Characterization of a nanoscale S-layer protein based template for biomolecular patterning

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Abstract—**Well organized template for biomolecular conjugation is the foundation for biosensing. Most of the current devices are fabricated using lithographic patterning processes and self-assembly monolayer (SAM) methods. However, the research toward developing a sub-10 nm patterned, self-regenerated template on various types of substrates is limited, mainly due to the limited functional groups of the building material. Bacterial surface layer proteins (S-layer proteins) can self-assemble into ordered lattice with regular pore sizes of 2-8 nm on different material supports and interfaces. The ordered structure can regenerate after extreme variations of solvent conditions. In this work, we developed a nanoscale biomolecular template based on S-layer proteins on gold surface for fabrication of sensing layer in biosensors. S-layer proteins were isolated from *Bacillus cereus*, *Lysinibacillus sphaericus* and *Geobacillus stearothermophilus*. Protein concentrations were measured by Bradford assay. The protein purities were verified by SDS-PAGE, showing molecular weights ranging from 97 – 135 kDa. The hydrophilicity of the substrate surface was measured after surface treatments of protein recrystallization. Atomic force microscopic (AFM) measurement was performed on substrate surface, indicating a successful immobilization of a monolayer of S-layer protein with 8-9 nm height on gold surface. The template can be applied on various material supports and acts as a self-regenerated sensing layer of biosensors in the future.**

I. Introduction

Matching in size scale between biomolecules and devices is an important consideration in fabrication of biosensors. Many molecules of biological significance are of a length scale of nanometers. Researchers have great interest in developing well-organized templates in nanometer range for biomolecular conjugation. Plenty of researches have been done on developing patterned templates by nanolithographic methods, such as e-beam lithography [1], dip-pen nanolithography [2] and nanocontact printing [3]. However, the fabrication cost and equipment price are high. There is also limitation in fabricating structures with sizes below 50 nm. Current researches introduce different approaches in fabricating template of sub-10 nm resolution. Some researches focus on surface functionalization with chemical groups which enable easy conjugation for biomolecules on templates. Self-assembly monolayers (SAMs) are commonly applied to form ordered organic films for this purpose [4], including alkanethiols [5], aromatic thiols [6] and carbon nanotubes [7]. However, SAMs immobilizes on particular substrates, depending on their functional head groups. For example, alkanethiols contain head sulfur atom group (i.e., HS), which mainly enhance immobilization on gold, silicon oxide and silver surface. Further chemicals or biological treatments are required for immobilizing on different substrates. The ordered structure cannot regenerate after chemical or thermal distortion. The research toward developing a self-regenerated, nanoscale template on various types of substrates is limited, mainly due to the limited functional groups of the building material. In this study, we used bacterial surface layer proteins (S-layer proteins) as building blocks to develop the template.

S-layer proteins are the outermost envelope and most common cell surface structures in prokaryotic organisms. S-layer proteins are commonly found in bacteria and archaea [8]. S-layer proteins are generally 5 to 25 nm thick [9]. They are composed of a single sort of protein or glycoprotein with molecular weight varying between 40 and 200 kDa. S-layer proteins can self-assemble into oblique (p1, p2), square (p4), or hexagonal (p3, p6) symmetry. Depending on the lattice type, morphological units forming the lattice consist of one, two, three, four or six identical (glyco) protein subunits. The identical subunits exhibit pores identical in size and morphology, with center-to-center spacing of approximately 2-8 nm. S-layer proteins are associated with cell envelope by hydrogen bonds, ionic bonds (divalent cations or direct interactions of polar groups) and hydrophobic interactions [9]. Isolation of S-layer proteins from Gram-positive bacteria into monomers can be achieved by treating the cells with hydrogen-bond breaking agents (e.g., guanidinium hydrochloride (GHCl)). There are a number of applications of S-layer protein lattice in researches, for example functioning as isoporous ultrafiltration membranes [10], vaccine development [11] and supports for lipid membranes [12] etc.

