Epifluorescence microscopic detection of photolithographically micropatterned aldehyde- and carboxy-terminated self-assembled monolayer

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Abstract. An aldehyde (CHO)-terminated self-assembled monolayer (SAM) was prepared on silicon substrates covered with native oxide (SiO₂/Si) by chemical vapor deposition of triethoxysilylundecanal (TESUD). The CHO-terminated SAM was subsequently converted to the COOH-terminated SAM by photooxidation using 172-nm vacuum UV light through photomasks with square windows of 10 × 10 and 2.5 × 2.5 µm². The chemical reactivity of each microspot composed of the COOH-terminated SAM was detected with high resolution by epifluorescence microscopy using Qdot® 655. These results demonstrated that the microspots (spot size: 2.5 × 2.5 µm²) composed of active COOH-terminated SAM were successfully fabricated on the SiO₂/Si substrate at a high density of 8 × 10⁴ spots/cm², which was four times higher than that of previously reported micropots.

1. Introduction
Microarray technology has become a crucial tool for large-scale and high-throughput biological science and technology, facilitating fast, easy, and simultaneous detection of thousands of addressable elements within a single experiment under identical conditions [1]. In recent years, microarrays of DNA, proteins, and cells are becoming indispensable for studies on genomics, proteomics, and cellomics. Spatially defined immobilization of DNA, proteins, and cells [2] on the surfaces of various microarray platforms is an important technology. Many strategies for constructing protein microarrays on a wide range of substrates have been proposed in the past decades. The method for immobilizing proteins on surfaces will determine the functional properties of protein microarrays. There are two approaches for developing protein microarrays: tailoring the surfaces to functionalize natural target proteins and modifying target proteins with tags such as histidine, streptavidin, and biotin in order to effectively bind them to surfaces [3]. The former approach is considered to be more practical because it can tailor microarray surfaces to be used for wide varieties of proteins without compromising their functional properties. Almost all types of proteins have reactive functional groups such as carboxy (COOH), amino (NH₂), thiol (SH), and hydroxy (OH) groups in their side chains. These functional side groups can be used as a starting point for immobilizing proteins on tailored microarray surfaces. One of the most promising approaches to regulate site-selective attachment of proteins is employment of patterned self-assembled monolayers (SAMs) terminated with chemically reactive groups that bind
to these functional side groups in proteins. Various constructing strategies and a wide range of substrates, such as glass, gold, polymer and other special formats, have been examined for constructing protein microarrays [4]. In a previous study [5], we reported the fabrication of well-defined microspots (5 × 5 μm²) composed of the COOH-terminated SAM by photooxidation of triethoxysilylundecanal (TESUD, [CHO]–[CH₂]₁₀[Si(OS₂H₅)₃]) SAM on silicon substrates by masked 172-nm vacuum UV (VUV) light. The chemical reactivity was confirmed with a novel method using epifluorescence microscopy of the Qdot® 655 streptavidin conjugate tagged to biotin hydrazide bound to the activated COOH groups. In this study, we report the fabrication of microspots (2.5 × 2.5 μm²) composed of the COOH-terminated SAM; these microspots were smaller than those previously reported using masked photooxidation of TESUD SAM [5]. Moreover, it was demonstrated these microspots could be detected with high resolution by epifluorescence microscopic detection using Qdot® 655.

2. Materials and Methods

2.1. Preparation of CHO-terminated SAM

The CHO-terminated SAM was prepared on silicon substrates covered with native oxide (SiO₂/Si) by chemical vapor deposition (CVD) of TESUD (Gelest Inc.), as previously described [3]. In brief, Si (100) wafers (1.5 × 1.5 cm²) were exposed for 30 min at 10³ Pa to 172-nm VUV light (λ= 172 nm, 10 mW/cm²) radiated from an excimer lamp (UER20-172V; Ushio Inc.); the distance of between the lamp window and the sample surface was 20 mm. The VUV/ozone-cleaned Si wafers were placed with 0.1 cm³ of TESUD diluted with 0.7 cm³ of absolute toluene in a Teflon container having a volume of 65 cm³ in a dry N₂ atmosphere with less than 10% relative humidity. The container was sealed and heated in an oven maintained at 130°C for 6 h. Each sample exposed to TESUD vapors was sonicated for 20 min successively in absolute toluene, absolute hexane, and acetone. Finally, the sample was rinsed with deionized water and blow dried with a N₂ gas stream.

2.2. Photooxidation of CHO-terminated SAM

Next, the CHO-terminated SAM was irradiated for 25 min at 10⁵ Pa by the masked 172-nm VUV light radiated from a distance of 90 mm between the window and the sample surface [5]. As shown in Figure 1, the CHO-terminated SAM on SiO₂/Si substrates was fabricated by site-selective photooxidation with activated oxygen species (O(^1)D), O(^3)P) through photomasks with square windows of 10 × 10 and 2.5 × 2.5 μm².

