubes adsorbed on the amine-functionalized plates through electrostatic attraction between NH_3^+ groups on the quartz plate and COO^- groups on the flagella. AFM images were recorded after the plates were air-dried for 10 min and the UV–visible absorbance spectrum was recorded with a Lambda 20 spectrophotometer (Perkin-Elmer, Wellesley, MA). The resulting plates were immersed in a pH 7.0, 0.01% solution of polyethylenimine in water for 5 min and washed three times with DI water. This yielded a second amine layer on the flagella through electrostatic attraction between carboxylate groups of flagella and protonated amine groups of polyethylenimine. This plate was then immersed in the Glu–Asp loop flagella solution for 5 min to assemble a second layer of flagella by electrostatic attraction, followed by AFM imaging and absorbance measurements. The same procedure was repeated for additional cycles to prepare multiple layers of the anionic flagella-cationic polymer composite material on the quartz slides.

$CaCO_3$ Mineralization on Flagella. Flagella were deposited at low density on an AEPTS-coated quartz plate through electrostatic interactions between anionic carboxylate and cationic amine groups by immersing the plate in a 0.5 mg/mL solution of Glu–Asp-loop flagella in 100 mM NaCl at pH 7.0 for 1 min. These quartz plates were placed in 75 mL of 5 mM $CaCl_2$ solution in a 100 mL beaker. This beaker was then placed in sealed chamber containing solid $(NH_4)_2CO_3$ for 4 h to promote the formation of $CaCO_3$ on the flagella. Each quartz plate was air-dried and AFM images were recorded in tapping mode.

Results and Discussion

Mixed thiol SAMs on a gold surface with alkyl- and amine-terminated groups were used to generate a biotin-functionalized Au(111) surface for the assembly of a first layer of streptavidin. In this case, the alkyl-terminated thiol was used to reduce the surface density of the resulting biotin SAM; furthermore, alkyl groups have a very low affinity for proteins, thus minimizing nonspecific binding of streptavidin. Washing treated plates with dilute PEG solutions and DI water also removed nonspecifically adsorbed protein from the surface. AFM images of a biotin-functionalized gold surface and a subsequent streptavidin-assembled surface are shown in Figure 1a–c. In Figure 1a, gold granules with step edges are clearly indicated, and densely packed streptavidin molecules are indicated in Figure 1b,c.

Streptavidin self-associates to form a cubic tetramer with two pairs of biotin binding sites on opposite faces. Therefore, each streptavidin molecule immobilized on the biotinylated surface has a second pair of available biotin binding sites on the solvent-exposed surface of the protein. These unbound biotin binding sites were used to assemble a second protein layer of biotinylated flagella on the surface of the streptavidin layer. AFM images of the assembled flagella layer are shown in Figure 2a,b. Flagella with surface-displayed cationic peptides containing three lysine residues interspersed with three arginine residues (Arg–Lys loop)[22] were chemically coupled to biotin molecules via a 2.9 nm (29 Å) long hydrophilic polyethylene oxide (PEO) spacer

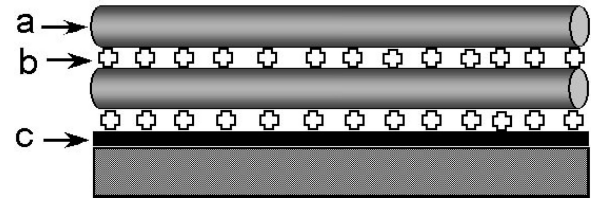

Figure 4. Schematic illustration of layer-by-layer assembly of the arginine–lysine peptide loop flagella through molecular complementarity shown in Figures 1–3: (a) top layer of biotinylated flagella; (b) middle layer of streptavidin; and (c) bottom layer on biotinylated amine-terminated mixed SAM on gold.

arm; theoretically, each flagellin molecule may have three or more biotin molecules covalently linked to its surface. Amine coupling of biotin via the attached PEO spacer arm was used to prevent any steric hindrance of the biotin during binding with streptavidin. Following this procedure, covalently linked biotin molecules were available on the flagella surface. These exposed biotin molecules were used to bind and assemble another layer of streptavidin on the flagella layer. AFM images of the streptavidin layer assembled on flagella are indicated in Figure 3a,b. In a phase image of Figure 3b, assembly of streptavidin was clearly indicated compared to a phase image of flagella in Figure 2b. Another biotinylated flagella layer was then assembled on the streptavidin layer and AFM images were recorded as indicated in Figure 3c. Figure 4 indicates a schematic representation of layer-by-layer assembly of biotinylated flagella nanotubes.