S-later proteins are favorable to act as building blocks of a self-regenerated, nanoscale template on various types of substrates. The isolated S-layer proteins can recrystallize at surfaces of solid supports (e.g., silicon, glass, polymer) and interfaces (e.g., air-liquid interface, liposomes, lipid films) [13], making it as a suitable material for well-organized template on different substrates. Recrystallized lattice surface also contains a high density of carboxyl groups at identical positions and orientations down sub-10 nm resolution. This promote easy and ordered conjugation to various biomolecules [14]. Another important aspect of building template surface with S-layer protein is their stability under extreme solvent conditions and temperature. Researches show that S-layer protein lattice is distorted when exposing to 80%

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ethanol, but can be recovered by treating buffer solution [15]. The lattice can also withstand a high temperature up to 60℃ and is stable in various pH values (i.e., 5, 7 and 9).

In the present study, we developed a nanoscale biomolecular template based on S-layer proteins on gold surface. S-layer proteins were isolated from *Bacillus cereus*, *Lysinibacillus sphaericus* and *Geobacillus stearothermophilus* using GHCl. The isolated S-layer proteins were characterized by Bradford assay and SDS-PAGE. The proteins were recrystallized on gold surface with cross linkers (i.e., 11-mercaptoundecanoic acid (11-MUA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)). Atomic force microscopic (AFM) measurement and water contact angle measurement were used to study the surface property. This research contributes to biosensor fabrication by developing a well-organized template down to sub-10 nm resolution. The template can be applied on various material supports and act as a self-regenerated sensing layer of biosensors in the future.

II. METHOD

A. Organism, isolation and characterization of S-layer proteins

B. cereus (ATCC 14579) was cultivated for 24 h on tryptic soy broth agar plate at 37°C. *L. sphaericus* (ATCC 4525) and *G. stearothermophilus* (ATCC 12980) were cultivated for 24 h on SVIII agar plate at 30°C and 55°C, respectively. Wet pellets of cell wall fragments were obtained by centrifugation at 10,000 g for 15 min. S-layer proteins were extracted from clean cell wall fragments with 10-fold volume GHCl (5 M in 50 mM Tris-HCl buffer, pH 7.2) for 2 h at 20°C. The peptidoglycan-containing cell wall layers were separated from the extracted S-layer protein by centrifugation at 40,000 g for 20 min at 4°C. Subsequently, the S-layer protein containing GHCl extracts were dialyzed against DI water for 24 h at 4°C by dialysis tubing, with molecular weight cut off of 11065 Da (D9527, Sigma-Aldrich, MO, USA). Self-assembly products formed were sedimented for 20 min at 40,000 g at 4°C. The clear supernatant was stored at 4°C for further recrystallization experiments. The molecular weights and the purities of S-layer proteins were determined by SDS-PAGE (Bio-Rad, CA, USA). Protein concentration was determined by the Bradford assay.

B. Recrystallization

10 nm and 70 nm layer of chromium gold were deposited onto 10 mm x 10 mm silicon respectively. Before the recrystallization of S-layer proteins, the substrate was cleaned with pure ethanol and Milli-Q-water. The substrate was immersed in 1 mM ethanol solution of 11-MUA for 24 h at room temperature. After removal out of solution, the sample was carefully rinsed with ethanol and Milli-Q-water and dried with nitrogen gas. The substrate was immersed in 15 mg mL^{-1} EDC in water for 1 h at room temperature. After removal out of solution, the sample was carefully rinsed with Milli-Q-water and dried with nitrogen gas. S-layer protein was diluted to 1 mg mL^{-1} using the appropriate amount of recrystallizing buffer (5 mM Tris, 10 mM $CaCl_2$ buffer, pH 9) right before recrystallization. The substrate with cross linkers was placed in the protein solution and incubated overnight at 23°C. Finally, the substrate was rinsed with recrystallizing buffer and Milli-Q-water to remove any excess of protein, and was dried under nitrogen gas.

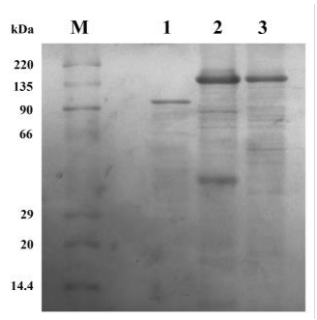

Figure 1. SDS-PAGE pattern of molecular mass standard (lane M) and S-layer protein samples isolated from *B. cereus* (lane 1), *L. sphaericus* (lane 2), and *G. stearothermophilus* (lane 3).

