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Gold cleaning methods for preparation of cell culture surfaces for self-assembled monolayers of zwitterionic oligopeptides

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Self-assembled monolayers (SAMs) have been used to elucidate interactions between cells and material surface chemistry. Gold surfaces modified with oligopeptide SAMs exhibit several unique characteristics, such as cell-repulsive surfaces, micropatterns of cell adhesion and non-adhesion regions for control over cell microenvironments, and dynamic release of cells upon external stimuli under culture conditions. However, basic procedures for the preparation of oligopeptide SAMs, including appropriate cleaning methods of the gold surface before modification, have not been fully established. Because gold surfaces are readily contaminated with organic compounds in the air, cleaning methods may be critical for SAM formation. In this study, we examined the effects of four gold cleaning methods: dilute aqua regia, an ozone water, atmospheric plasma, and UV irradiation. Among the methods, UV irradiation most significantly improved the formation of oligopeptide SAMs in terms of repulsion of cells on the surfaces. We fabricated an apparatus with a UV light source, a rotation table, and HEPA filter, to treat a number of gold substrates simultaneously. Furthermore, UV-cleaned gold substrates were capable of detaching cell sheets without serious cell injury. This may potentially provide a stable and robust approach to oligopeptide SAM-based experiments for biomedical studies.

[**Key words:** UV cleaning; Gold substrate; Self-assembled monolayer; Oligopeptide; Non-fouling surface; Electrochemical cell detachment]

Understanding the mechanisms of the biointerface between materials and cells is important in diverse fields ranging from fundamental cell biology to artificial organ and regenerative medicine applications. A typical approach to examining cell–substratum interactions is the use of self-assembled monolayers (SAMs) of alkanethiol molecules prepared on gold surfaces. Because alkanethiol SAMs provide stable and well-defined surfaces not only under vacuum conditions but also in aqueous solutions, this approach has been used to study the effects of surface chemistry on cellular behaviors, leading to precise control over cell adhesion and repulsion on culture surfaces. Spatial patterning of cell-adhesive and -repulsive regions can also be fabricated down to subcellular scales on a surface by means of soft lithography, with which cellular responses against local adhesion regions, such as growth, apoptosis, and differentiation, have been revealed (1–4). Furthermore, surfaces that are dynamically switchable from cell-adhesive to -repulsive in culture by external stimuli have been proposed and applied to fabricate three-dimensional tissues for regenerative medicine applications (5–8).

One potential drawback of the alkanethiol SAM-based approach is that the molecules potentially cause cytotoxic effects and acute inflammatory reactions *in vivo* (9,10). To alleviate this issue, oligopeptide SAMs have been generated. Oligopeptides are biodegradable and in general biocompatible, because they only generate amino acids when degraded. However, basic procedures for the preparation of oligopeptide SAMs have not been fully established. No appropriate cleaning protocols of gold surfaces before modification with oligopeptides have been reported. Such protocols are necessary because gold surfaces are readily contaminated with organic substances in the air (11). In the case of alkanethiols, contaminants exert no significant adverse effect on the formation of SAMs, because alkanethiol molecules push out contaminants from the gold surface in the process of SAM formation driven by van der Waals forces between alkyl chains (12,13). However, oligopeptides form SAMs by a different mechanism. Oligopeptides have typically been designed to form SAMs with electrostatic forces between positively and negatively charged amino acids. Thus, contaminants possibly compromise the formation of oligopeptide SAMs, and cleaning methods of gold surfaces may be critical for preparation of precisely controlled oligopeptide SAMs.

Several gold cleaning methods have been established in the field of semiconductor research and in industry. These are divided into wet and dry cleaning methods. Washing with dilute aqua regia is a typical wet cleaning method that etches gold layers due to its highly corrosive nature (14). Another wet cleaning method is the use of ozone water, which effectively decomposes organic matter and is non-corrosive to gold. Wet cleaning is applicable to both flat surfaces and complicated structures, but disposal of a relatively large amount of these chemical solutions is a labor-intensive and environmentally burdensome process. Widely used dry cleaning

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methods include high-power UV and plasma exposures (15,16) that degrade organic compounds into volatile compounds. In general, dry cleaning methods are environmentally friendly and cost-effective, but inapplicable to substrates with complicated configurations.