Electrostatic interactions were also used to prepare a layer-by-layer assembly of negatively charged bioengineered flagella nanotubes. In this case, flagella were assembled on a positively charged quartz surface coated with AEPTS. At pH values below 9, the AEPTS amine groups are protonated, resulting in a positively charged surface. The positively charged polyethylenimine was used to electrostatically bind to the negatively charged flagella nanotubes. Figure 5a–d indicates each step in the electrostatically driven layer-by-layer assembly of flagella. Figure 6 is a schematic illustration of the electrostatic layer-by-layer assembly process. The UV–visible absorbance spectra were recorded after each step in the assembly process; the raw absorbance data are indicated in Figure 7. An increase in absorbance in the visible region of the spectrum, where the protein does not absorb, could be attributed to light scattering by multiple layers of protein nanotubes. An absorbance increase in the UV region was due to the aromatic amino acid residues present in the flagellin protein. By comparison, the first layer of flagella generated by electrostatic interaction was more tightly packed than the flagella assembled with biotin–streptavidin linkages. This can be easily seen by differences between Figure 2a,b versus Figure 5a,b. Electrostatic interactions with the

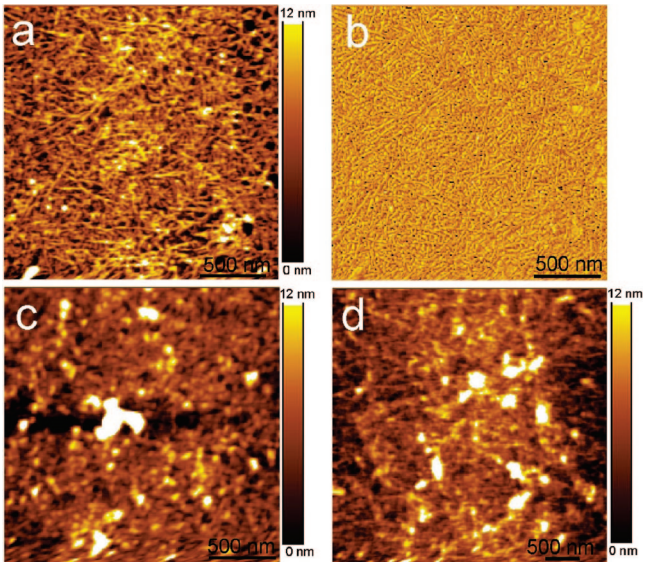

Figure 5. AFM images of anionic flagella layers assembled on the amine–quartz surface by electrostatic interactions: (a) topographic AFM image of glutamic acid–aspartic acid peptide loop flagella monolayer assembled on 3-[2-(2-aminoethylamino)ethylamino]propyltrimethoxysilane coated quartz surface; (b) phase AFM image of (a); (c) topographic AFM image of polyethylenimine assembled on (a); and (d) topographic AFM image of the second layer of glutamic acid–aspartic acid peptide loop flagella on (c).

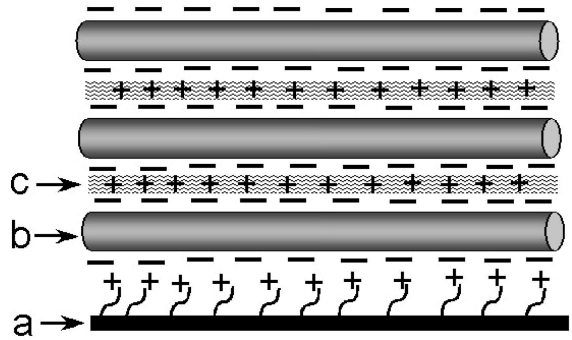

Figure 6. Schematic illustration of layer-by-layer assembly of flagella by electrostatic attraction: (a) quartz with positively charged amine groups; (b) flagella with glutamic acid–aspartic acid peptide loops assembled on (a) by electrostatic attraction; and (c) polyethyleneimine adsorbed on (b) through electrostatic attraction.

flagella bionanotubes occurred over the entire surface of the surface-functionalized quartz, but biotin–streptavidin interactions resulted in only partial binding and assembly of flagella on the streptavidin–gold surface. This suggests that electrostatic interactions are more suitable for generating nanomaterial surfaces with smaller and fewer pores and a more tightly packed flagella monolayer, while the opposite trend holds for flagella monolayers assembled via molecular complementarity.