C. AFM measurement

Images were recorded in tapping mode at 1 Hz at room temperature using a Nanoscope V controller multimode AFM (Bruker AXS, Santa Barbara, USA). A silicon AFM probe (Tap300E, BudgetSensors, Bulgaria) with spring constant of 40 N m^{-1} and resonant frequency of 300 kHz was used. Image processing was performed with the Nanoscope v5 (Bruker AXS, Santa Barbara, USA) program for background removal (flattening) and free software Gwyddion developed by Nečas [16].

D. Contact angle measurement

10 μL of DI water droplet was pipetted on substrate surface. All measurements were performed inside a laminar flow hood to reduce air motion from the surrounding. Contact angle were captured using a digital microscope (AM4131 Dino-Lite Pro, Dino-Lite Digital Microscope, Taiwan). Angles were analyzed with a free software Drop Shape Analysis developed by Stalder [17]. Angles obtained were the mean values of both sides of each droplet. The process was repeated three times to ensure the accuracy.

III. RESULT AND DISCUSSION

A. Purification of S-layer protein

Extracts of *B. cereus*, *L. sphaericus* and *G. stearothermophilus* were analyzed by SDS-PAGE, showing dominant bands of 97, 140 and 135 kDa respectively (Figure 1). The molecular weights were close to the published data of that for *B. cereus* (i.e., 97 kDa [18]), *L. sphaericus* (i.e., 120 kDa [19]) and *G. stearothermophilus* (i.e., 122 kDa [20]). The result indicated the presence of S-layer proteins in the crude extracts. A few faint bands were visible in lane 1, 2 and 3, showing the presence of impurities in protein extracts. The proteins were further purified by use of ion-exchange columns (Macro-Prep High Q and S cartridge, Bio-Rad, CA, US) following the protocol provided by manufacturer. Protein

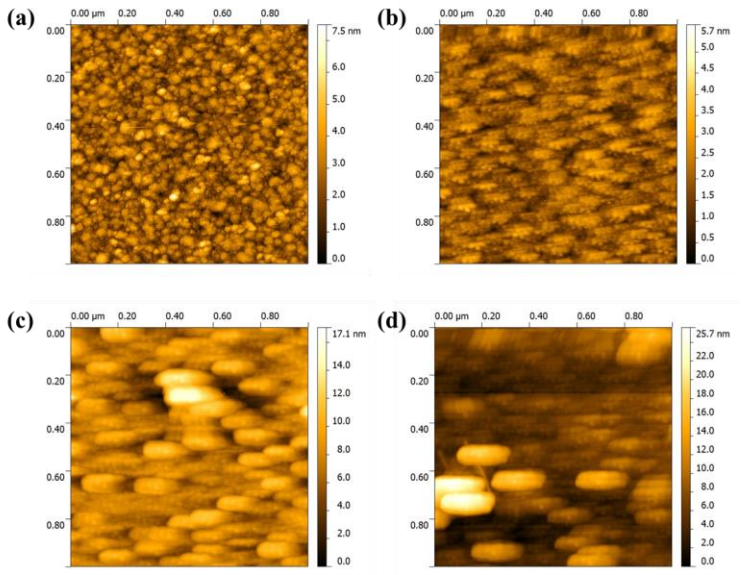

Figure 2. AFM height image of (a) Au, (b) Au \ 11-MUA \ EDC, (c) Au \ 11-MUA \ EDC \ S-layer protein extract of *B. cereus* and (d) Au \ 11-MUA \ EDC \ S-layer protein extract of *G. stearothermophilus*. All the images are of size 1 µm x 1 µm.

concentration was determined by Bradford assay. Extracts of *B. cereus, L. sphaericus* and *G. stearothermophilus* contained 2664, 932, 1525 µg mL^{-1} S-layer protein respectively.

B. Contact angle measurement

Substrate surface was characterized by contact angle measurements. The result showed that gold surface had an angle of 71˚ ± 6˚. The value was close to the published value [21]. The contact angle decreased to 64˚ ± 7˚ after treated with 11-MUA. 11-MUA acted as a cross linker for S-layer protein to gold surface. It was a methylterminated long alkane thiols (n>6) for forming a well-ordered, closely packed monolayers on gold [22]. Its head sulfur atom group (i.e., HS) bound to gold while highly polar terminal carboxylic acid group (i.e., COOH) bound to S-layer protein, leading to a decrease in hydrophobicity. However, the experimental contact angle value did not match with the published value of 16˚ ± 2˚ [21]. The presence of 11-MUA on gold surface was further characterized by AFM in this study.

