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Replication of surface microstructure made of bacterial colony towards functional surface fabrication

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Abstract
Surface microstructures are important for realizing valuable optical, fluidic, thermal, and tribological functionalities. Among existing fabrication methods of surface microstructures, self-organization is a key principle for low-cost and large-area fabrication. However, conventional techniques have limitations in the controllability of fabricated structures and applicable range. For example, in colloidal lithography, which is a representative technique for the fabrication of micrometer-scale structures based on the spontaneous arrangement of microparticles, the fabricated structures are limited to two-dimensional close-packed structures, and it is difficult to apply to non-planar surfaces.
To overcome the limitations of the conventional techniques, we propose a new method based on the self-organization in bacterial colonies. The proposed method has the potential for flexible control of fabricated structures because of the responsive behavior of bacteria to environmental changes. In addition, the autonomous covering of bacterial colonies on the object surface indicates the applicability to non-planar surfaces. On the other hand, because the bacterial cell is not durable and stable, some additional process is required for the practical realization of functionalities. From this perspective, we replicated surface microstructures of bacterial colonies by directly casting polydimethylsiloxane (PDMS), which had never been applied to bacterial colonies as far as the author knows. Negative and positive replicas were produced using the standard procedure of two-step molding. As a result of the microscopic observation, the correspondence of microstructures down to the sub-micron scale between the template colony and the PDMS replicas was confirmed, which means that the surface microstructure of the bacterial colony was successfully transferred onto the PDMS by a standard replication protocol. Also, the three-dimensional measurement by atomic force microscopy (AFM) revealed that a dot pattern, with each diameter of about 800 nm and height of 50 to 100 nm, derived from the individual bacterial cell exists on the PDMS replica.

Surface, microstructure, processing, nano technology

1. Introduction

Since the confirmation of the superhydrophobicity of the surface microstructure of lotus leaves in the 1990s [1], functional surfaces, where surface microstructures provide specific functionalities, have attracted significant attention. Functional surfaces inspired by natural surface microstructures, such as those found in insects and plants, have been studied for their ability to achieve valuable functionalities beyond the intrinsic chemical properties of the materials [2–5]. These functionalities, including wettability control and optical effects, are typically achieved using surface microstructures with lateral feature lengths of approximately 500 to 1000 nm (Fig. 1). However, implementing these functional surfaces has been limited to a few industrial products, such as airplanes, swimsuits, and food packaging, despite the potential for many industrial products to enhance their performance through surface functionalization. This limitation arises from constraints in existing fabrication methods.

The fabrication methods for functional surfaces can be broadly classified into two groups: top-down methods and bottom-up methods [6]. Top-down methods involve the addition, removal, or deformation of material from macroscopic objects, while bottom-up methods rely on the assembly and arrangement of microscopic components. In general, top-down methods offer better controllability of the fabricated structures but are associated with high manufacturing costs and process complexity. Among the existing techniques for fabricating surface microstructures, self-organization stands out as a key principle for low-cost and large-area fabrication, as it utilizes spontaneously emerging microstructures without the need for precise control over microcomponents. However, conventional bottom-up techniques have inherent limitations in the controllability of fabricated structures and their range of applicability.

For instance, colloidal lithography [7], a widely used bottom-up technique for fabricating micrometer-scale structures based on the spontaneous arrangement of microparticles, is constrained to producing two-dimensional close-packed structures. Furthermore, it is challenging to apply this technique to non-planar surfaces [8–10].

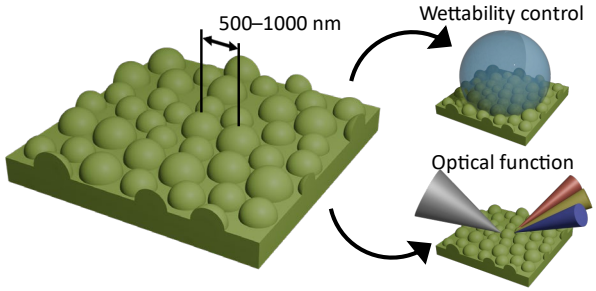

Figure 1. Schematic illustration of functional surface microstructure.