Glutamic acid and aspartic acid-rich acidic proteins are known to function as templates for natural processes involving in vivo biomineralization of calcium carbonate to form durable bioinorganic materials, such as the shells of marine organisms.[31,32] The study of in vitro biomineralization is important because the resulting hybrid inorganic-biomineralized materials have potential applications as artificial bones, teeth, and cartilage.[33] As a final demonstration of bottom-up assembly of a potentially biocompatible nanomaterial scaffold, calcium carbonate mineralization was performed with previously assembled composites of anionic Glu–Asp peptide loop flagella layered onto positively charged quartz plates. A well-separated, low surface density

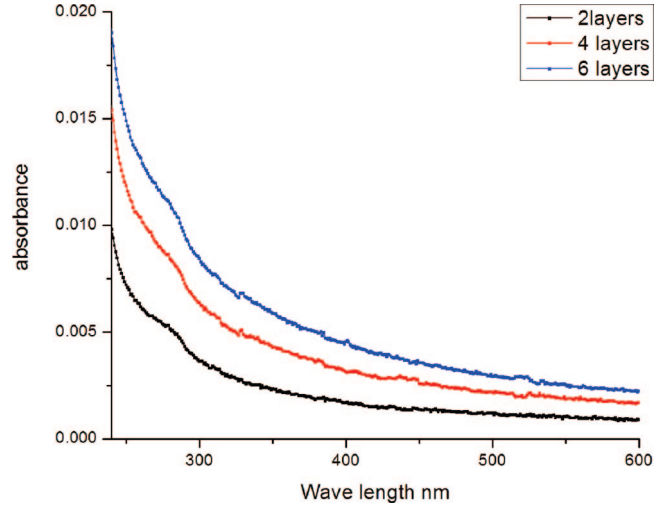

Figure 7. UV–visible absorption spectra of layer-by-layer assembly of glutamic acid–aspartic acid peptide loop flagella on an amine-functionalized quartz plate.

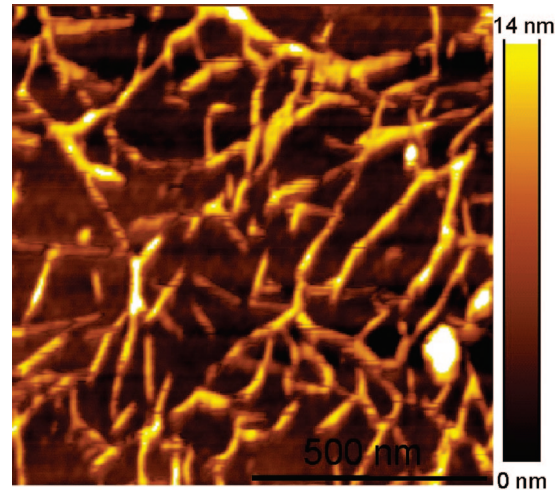

Figure 8. Topographic AFM image of calcium carbonate mineralized on glutamic acid–aspartic acid peptide loop flagella that were previously assembled on an amine-functionalized quartz plate.

layer of Glu–Asp loop peptide flagella was assembled on quartz by brief exposure to the flagella solution. In the biomineralization process, Ca^{2+} ions are thought to bind to the carboxylate side chains of the aspartic acid and glutamic acid residues. This process may then assist in initiating nucleation of $CaCO_3$ mineralization by generation of a region of local supersaturation, with diffusion of CO_2 (HCO_3^-) to the vicinity of the flagella surface. AFM images of $CaCO_3$ mineralized on flagella are shown in Figure 8. We have previously demonstrated the biomineralization of hydroxyapatite on glutamic acid–aspartic acid peptide loop flagella and the formation of hydroxyapatite nanocrystals by TEM.[19]

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