In the present study, we examined four typical wet and dry methods for gold cleaning, namely, aqua regia washing, ozone water washing, UV exposure, and atmospheric plasma exposure, on the preparation of oligopeptide SAMs for cell culture (Fig. 1A). The purpose of this study was to establish a gold cleaning method to form a dense peptide SAM. We first characterized gold surfaces using water contact angle, optical microscopy, and atomic force microscopy after treatment with the cleaning approaches. For cell culture experiments, two different zwitterionic oligopeptides were employed. One serves as a cell-repulsive background (17), and the other is dynamically switchable from cell-adhesive to -repulsive upon application of electrochemical potential (17,18). Because contaminants may prevent self-assembly of oligopeptides on gold surfaces and make it difficult to prepare cell-repulsive and cell-releasing surfaces, we evaluated the effects of the cleaning approaches by counting the number of cells attached to the cell-repulsive surfaces and remaining on the cell-releasing surfaces after the potential application.

MATERIALS AND METHODS

Cell preparation Red fluorescent protein-expressing human neonatal dermal fibroblasts (RFP-HNDFs; Angio-Proteomie, Boston, MA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Yokohama, Japan) supplemented with 10% fetal bovine serum (FBS; Sigma–Aldrich, Tokyo, Japan) and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA). Green fluorescent protein-expressing human umbilical vein endothelial cells (GFP-HUVECs, Angio-Proteomie) were maintained in endothelial growth medium (EGM-2) containing endothelial basal medium (EBM-2; CC-3156, Lonza, Tokyo, Japan) and SingleQuot growth supplement (CC-4176, Lonza). The culture media were exchanged every two days and the cells were passaged with 0.25% trypsin-EDTA (Thermo Fisher) at 80% confluency. When GFP-HUVECs and RFP-HNDFs were mixed, the culture medium for RFP-HNDFs was used.

Preparation and cleaning of gold substrates Gold substrates were prepared by sputter coating a few nanometers of chromium and 30–40 nm of gold using a sputtering machine (CFS-4EP-LL, Shibaura Mechatronics, Yokohama, Japan) on glass substrates (24 mm × 24 mm, No. 4, Matsunami, Osaka, Japan). After storage for at least one week under ambient conditions, the substrates were cleaned with four cleaning methods, namely, dilute aqua regia (HCl:HNO_3:deionized water, 1:1500:500 v/v) for 75 s, 10 ppm ozone water for 3 min, UV exposure (MEUT-1400, M. D. COM, Yokohama, Japan) at 100 mW/cm^2 for 3 min, and atmospheric plasma (P500-SM, Sakigake-Semiconductor, Kyoto, Japan) for 175 s (10-mm distance from the plasma source, 5 mm/s scan rate). Then, the surface wettability was measured with a portable contact angle meter (PCA-11, Kyowa, Tokyo, Japan). The changes in gold surface topography before and after the cleanings were observed with an optical microscope (NewView 6000, Zygo, Tokyo, Japan) and an atomic force microscope (AFM; SPA-400, Yamato Scientific Co., Tokyo, Japan).

Cell adhesion tests on a surface modified with cell-repulsive oligopeptides The cell-repulsive oligopeptide (CGGGKEKEKEK, > 95% purity, Scrum, Tokyo, Japan) contains cysteine at the N-terminal and the alternating zwitterionic KE sequence, which chemically adsorbs onto the gold surface via gold-thiol bonding and forms a dense self-assembled monolayer (SAM) by electrostatic interactions as described previously (17,19). The gold-coated substrates were stored for 1 week under ambient conditions and cleaned by the four cleaning methods described above, and then were immersed into a 5-μM oligopeptide solution overnight at 4°C. The substrates were then rinsed with phosphate-buffered saline (PBS) twice and placed in a 6-well plate (BD Falcon, Tokyo, Japan). To examine cell repulsion on the surfaces against two different cell types simultaneously, GFP-HUVECs and RFP-HNDFs (ratio 1:1) were mixed and seeded at a density of 1.0 × 10^5 cells/2 mL. The culture plate was then placed in an incubator (SANYO, Osaka, Japan) with humidified air containing 5% CO_2 at 37°C. After 3 h, the substrates were washed twice with PBS to remove non-attached cells. Fluorescent microscopic images were taken at 5 distinct positions, and the number of each cell type was counted using the Image J software. A bare gold surface without oligopeptide modification (w/o peptide) and a gold substrate modified with the oligopeptides without cleaning (no treatment) were used as negative controls.