To address the limitations of conventional techniques, we have proposed a new method based on self-organization in bacterial colonies [11]. In this approach, bacteria serve as the microcomponents for forming self-organized functional microstructures. As bacterial colonies grow, they cover the object's surface, and surface microstructures spontaneously emerge within the colonies. This method offers the potential for flexible control of fabricated structures due to the responsive behavior of bacteria to environmental changes. Moreover, the autonomous growth of bacterial colonies on object surfaces suggests applicability to non-planar surfaces. However, since bacterial cells lack durability and stability, additional processes are required for the practical realization of functionalities.

In this context, we explored replicating the surface microstructures of bacterial colonies through direct molding. While several studies have demonstrated the successful replication of surface microstructures from biological tissues, such as plant leaves and gecko feet [12–14], the replication of bacterial colonies has not been reported to the best of our knowledge. Thus, the feasibility of replicating bacterial colonies via direct molding needs experimental verification.

The following sections outline our investigation. Section 2 describes the replication method for bacterial colonies, Section 3 presents the replication results, and Section 4 provides the conclusion.

2. Methods

Experiments were conducted to verify the feasibility of replicating the surface microstructures of bacterial colonies via direct molding. *Staphylococcus epidermidis*, a spherical bacteria with a diameter of approximately 800 nm, commonly found in the human skin microbiome was used in the experiment. Polydimethylsiloxane (PDMS), a widely used silicone elastomer in microfluidics and MEMS, was employed as the transfer agent. The accuracy of the replication and the resulting shape were evaluated through optical microscopy and atomic force microscopy (AFM). The replication process is summarized in Fig. 2.

2.1. Preparation of agar plates and bacterial cultures

LB agar plates containing 10 g/L of LB Broth (Sigma-Aldrich, L3011) and 10 g/L of agarose (Sigma-Aldrich, A4018) were prepared in ϕ50 mm Petri dish. To prepare the medium, 100 mg of LB Broth and 100 mg of agarose were dissolved in 10 mL of water, autoclaved for 20 minutes, poured into a Petri dish, and left at room temperature to allow gelation. Cultures of *S. epidermidis* (wild strain) at 10^7 colony forming units per microliter were inoculated onto the agar plate and incubated at 37 °C for 17 hours.

2.2. Replication by direct molding with PDMS

To inactivate the bacteria while preserving the morphology of the colonies, a vapor fixation method commonly used for fungal mycelium was employed. In this method, the sample is exposed not to the liquid but to the vapor of the fixative agent. A 2 % formaldehyde fixative agent (2 w/w% paraformaldehyde, 10 v/v% PBS, 50 mM NaOH) was used. The fixative agent was dropped to gauze spread on the lid of a Petri dish, and the dish containing the bacterial colony was inverted and placed over the lid. The assembly was incubated at 37 °C for 24 hours.

To create a negative replica, 15 g of PDMS (Dow, SILPOT 184) and 1.5 g of catalyst were mixed at 2000 rounds per minute for 120 seconds using a planetary centrifugal mixer (THINKY, ARE-250), defoamed for 30 seconds, and poured onto the bacterial colonies. The PDMS was cured by incubating at 37 °C for at least 18 hours. After curing, the PDMS was peeled off and washed

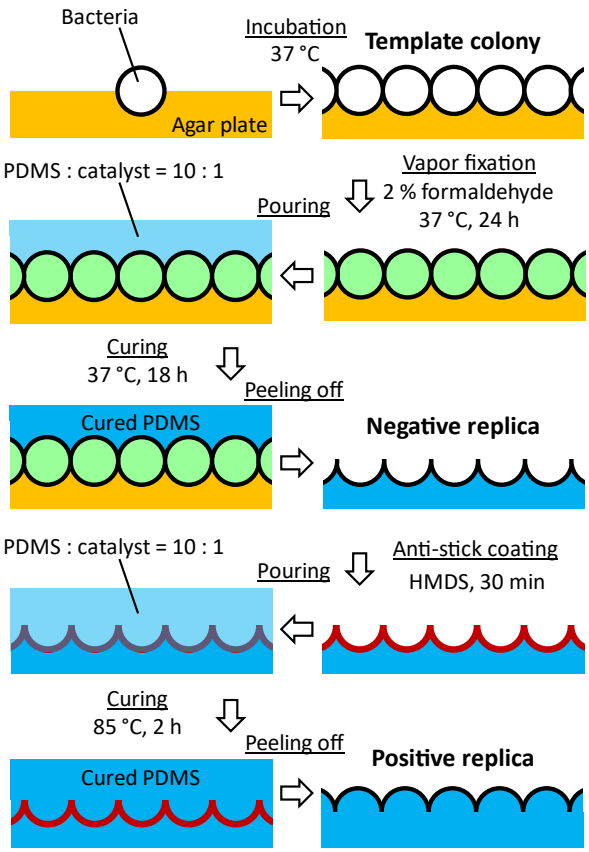

Figure 2. Replication process of surface microstructures of bacterial colony by direct molding with PDMS.

with running water and scotch tape to complete the negative replica.

