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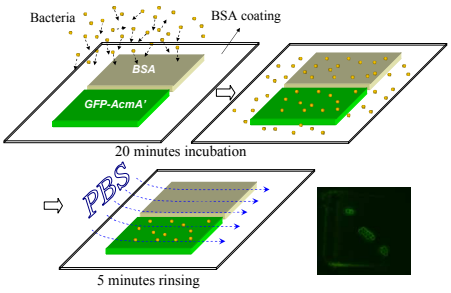

The serviceable bioprobes fabricated by laser-induced cross-linking technology for simple and direct screen of Gram-positive bacteria on a biochip.

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ARTICLE TYPE

Laser-induced cross-linking GFP-AcmA' bioprobe for screening Gram-positive bacteria on a biochip

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A serviceable bioprobe is one of the important components for the developments of microfluidic and lab-on-a-chip systems. In this paper, we report a novel bioprobe fabricated by laser-induced cross-linking technology for simple and direct screen of Gram-positive bacteria on a biochip. The AcmA' protein is known to bind specifically to peptidoglycan (PG) that forms the thick outside layer of Gram-positive bacteria. Moreover, the AcmA' protein has a much broad spectrum of bacterium types than do antibodies that are more specific to only one bacterium type because the AcmA' protein is a generic characteristic of Gram-positive bacteria. Green fluorescent protein (GFP) is generally used as a mecular marker. In this study, the GFP was fused with the AcmA' protein to act as an indicator for tracing the AcmA' binding activity on PG by green fluorescence. The GFP-AcmA' protein was three-dimensionally structured by laser induced cross-linking photochemistry technology to fabricate a bioprobe for capturing Gram-positive bacteria. The positive and negative tests on Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus* and *Streptococcus agalactiae* were demonstrated respectively. Screening is readily performed using optical microscopy observation. The experiments show that only Gram-positive bacteria were bound on GFP-AcmA' probes after minutes of incubation and phosphate buffered saline (PBS) rinsing. No binding was observed in the Gram-negative bacteria or in reference probes composed of neutral bovine serum albumin (BSA). The repeated experiments indicate that our bioprobes are reusable. Finally, a 3D wedge-shaped GFP-AcmA' probe was demonstrated in a microfluidic channel. This study provides a novel platform for convenient Gram-positive bacteria screen that could potentially be used in lab-on-a-chip applications.

Introduction

Gram-positive bacteria, especially *Staphylococcus* and *Streptococcus*, are serious pathogens to threaten human health. For instance, the incidence of nosocomial by *S. aureus* was reported by 14 hospitals in Europe, North America, and South America during a 10-year period (1998–2007) [1]. *S. agalactiae*, known as Group B *Streptococcus* (GBS), is a prevalent cause of illness in newborn infants and pregnant women [2,3]. In newborns, GBS infection is the major cause of sepsis, meningitis, bone and soft tissue infections, and pneumonia. In pregnant women, colonization with GBS (20%–30% of women) in the genitourinary tract is a risk factor for premature delivery, and ascending GBS spread can cause chorioamnionitis and maternal sepsis [3]. *S. agalactiae* is present in approximately one-third of women of childbearing age, and 1.8 cases per 1000 live births are affected by GBS infection [4].

The gold standard method of pathogenic bacteria diagnosis is the Gram stain with bacteria that are cultured during 48 h to 72 h [5]. However, the false negative results obtained are within the range of 7.2% to 21.2% [6,7]. New molecular diagnostic methods, including the conventional 16S ribosomal RNA sequencing and polymerase chain reaction (PCR), have high sensitivity and specificity [8]. However, they require high-tech equipments, such as a DNA sequencer and thermal cycler [9]. Recent developments in microfluidic and lab-on-a-chip systems [10-17] are targeting fast, sensitive, compact, and low-cost biosensors for point-of-care tests. In this case, a serviceable bioprobe for simple and direct screening Gram-positive bacteria is still unsatisfactory.

The AcmA' protein is known to bind PG specifically, which is a generic characteristic of Gram-positive bacteria [18-20]. Thus, the AcmA' protein has a much broad spectrum range of bacterium types than do antibodies that are more specific to only one bacterium type. Previously, we reported a modified protein, GFP-AcmA', obtained from GFP fused with an active AcmA'-based protein. The GFP acts as an indicator to trace the AcmA' binding activity on PG by green fluorescence [21].

