Anti-biofouling Coating by Wrinkled, Dual-roughness Structures of Diamond-like Carbon (DLC)

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Abstract
The textured surface with superhydrophobic nature was explored for an anti-biofouling template. Hierarchical structures composed of the nano-scale wrinkle covering on micro-scale polymer pillar patterns were fabricated by combining the deposition of a thin coating layer of biocompatible diamond-like carbon (DLC) and the replica molding of poly-(dimethylsiloxane) (PDMS) micro-pillars. The as-prepared surfaces were shown to have extreme hydrophobicity (static contact angle >160°) owing to low surface energy (24.2 mN/m) and dual-roughness structures of the DLC coating. It was explored that the hierarchical surfaces showed poor adhesion of the Calf Pulmonary Artery Endothelial (CPAE) cells for cultures of 7 days suggesting that the 3-dimensional (3-D) patterned superhydrophobic DLC coating exhibits excellent anti-biofouling properties against non-specific cell adhesion. In particular, the reduced filopodia extension during cell growth was caused by disconnected focal adhesions on the pillar pattern. This limited cell adhesion could prevent undesired growth and proliferation of biological species on the surface of biomedical devices such as stents, implants or even injection syringes.

Keywords: Diamond-Like Carbon (DLC), Cell adhesion, Superhydrophobicity, Dual-scale structure, Anti-biofouling

Introduction
The interaction behaviors between biological environment with solid surfaces have been extensively studied in recent years. In general, undesired biological attachment on engineered solid surface has produced severe problems in various fields, ranging from the macroscopic level such as microbiology-induced corrosion in marine structures and ships to the microscopic level such as malfunction of biomedical devices. The biofouling mechanism has involved a complex interplay between chemistry, surface morphology and polarity. In biomedical field, the failure of some intravascular biomedical devices during operation is caused by proliferation of cells on such devices. This biofouling is accelerated by adherence of proteins, glycoproteins, or bacteria while the vascular flow is insufficient to clean the surface. Particularly, the current restenosis rate of the implanted stent after one month is about 30-40% due to the proliferation of cells and the resulting accumulation of sludge inside the stent. Several methods have been developed to prevent cell proliferation on biomedical devices, such as radiotherapy, dietary program, and drug eluting surfaces. While these methods are useful, there are potential drawbacks involving complexity of fabrication and operation. Therefore, it is of great importance to develop a device with self-cleaning and anti-biofouling characteristics, together with long-term stability, in vivo environments.

Recently, surface texturing to improve superhydrophobicity has been extensively studied both experimentally and theoretically. Furthermore to fabricate a water-repellent superhydrophobic surface, it can be desirable to combine the methods of top-down and bottom-up processes, which has also been demonstrated using vertically aligned carbon nanotubes, or micelle aggregation. Among the fabrication methods reported so far, however, such as superhydrophobic surfaces with biocompatibility-an essential aspect of biomedical applications-have been relatively unexplored. The use of traditional engineering materials for nano and microfabrication, such as silicon, metals, or non-biocompatible polymers, in biomedical applications, may result in degradation of such materials and consequently improper functionality on cellular and systemic activities under the host environment. Therefore, use of biocompatible materials for surface finishing is necessary for long-term stability of the system.

In this work, we explore the potential of surface textures coated with a material of low surface energy as
well as of biocompatibility as a means to prevent biofouling at cellular level. Hierarchical surfaces was fabricated with a nanoscale wrinkle formation of the diamond-like carbon (DLC) coating on a micro-scale patterns by soft-lithographic method as shown in Figures 1 and 2. DLC coating was chosen for surface finishing layer due to its superior tribological and mechanical properties as well as chemical inertness in biological environment with biocompatibility and hemocompatibility. Furthermore, poly-(dimethylsiloxane) (PDMS) is a powerful material for building micro- and nanostructures with the vast advantages of soft lithography to provide a convenient, effective, and low-cost method for many biological assays application. A Non-linear wrinkle pattern can be formed in DLC film on soft polymer such as PDMS, which is caused by the difference in elastic properties between DLC film and polymer and the high compressive stress in DLC film. The 3-dimensional (3-D) patterned, hierarchical-roughness structures are used as an anti-biofouling coating to resist cell adhesion using bovine endothelial cells. The experimental results revealed that the adhesion and proliferation of CPAE cells is highly restricted on such superhydrophobic surfaces when the spacing between micro-pillars is smaller than the diameter of the cells. In addition, the effects of various micro-pillar spacing ratios on the interplay between superhydrophobicity and cell adhesion are investigated as described below.