A positive replica was also fabricated using the negative replica as a mold. An anti-stick coating was applied by placing the negative replica in the center of a ϕ90 mm Petri dish surrounded by 5–6 drops of hexamethyldisilazane (HMDS) and exposing it to HMDS vapor for 30 minutes. PDMS and catalyst were prepared as described above, poured onto the negative replica, and cured on an 85 °C hotplate for 2 hours. The cured PDMS was then peeled off after square cutting, resulting in a positive replica.

2.3. Evaluation of replica morphology

To assess the replication accuracy, corresponding regions of the bacterial colony and its replicas were observed and compared using an optical microscope (Keyence, VHX-8000), and obtained images were compared to evaluate structural fidelity. Additionally, the macroscopic profiles of the samples were measured using the same optical microscope. This involved horizontally scanning the samples and recording the z-coordinates of the objective lens at each position where the sample was in focus.

The surface morphology of a specific area of the replica was further analyzed using atomic force microscopy (AFM) (Bruker, Innova) operated in tapping mode. A high-aspect-ratio cantilever (Bruker, RTESPA-300) was used to ensure accurate measurement of the replica's microstructures.

3. Results

Figure 3 presents images and microscopic views of the *S. epidermidis* colony and its replicas. After 17 hours of incubation, a circular colony approximately 40 mm in diameter formed on the agar plate (Fig. 3(a, b)). As shown in Fig. 3(c), the bacterial cells collectively organized into a micropattern resembling typical functional surface microstructures depicted in Fig. 1. The