Several studies have applied protein patterning for bioapplications, such as controlled cellular migration, cellular growth, and enzymatic assays [22]. The proteins are immobilized on surfaces by using various technologies [23]. Recently, multiphoton-absorption-based photochemical cross-linking has

been reported to fabricate three-dimensional (3D) structures with a variety of proteins, such as BSA, collagen, fibrinogen, and enzymes [24-31]. Cross-liking occurs only at the laser focal point, and complex structural shapes can be obtained by scanning the laser spot [32]. This technology has particular advantages for biological applications because of the 3D capability [33], harmless visible to near-infrared laser wavelength, and processing conditions that are mild without the need for harsh reagents such as organic solvents [24]. Such a multiphoton-based fabrication of 3D protein microstructures have recently been reported for biological applications, such as stem cell migration study [34], pH-sensitive circular enclosure and bendable rods [35], microchamber for bacteria steering [36], and scaffolds for tissue engineering [37]. However, to fabricate a bioprobe by cross-linking protein for employing on capturing bacteria is still rare thus far.

This paper reports a novel application of protein cross-linking technology for applying to biochips. We demonstrated the fabrication of a pad-shaped bioprobe based on GFP-AcmA' protein to capture Gram-positive bacteria. The bacteria screen is readily performed using simple optical microscopy observation. Gram-positive (*S. aureus* and *S. agalactiae*) and Gram-negative (*Escherichia coli*) bacteria were effectively distinguished using the GFP-AcmA'-based bioprobe. Eventually, an example of an inclined GFP-AcmA' pad was fabricated in a microfluidic channel for testing the possibility of the bacteria capture in a laminar flow.

GFP-AcmA'

1. Plasmid construction

The process used for generating the recombinant protein of GFP-AcmA', plasmid-expressing GFP fused with the AcmA' protein, which serves as an anchoring protein onto Gram-positive bacteria was described by Lin et al. [22] and Steen et al. [38]. The map and structure of the GFP-AcmA'-pET24a plasmid expressing the GFP-AcmA' recombinant protein is presented in Fig. 1. Plasmid AcmA'-pET24a contains fragments of Lys motif domain (AcmA'; 261 bp) with bacteria cell wall-anchored ability upstream of the hexahistidne tag (H6). The 813-bp DNA fragment of the GFP for displaying on *S. aureus* was inserted upstream of AcmA'. The expression of the recombinant protein is controlled by the T7 RNA polymerase promoter (T7 p). Restriction enzymes used for cloning and other cis-elements on a vector backbone are indicated, such as the lac operator (lacO), ribosome binding site (rbs), and stop codon (stop) [22].

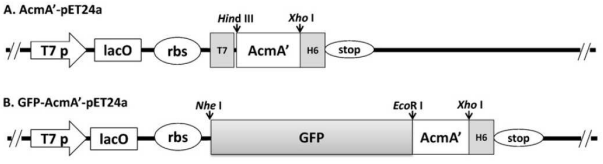


Fig. 1 Schematic illustration of the constructs expressing AcmA'-fusion proteins

2. Recombinant protein expression, purification, and cell wall binding assay

The GFP-AcmA'-pET24a plasmid was transformed into *E. coli* strain BL21 (DE3). The recombinant protein expressed condition and purified steps were described by Lin et al. [22]. The recombinant protein concentration was determined using the Bradford method (Bio-Rad). The binding activity of recombinant GFP-AcmA' to cell wall of Gram-positive bacteria was analyzed in previous work [21].

Fabrication of GFP-AcmA' bioprobe

Eosin-Y (40 μL, 1 wt% in water) was used as a photoinitiator to add to 160 μL of GFP-AcmA' (1.6 mg/mL). A small quantity (approximately 30 μL) of the sample was dropped on a microscope glass cover slide that was precoated with BSA on the surface. The protein probes were fabricated using a commercial laser-induced 3D microfabrication machine (Teem Photonics Inc.) equipped with a passively Q-switched Nd-YAG microchip laser featuring a wavelength of 532 nm. The laser beam is expanded by a telescope, coupled to an inverted microscope (Olympus IX51), and focused using a microscope objective lens (100×, NA = 1.3). Laser-induced cross-liking of proteins occurs at the focal point in the sample at a laser power of approximately 0.1 mW and an exposure time of 1 ms. The schematic of the experimental setup and the dimension of the pad-shaped probe are shown in Fig. 2.