**Results and Discussion**

A schematic illustration of the fabrication procedure of the dual-roughness DLC structure with combining both top-down and bottom-up processes shown in Figure 1. For the top-down process, direct replica molding of PDMS from a silicon master was used to produce micrometer scale pillars of 4 μm diameter and 5 μm height, separated in equally spaced square array. Specimens of ten different inter-pillar gap distances were tested for the wetting and bio-adhesion experiments, with ten different integer values of spacing ratio ranging from 1 to 10. The spacing ratio is defined as the ratio of the nearest pillar-pillar separation distance to the pillar diameter (shown as b/a in Figure 1). To fabricate nanostructures on the micro-pillar surface structures, a bottom-up process was ado-
pted by using a radio frequency-plasma assisted chemical vapour deposition (RF-PACVD) of DLC films from hexametyldisiloxane (HMDSO) monomer as a precursor. HMDSO was selected due to its very low surface energy ($24.2 \text{ mN/m}$) close to that of poly(tetrafluoroethylene) (PTFE) ($18.5 \text{ mN/m}$)\(^26\).

As shown in Figure 2, random orientation of a highly contorted hierarchical wrinkle structure is clearly seen after deposition of a DLC thin film on pre-patterned pillars of PDMS. For the deposition for $10 \text{s}$, an isotropic wrinkle pattern was formed on the top as well as on the bottom valley surfaces of the pillars. With increasing deposition time to $1 \text{ min}$, a dual-mode wavy structure with the primary mode of $\sim 1.1 \text{ μm}$ and the secondary mode of $\sim 120 \text{ nm}$ appeared. Based on these results, an optimum deposition condition for superhydrophobicity was obtained\(^25\): deposition time of $30 \text{ s}$ and base and deposition pressures of $0.01$ and $10 \text{ mTorr}$, respectively, at a bias voltage ($V$) of $-400 \text{ V}$. This process condition allows for a distinct hierarchical structure with microscale PDMS pillars covered with a uniform nanoscale DLC coating. For the optimum condition of nanoscale wrinkle formation on microscale pillars, it has been shown theoretically and experimentally that the spacing ratio of 1 to 4 gives superhydrophobicity properties on the surfaces as described in our previous work\(^25\).

Recent studies have shown that there are strong correlations between cell adhesion and the distribution of extra cellular matrix (ECM) proteins\(^27-29\), surface geometrical features\(^30,31\), and surface free energy\(^32,33\). Our primary interest is to potentially apply the superhydrophobic surfaces to implanted biomedical devices. To this end, we first need to examine the cell adhesion on superhydrophobic surfaces for a prolonged period of time. We believe that the superhydrophobic surfaces would lower the adhesion strength of the cells, due to the limited contact between the cell medium and the

**Figure 2.** SEM images of patterned PDMS by DLC coating. Wrinkle evolution is observed after DLC coating for $10 \text{s}$ (a), $30 \text{s}$ (b), $1 \text{ min}$ (c), $10 \text{ min}$ (d). The micropatterned of PDMS before (e) and after (f) DLC coating is shown. The picture in (g) shows a large area view along with a magnified view in the inset. Contact angles of water droplet show that the surface is highly water-repelling after DLC coating (contact angle $\sim 160^\circ$ in (f)). Scale bars are $500 \text{ nm}$ in Figure 2 (a-d), $5 \text{ μm}$ in Figure 2 (e, f) and $20 \text{ μm}$ in Figure 2 (g).
The initial cell attachment is restricted by less ECM proteins adsorbed on the surface, in which the initial superhydrophobicity plays a key role. As time goes by, the cell attachment can be enhanced as the cells recognize the surface by secreting their own ECM proteins or as the surface loses its original hydrophobicity. Nonetheless, the cell proliferation would be highly restricted on the hierarchical structures in that the focal adhesions are only formed at the peak of nanoscale wrinkles, while these adhered cells have difficulty in crawling into the spaces between micropillars. From a surface free energy point of view, cell adhesion is driven by the balance between dispersive and polar components of the total surface energy. Baier et al.\textsuperscript{32} reported that cell adhesion could be minimal when surface free energy is between 20 to 30 mN/m, which is very close to that of the DLC coating (24.2 mN/m) used in our experiments.

Figure 3 shows the adhesion behavior of CPAE cells on surfaces with different degrees of hydrophobicity when cultured over 7 days. On hierarchical structures with the spacing ratio of 1 to 4 (superhydrophobic surfaces), the cells remained less spread-out with a spherical shape after cultures of several days. The cell density was $<50$ cells/mm$^2$ even for a culture period of 1 week, which was smaller than that on the Petri dish control by one order of magnitude. As mentioned above, we attribute the reduction in the initial cell attachment on superhydrophobic surfaces to the reduced amount of ECM proteins. In addition, the cells cannot easily crawl into the spaces between micropillars, yielding suspended cell attachment and limited cell proliferations.