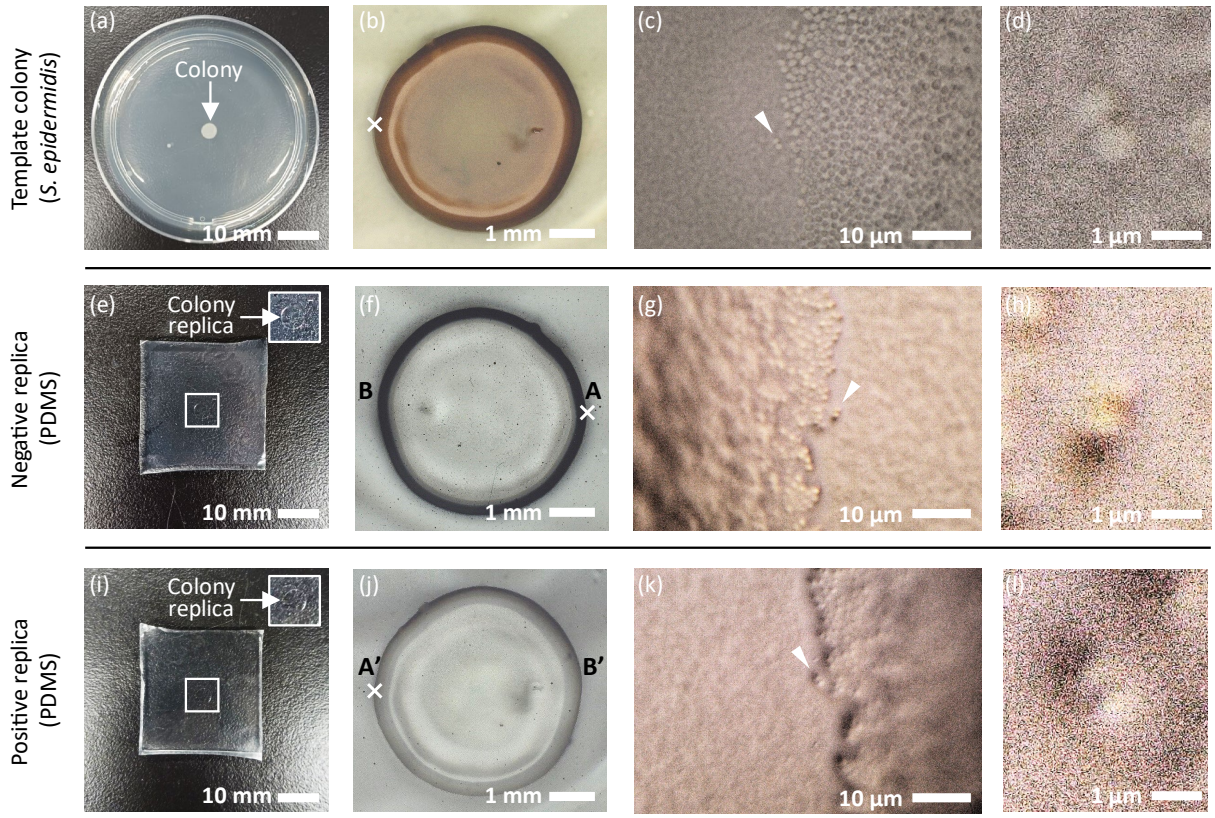

Figure 3. Replication results of the *S. epidermidis* colony. Corresponding images of the template colony (a, b, c, d), the negative replica (e, f, g, h), and the positive replica (i, j, k, l). Overall pictures of the sample (a, e, i), microscopic images of the whole colony (b, f, j), magnified images of the edge part denoted by white crosses (c, g, k), and magnified images of the doublets of the bacterial cell denoted by white arrows (d, h, l).

replication process was conducted successfully, resulting in two PDMS replicas (Fig. 3(e, i)). Notably, after peeling off the replicas, most bacteria adhered to the PDMS rather than the agar plate, but these residual bacteria could be removed using running water and scotch tape. As shown in Fig. 3(b, f, j), the macroscopic contours of the colony were almost perfectly transferred onto the two replicas, with an inversion of the left and right sides in the negative replica. Additionally, a comparison of the macroscopic profiles along the center line of the negative and positive replicas confirmed replication accuracy in the height dimension to be within 10 µm (Fig. 4).

Microscopic observation revealed that replication accuracy at the single-cell level, approximately 800 nm, was achieved. For instance, a doublet of bacterial cells at the colony's edge (Fig. 3(d)) could be observed at corresponding positions on both replicas (Fig. 3(h, l)). These findings demonstrate the feasibility of replicating the surface microstructure of bacterial colonies by direct molding with sufficient accuracy.

Figure 5 shows the AFM measurement results for a 20 µm × 20 µm area located in the central region of the negative and positive replicas. Both replicas were found to be covered with dot patterns, where each dot, with a diameter of 500–1000 nm, corresponds to the size of a bacterial cell. This suggests that each dot originates from an individual *S. epidermidis* cell. These results confirm that the replication of bacterial colonies can successfully capture the lateral feature dimensions typical of functional microstructures. Also, it is revealed that the height of the dots ranges from 50 to 100 nm. This is lower than the radius of a bacterial cell, which is about 400 nm, but this discrepancy could be improved by incorporating additional processes, such as degassing.

From the above results, it can be concluded that the feasibility of replicating the surface microstructures of bacterial colonies has been fundamentally verified.

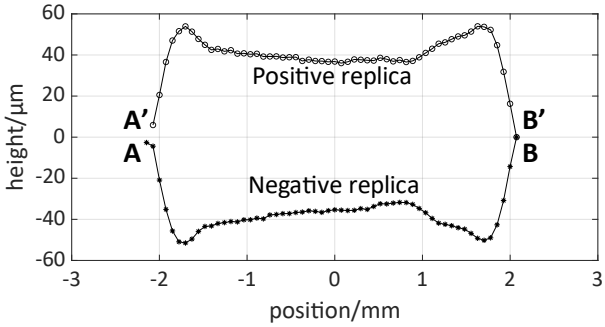

Figure 4. Macroscopic profile along the center line of the negative and positive replicas measured by focus scanning method. The profile of the negative replica is horizontally inverted for easy comparison.

4. Conclusions

The goal of this study is to establish a fabrication method for functional surfaces based on the growth and self-organization of bacterial colonies. To address the chemical and mechanical instability of bacterial cells, this study explores the replication of bacterial colony surface microstructures. The replication experiments were performed using direct molding with PDMS, incorporating a specialized fixation method, vapor fixation, to preserve the fragile structure of the bacterial colonies.