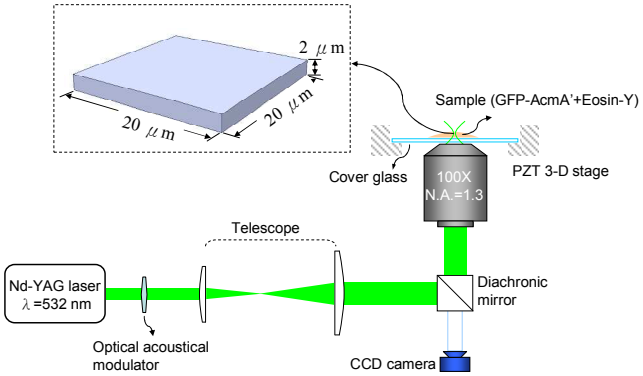


Fig. 2 Schematic of the laser-induced micro-fabrication setup and the dimension of the pad-shaped bioprobe

Demonstration of the GFP-AcmA' bioprobe

1. Biobinding capability

First, the GFP-AcmA' and BSA bioprobes were fabricated on the BSA-coated cover slide by applying the laser-induced cross-linking fabrication technology. The *S. aureus* was chosen for verifying the biological function of the laser-induced GFP-AcmA' probes. A very small drop (approximately 20 μL) of the *S. aureus* with PBS (~1.5×10^8 CFUs/mL) was dripped on the pad-shaped probe surface. After 20 min of incubation for waiting the bacteria sank down on the surface of the pad-shaped probe and 5 min of PBS rinsing, the probe was observed using an optical microscope. Schematic figures of the demonstration of assessing *S. aureus* is shown in Fig. 3. The demonstration result shows that the *S. aureus* were bound only on the GFP-AcmA' probe (Fig. 4 (a)) but not the BSA probe (Fig. 4 (b)). In other words, the structured GFP-AcmA' remained its biological function (binding activity with Gram-positive bacteria) after the laser cross-linking. Finally, Rabbit anti-*S. aureus* polyclonal Ab-conjugate fluorescein (OmnitopeTM, Viro-Stat, Inc.) was diluted 10 times with PBS for a fluorescent test to verify the *S. aureus*. Figure 4(c) shows another capture experiment with the GFP-AcmA' probe and fluorescent test result.

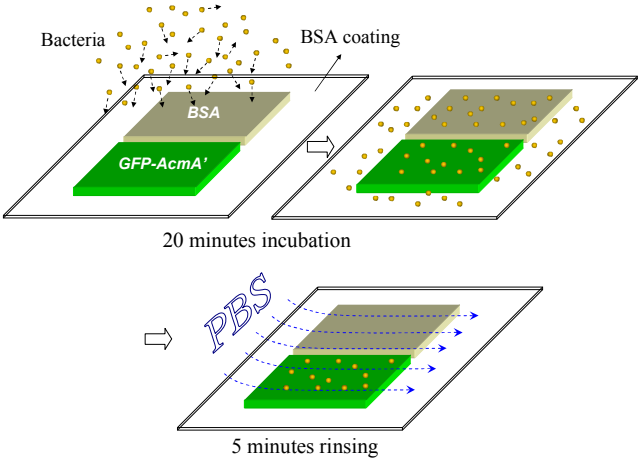
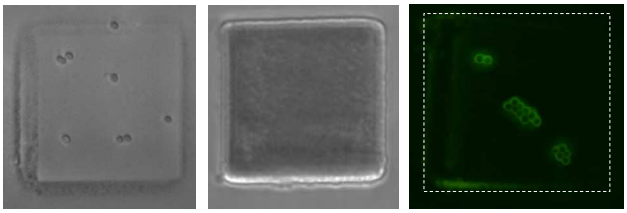


Fig. 3 Schematic figures of the demonstration of assessing *S. aureus*



(a) GFP-AcmA' probe (b) BSA probe (c) GFP-AcmA' probe (fluorescent test)

Fig. 4 *S. aureus* attached on the GFP-AcmA' probe (a) but not the BSA probe (b); (c) Fluorescent test on the GFP-AcmA' probe

2. Screen demonstration of Gram-positive bacteria

In this experiment, the *S. aureus* ATCC 25923, *S. agalactiae* M1 (Gram-positive), and *E. coli* BL21 (Gram-negative) were used as the model bacteria. The *S. agalactiae* M1 was isolated from bovine mastitis, and the *S. aureus* ATCC 25923 and *E. coli* BL21 were respectively purchased from Bioresource Collection and Research Center, Hsinchu, Taiwan and Invitrogen Inc. The *S. aureus* 25923, *S. agalactiae* M1, and *E. coli* BL21 were streaked onto brain heart infusion agar, tryptic soy agar with 5% defibrinated sheep blood, and Luria–Bertani (LB) agar plates, respectively, and then incubated at 37 °C for 16 h. All bacterial single colonies were selected and inoculated in a 5-mL medium at 37 °C for 16 h. The bacterial cultures were then aliquot to ~1.5 × 10^8 CFUs/mL in PBS for the bacteria binding experiment as the same process illustrated previously.