When the micropillar spacing is larger than the diameter of cells (10-20 μm), i.e., spacing ratio $>4$, the attachment and growth of cells becomes distinctively larger, since the cells can crawl in between the larger micropillar spaces and anchor themselves to the bottom surface. As shown in Figure 3, the cells were relatively well spread with many interconnections for the spacing ratio of 5 and 10. On the petri dish surface (control), the cells formed a confluent layer within a
week. The details of cell morphology on the surfaces with different hydrophobicity are shown in Figure 4. It is clearly seen that CPAE cells were trying to make a confluent layer on the flat surface (Figure 4a). As the hydrophobicity increased by the application of micro- and nanoscale roughness, cells were more difficult to spread onto the surfaces. Finally on the surfaces in the superhydrophobic regions (hierarchical

**Figure 4.** Adhesion behavior of CPAE cells on: (a) flat PDMS, (b) hierarchical wrinkled structure with high spacing ratio of 5 (hydrophobic), (c) hierarchical wrinkled structure with low spacing ratio of 1 (superhydrophobic), (d-f) details of cell morphology on the surfaces shown in Figure 5c. Arrows indicate the detachment of filopodia of CPAE cells from wrinkled pillar surfaces. Scale bars are 10 μm (Figure 4a-c) and 5 μm (Figure 4d-f).

**Figure 5.** Adhesion behavior of CPAE cells on surfaces with different degree of hydrophobicity when cultured over 7 days. The cell density (a) and the area (b) are shown as a function of spacing ratio, where samples with spacing ratio of 1 to 4 show superhydrophobic surfaces.
Conclusions

We have presented dual-roughness, hierarchical structures of DLC as an anti-biofouling coating against non-specific adhesion of CPAE cells. The structures were fabricated by depositing a thin coating layer of biocompatible DLC on PDMS micro-pillars created from replica molding. Our experimental found that superhydrophobicity could be achieved on the 3-D hierarchical surface structure with a low microstructure spacing ratio of 1 to 4. The adhesion assays on surfaces with different degree of hydrophobicity revealed that the proliferation of CPAE cells was highly restricted on superhydrophobic surfaces with the spacing smaller than the diameter of the cells; the limited spacing restricts the entry of the cell, resulting in suspended cell attachment and limited growth. The low surface free energy of DLC coating further contributed to the reduced adhesion and growth of the cells.

The simple fabrication method presented here provides an effective way of creating 3-D hierarchically structures with superhydrophobicity and anti-biofouling properties. These properties would be particularly useful for biomedical devices such as stents, implants or even injection syringes.

Materials and Methods

Preparation of Dual-roughness Structures of DLC

Details on the preparation of dual-scale DLC structures can be found elsewhere. Briefly, PDMS (Sylgard 184 Silicone elastomer, Dow Corning) was used as the soft base material (Young’s modulus of PDMS ~20 MPa). To make pillar structures with different spacing ratio, the standard soft lithographic technique called replica molding was used. The array of micro-pillars with spacing ratio from 1 to 10 was replicated from 4 inch diameter of silicon master. Each array had 1 × 1 cm² area, containing 4 µm diameter and 5 µm height of micropillars. To generate nanometer scale wrinkle structures, RF-PACVD was used to deposit a very thin layer of DLC on pre-patterned PDMS surfaces. The precursor gas of hexamethyldisiloxane (HMDSO) was decomposed into a DLC film at a base pressure and a deposition pressure of 10⁻⁶ and 10⁻² Torr, respectively.

Contact Angle (CA) and Contact Angle Hysteresis (CAH) Measurements

Deionized (DI) water with a droplet volume set at 5 µL was used in the measurement of static CA with sessile droplet mode. For CAH measurement, advancing and receding CAs were measured when a water droplet with total volume of about 50 µL was drawn in and out to the surfaces. The data was averaged over at least 5 different locations using a contact angle analyzer (KRUSS DSA 100).

Cell Culture and Viability Test

Calf pulmonary artery endothelial (CPAE) cells were purchased from the American Type Culture Collection (ATCC) and cultured on the surfaces with hierarchical structures. The surfaces were cleaned by 70% ethanol, DI water and phosphate-buffered saline (PBS) (pH 7.4) at room temperature. CPAE cells were then seeded at a cell density of 1.5 × 10⁵ cell/mL and maintained with the culture medium (Dulbecco’s modified Eagles medium/DMEM, 10% fetal bovine serum, and 1% penicillin-streptomycin) at 37°C in a humidified 5% CO₂ incubator. For measuring cell morphology, the cells were incubated with calcein-AM (2 µM, green fluorescence) in PBS at 37°C in humidified 5% CO₂ incubator. The cells were washed in PBS and immediately viewed with an optical inverted microscope (Olympus).
IX71) equipped with a reflected light fluorescence unit.

Observation
The details of hierarchical structures and cells morphology were observed under a scanning electron microscope (SEM) (NanoSEM 200, FEI company). Prior to SEM observation, the cells were dehydrated using multistage ethanol solutions (30, 50, 80 and 99 %) after paraformaldehyde (PFA, 4% in PBS) incubation for 15 min. The cells were then fixed using hexamethyldisilazane (HMDS) for 30 min. 10 nm Pt layer was deposited using a vapor deposition method for preventing the electron charging during SEM observation with the accelerating voltage of 5 kV.

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