As a result, PDMS replicas were successfully fabricated. Microscopic observations revealed that the replication accuracy reached the single-cell level, approximately 800 nm. This finding demonstrates the feasibility of the direct molding protocol for transferring the surface microstructure and potential functionality of bacterial colonies onto PDMS. This outcome is significant not only because the replicated structures can be applied to other materials but also because it enables the

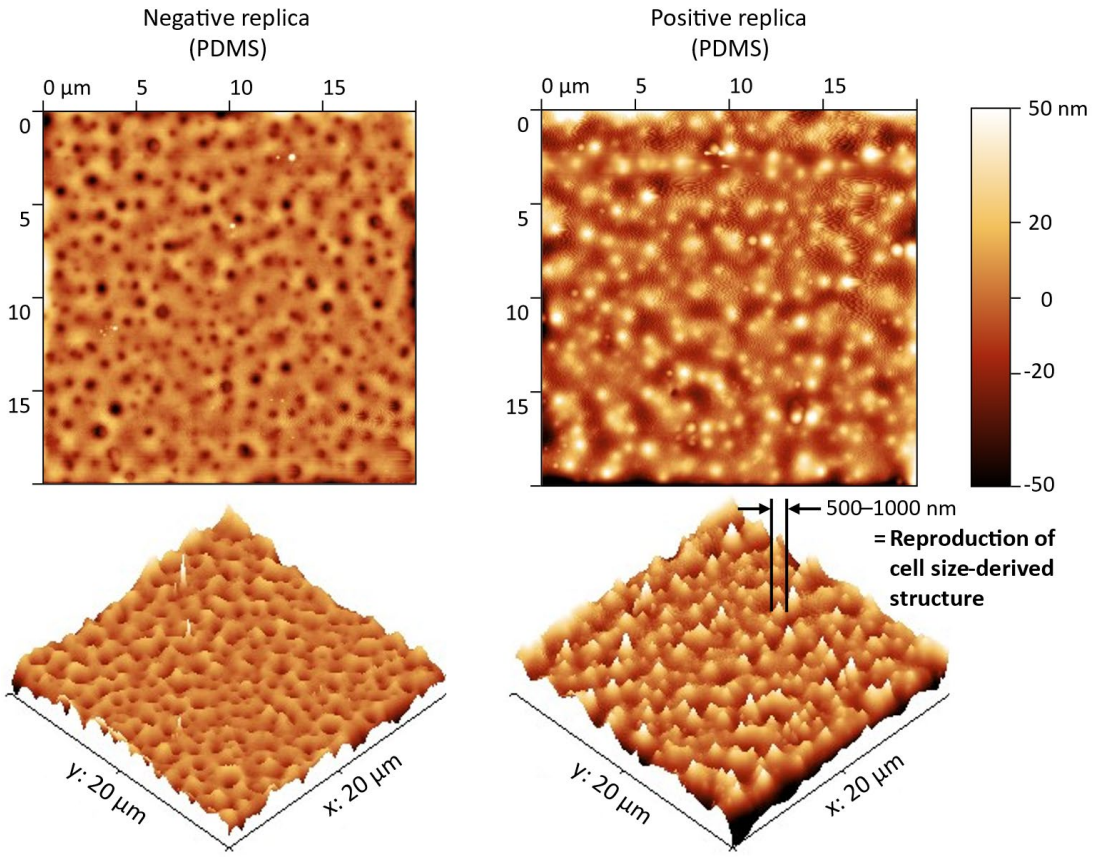

Figure 5. Results of AFM measurement of the central area of the negative and positive replicas.

evaluation of bacteria-derived structural functionalities on substrates with well-characterized properties. Furthermore, beyond the scope of functional surface fabrication, the direct transfer of bacterial colony morphology could find other applications such as the morphological analysis of biofilms.

Additionally, AFM measurements revealed a dot pattern covering the surface of the *S. epidermidis* colony replicas. The width of the dot pattern, which is considered to be derived from the arrangement of bacterial cells, is measured to be 500 to 1000 nm, which is consistent with the typical dimension of functional surface microstructures. This suggests that the replication technique established in this study is applicable to the fabrication of practical functional surfaces.

In the future, the valuable functionalities enabled by bacteria-derived structures should be demonstrated. To achieve this, efforts will focus on controlling the fabricated structures through bacterial behavior and evaluating the functionalities of these structures using the replication method established in this study.

Acknowledgment

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