Figure 5 shows a comparison of the binding phenomena of Gram-negative bacteria, *E. coli*, and Gram-positive bacteria, *S. aureus* and *S. agalactiae*. After 20 min of incubation and 5 min of PBS rinsing, only *S. aureus* and *S. agalactiae* were bound on the probes. The result verifys that the GFP-AcmA' probe has specificity to bind the Gram-positive bacteria, but not Gram-negtive bacteria. Moreover, the GFP-AcmA' bioprobe also provides a convenient morphology to type the pathogenic bacteria of *S. aureus* and *S. agalactiae*.

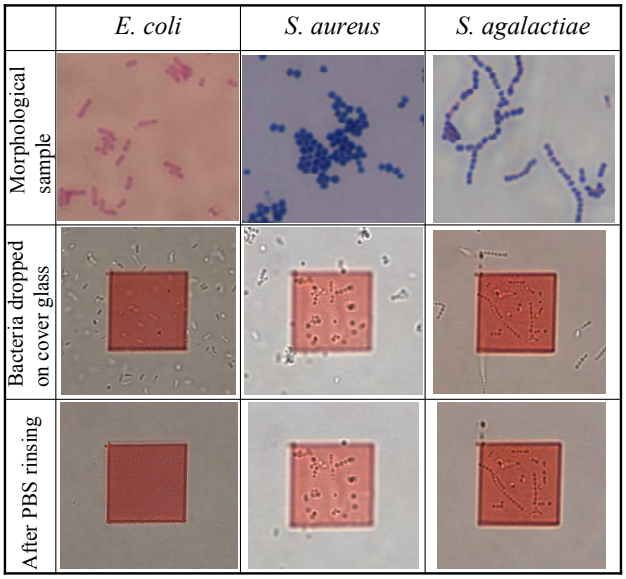


Fig. 5 Comparison of binding activity of Gram-negative bacteria, *E. coli*, and Gram-positive bacteria, *S. aureus* and *S. agalactiae*

3. Demonstration in a microchannel

To evaluate the microfluidic biochips integrating 3D functional microstructures, we fabricated the GFP-AcmA' probe in a microchannel. The microchannel was fabricated using polydimethylsiloxane (PDMS) anchored on a glass microscope slide by using the soft lithography and replica molding techniques [32]. A schematic design figure of the microchannel chip and the dimension of the wedge-shaped probe are shown in Fig. 6. The width and the depth of the channel are 50 μm and 60 μm, respectively. The apertures at both ends of the channel enable the entry and exit of fluid. The PDMS and glass surfaces were treated in advance with a plasma cleaner and then immediately brought into contact to create a permanent bond. First, a small drop of the GFP-AcmA' containing Eosin-Y was introduced into the microchannel by using a syringe. The structure was locally fabricated in the microfluidic channel by applying the laser-induced fabrication technique. PBS was then flowed into the microchannel by using a syringe to dissolve the nonpolymerized portion and to reveal the structure in the microchannel. Figure 7 illustrates the GFP-AcmA' probe fabricated in the microchannel by laser-induced cross-linking technology.

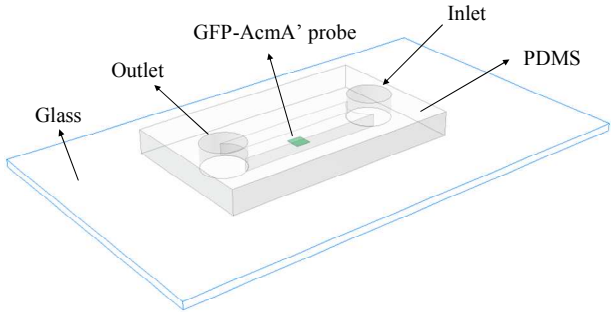


Fig. 6 3D schematic illustration showing microchannel design

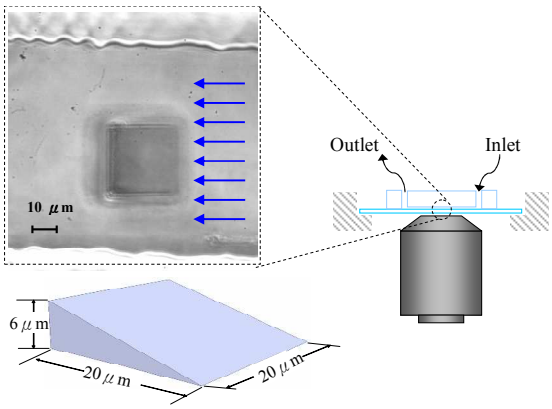


Fig. 7 Illustration of the laser-induced GFP-AcmA' probe in a microchannel and the dimension of the wedge-shaped probe