Automated UV cleaning apparatus We fabricated a UV cleaning apparatus with a rotation table, HEPA filter, and a touch-panel (Fig. 1B). The rotation table holds up to 8 gold substrates and rotates at 0–30 rpm during exposure to UV light. The UV light source (wavelength 172 nm) generates ozone at 15 mg/min. The distance between samples on the table and the light source is adjustable. When the distance was set at 1.0 mm, the UV intensity was ~7 mW/cm^2. In this study, we examined 8 different combinations of conditions, including exposure times of 1, 5, and 15 min; rotation speeds of 5 and 30 rpm; and distances of 1.0 and 50 mm. After the treatments, the gold substrates were immersed in the cell-repulsive oligopeptide solution and left overnight at 4°C. After washing with PBS twice, cell suspensions of GFP-HUVECs and RFP-HNDFs (both 5 × 10^4 cells/mL) were poured onto the substrates, and the number of attached cells were counted after 3 h of incubation.

Evaluation of cleaned gold substrates using electrochemical cell detachment The cell-adhesive oligopeptide (CGGGKEKEKEKGRGDSP, >95% purity, Scrum) contains the RGD domain for integrin-mediated cell binding (20). A gold substrate was modified with this oligopeptide just as described above for the cell-repulsive oligopeptide modification. A gold substrate was stored for 1 week, cleaned by UV exposure (30 rpm for 1 min at a 1-mm distance from the UV source), and modified with a 5-μM oligopeptide solution. The substrates were then sterilized with 70% ethanol for 5 min and washed with PBS twice before cell culture. GFP-HUVECs were seeded on the substrates at a density of 1.0 × 10^5 cells/2 mL. After 1 day of culture, the gold substrates were rinsed twice with PBS to remove nonadherent cells. The gold substrate (working electrode, W. E.), an Ag/AgCl reference electrode (R. E.), and a platinum counter electrode (C. E.) were connected to a potentiostat (HA-151, Hokuto Denko, Tokyo, Japan). After applying −1.0 V vs Ag/AgCl electrode for 1, 2, or 3 min and gently rinsing with PBS, phase-contrast microscopic images were acquired to quantify the number of adherent cells remaining on the gold substrates using the ImageJ software. As a positive control, the same experiments were conducted with a gold substrate modified with the cell-adhesive oligopeptide immediately after sputtering (with peptide). Negative controls were a bare gold substrate without peptide modification (w/o peptide) and a gold substrate modified with the oligopeptide without cleaning (no treatment).

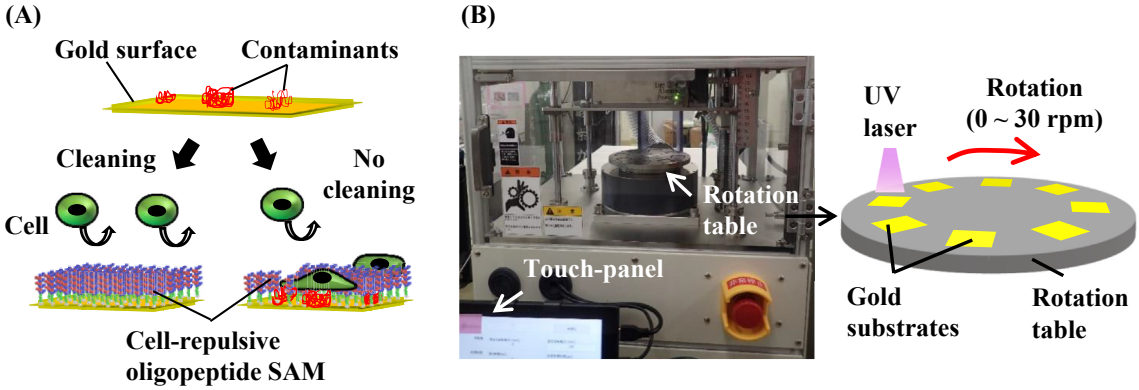

FIG. 1. Concept of cleaning gold substrates and oligopeptide modifications. (A) Hypothesis of surface cleaning effect on oligopeptide SAM formation for cell culture. (B) Appearance of the apparatus and schematic of the rotation table.

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