Carboxylic acid groups of 11-MUA were activated by EDC. Activated carboxylic acid groups reacted with free amino groups of S-layer protein, leading to stable peptide bonds between 11-MUA and S-layer protein. EDC made the surface become more hydrophobic, leading to an increase in contact angle to 66˚ ± 4˚. The value matched with the published value [21]. S-layer protein adsorption was influenced by the presence of ions [23]. Divalent ions (e.g., calcium ions) were essential for protein recrystallization [24]. Ca^{2+} cations served as a bivalent lateral linking agent. S-layer protein monomer preferentially formed 2D-crystals. These Ca^{2+} cations functioned as salt bridges between 11-MUA and S-layer protein. S-layer protein extracts of *L. sphaericus* was recrystallized on substrate surface. The contact angle became 68˚ ± 8˚, which was close to the published value [25].

C. AFM measurement

Different layers were immobilized on substrate surface during S-layer protein recrystallization (i.e., Au \ 11-MUA \ EDC \ S-layer protein). Each layer was characterized by AFM measurements (Figure 2). Cross linkers (i.e., 11-MUA and EDC) had an average thickness of 2 nm and roughness of 0.5 nm (Figure 2b). The experimental result matched with the published data [26], which EDC of negligible length and 11-MUA of 1.9 nm.

Figure 2c and d showed height images of S-layer proteins immobilized on the substrate. S-layer protein extracts of *B. cereus* and *G. stearothermophilus* had an average thickness of 7.7 nm and 7.2 nm roughness of 2 nm and 4 nm respectively. The thickness values were close to the published data of approximately 8-9 nm for monolayer [27]. However, S-layer protein lattice (i.e., p2 lattice for S-layer protein extracts of *Bacillus cereus* and *G. stearothermophilus*) was not observed in this study. Chung described AFM height value changes during four different stages of S-layer protein recrystallization: (i) adsorption of extended S-layer protein monomers onto the substrate, (ii) condensation into amorphous or liquid-like clusters, (iii) rearrangement and folding into crystalline arrays of tetramers, and (iv) growth by new tetramer formation at edge sites of the crystalline clusters [28]. In first stage, after introduction of protein solution, S-layer protein monomers with an average height of about 2 nm began to adsorb onto the substrate. In the second stage, amorphous clusters of heights (10–12 nm) formed on the substrate. The clusters did not reveal any internal structure nor change their dimensions significantly. Internal structure and ordering gradually began to emerge in each of the amorphous clusters. In third stage, nearly all of the amorphous clusters were transformed into crystalline clusters. Individual lattice units also appeared with height of 8-9 nm. In the last stage, each crystalline cluster grew by creation of new tetramers at its edges until nearly all of the adsorbed proteins were

consumed. In this study, S-layer protein extracts of *B. cereus* and *G. stearothermophilus* with average thickness of 7.7 nm and 7.2 nm respectively showed that third stage protein recrystallization was achieved. Further studies will be done in controlling the parameters on recrystallization to show the lattice structure.

IV. Conclusion

S-layer proteins can act as building blocks to develop a well-organized and self-regenerated template on various material supports. In this study, we developed a nanoscale biomolecular platform based on S-layer proteins on gold surface. We used Bradford assay and SDS-PAGE to characterize the isolated S-layer proteins. The substrate surface property was monitored by tapping mode AFM and water contact angle measurements. In future study, we will couple the platform with microfluidics to perform S-layer protein patterning. S-layer protein can be immobilized at particular positions on substrate by simply passing through microchannels on top. Gold electrodes containing working, counter and reference electrodes will be fabricated underneath the microchannel to permit an integration of electrical measurements on various biomolecules on the template. The mechanism of protein recrystallization on gold can also be studied by electrochemical measurements. This research contributes to biosensor fabrication by developing a well-organized template down to sub-10 nm resolution. The template can be applied on various material supports and acts as a self-regenerated sensing layer of biosensors in the future.

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