The flowing behavior of bacteria-like beads in the microchannel was simulated using COMSOL Multiphysics software with a computational fluid dynamics module. In the simulation model, the density and diameter of the beads respectively are 1.2 g/cm3 and 1 μm, which are approximately according to the *S. aureus*. The medium was set as water, and the flow rate of 1 μL/min was same as that used during the later experiment. The simulation result (Fig. 8(left)) indicated that the beads flowed near the channel bottom and a wedge-shaped bioprobe could facilitate the bacteria capture.

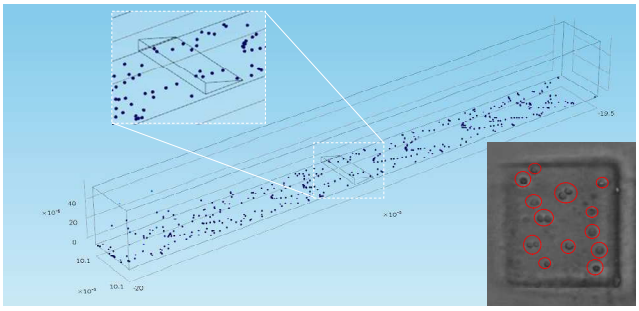


Fig. 8 Simulation result of the beads flowing in the microchannel and the experimental result of capturing cocci.

The *S. aureus* was prepared for the demonstration as previous illustration. It was dropped in the microchannel by using a syringe. The flowing velocity of the beads was determined to be approximately 1 μm/s based on the optical microscope observations with a 100× objective. After the bacteria flowed through the wedged-shaped probe, the channel was rinsed with PBS for 5 min by using a syringe. The images of the capture result (Fig. 8(right)) were directly observed using the same microscope. Figure 9 shows the capture numbers obtained in 10 repeated measurements. We must point out that the measurements were performed using the same bioprobe in the microchannel. That means the bioprobe is resuable. The average capture result by the wedged-shaped probe is 16 cocci which is two times better than the flat pad probe. The small scatter in data indicated that the results are highly reproducible. No bacterium binding on the wedge-shaped BSA probe was observed using the same experimental process. Based on this study, the high-throughput screen of Gram-positive bacteria could be addressed by fabricating large scaled 3D complex probes.

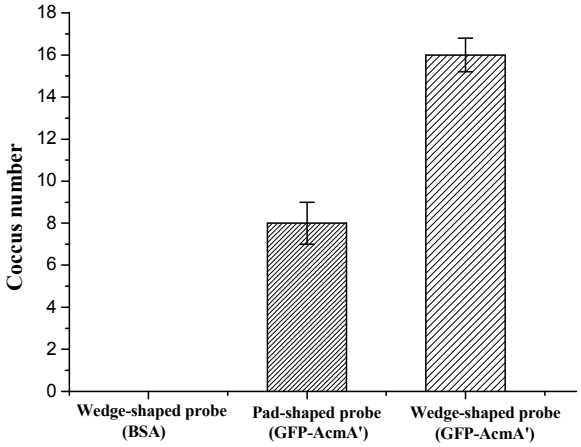


Fig. 9 Captured cocci numbers of the GFP-AcmA' probes (flat and wedge-shaped) and BSA probe (reference) on biochip

Conclusions

This paper reports the Gram-positive bacteria screen by using laser-induced cross-linking GFP-AcmA' bioprobe. The bioprobes are reusable. The biological capture was demonstrated using negative and positive tests with Gram-negative *E. coli* and Gram-positive *S. aureus* and *S. agalactiae*, respectively. Screen was readily performed by optical microscopy observation. The novel bioprobe can rapidly screen the Gram-positive bacteria, and also type the bacteria at the same moment. The demonstration in a microfluidic channel integrating 3D functional microstructures was achieved using a 3D wedge-shaped GFP-AcmA' probe. Thanks to the particular advantages of the 3D capability, the laser-induced cross-linking technology provides a flexible fabrication of bioprobe for the potential developments in microfluidic and lab-on-a-chip systems.

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Notes and references

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