2.3. Chemical Reactivity of 172-nm VUV irradiated microspots and unirradiated regions.

To investigate the chemical reactivity in the 172-nm VUV unirradiated regions composed of the CHO-terminated SAM (Figure 2A), the micropatterned sample was immersed overnight in 1 ml of 50 mM phosphate buffer (pH 5.8) containing 5μg/ml biotin hydrazide at 4°C. Excess physisorbed biotin hydrazide was removed by washing with 50 mM phosphate buffer (pH 7.4) containing 1 M NaCl and

![Figure 1. Fabrication of micropatterned CHO- and COOH-terminated SAM on the SiO₂/Si surface by photooxidation using masked 172-nm VUV light.](image-url)
0.05\% Tween 20. Next, the sample was incubated at 37°C for 2 h in a 2\% phosphate buffer (pH 7.4) solution of the Qdot\textsuperscript{®} 655 streptavidin conjugate (average diameter of ~15-20 nm, Invitrogen). On the other hand, to investigate the chemical reactivity of the 172nm-VUV irradiated microspots (Figure 2B), another micropatterned sample was immersed overnight in 1 ml of 50 mM phosphate buffer (pH 7.4) containing 1\µg/ml bovine serum albumin (BSA) at 4°C to block the CHO-terminated regions. Next, the sample was washed with 50 mM phosphate buffer (pH 7.4) containing 1 M NaCl and 0.05\% Tween 20. The sample was then incubated at room temperature for 2 h with 25 mM N-hydroxysuccinimide (NHS) and 20 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) in ethanol to activate the 172-nm VUV irradiated microspots. Immobilization of biotin hydrazide and subsequent labeling with the Qdot\textsuperscript{®} 655 streptavidin conjugate were conducted in a manner similar to that performed in the 172-nm VUV unirradiated regions.

2.4. Epifluorescence microscopy

Fluorescence (Em: 655 nm) from Qdot\textsuperscript{®} 655 tagged to biotin hydrazide immobilized on each sample surface (Figure 2A and 2B) was detected with an epifluorescence microscope (Olympus, BX51) equipped with a CCD camera (Olympus, DP70) and a U-MWG2 filter set (Olympus, excitation filter: 510–550 nm; emission filter: 590 nm).

![Figure 2. Labeling micropatterned CHO- (A) and COOH- (B) terminated SAM on the SiO\textsubscript{2}/Si surface with the Qdot\textsuperscript{®} 655 streptavidin conjugate.](image)

3. Results and Discussion

As shown in previous studies [5, 6], the hydrophobic TESUD SAM surface on a silicon substrate became hydrophilic through the 172-nm VUV light irradiation and the water-contact angle decreased from approximately 80\° to approximately 33\° during irradiation for the first 25 min, while the thickness remained unchanged at approximately 1.1 nm, suggesting no alkyl chains were reacted during the VUV irradiation. To investigate the chemical reactivity in the 172-nm VUV irradiated and unirradiated regions, the photolithographically micropatterned samples using photomasks with the square windows of 10 \times 10 and 2.5 \times 2.5 \µm\textsuperscript{2} were treated with biotin hydrazide, which was subsequently tagged with the Qdot\textsuperscript{®} 655 streptavidin conjugate and fluorescence from Qdot\textsuperscript{®} 655 was detected with epifluorescence microscopy. As shown in Figure 3A, Qdot\textsuperscript{®} 655 (red fluorescence) was selectively immobilized in the unirradiated regions but did not adsorb on the irradiated microspots (no fluorescence). Because NH\textsubscript{2} groups in biotin hydrazide react with CHO groups in TESUD SAM to
form a Schiff base, we concluded that the area-selective adsorption of biotin hydrazide in the unirradiated regions is based on the chemical reaction between NH₂ and CHO groups. Moreover, the fact that Qdot® 655 did not adsorb on the irradiated microspots indicated the area-selective photooxidation of CHO groups in TESUD SAM. If the photooxidation condition is precisely controlled for conversion of CHO groups to COOH groups, these irradiated microspots are expected to again become reactive to biotin hydrazide by activating COOH groups with NHS/EDC treatment. To confirm this, blocking the CHO groups in the unirradiated regions was carried out by BSA adsorption and subsequent activation of the irradiated microspots of 10 × 10 and 2.5 × 2.5 μm² with NHS/EDC treatment. As shown in Figure 3(B), well-resolved fluorescence from Qdot® 655 (see insets in Figure 3(B)) was detected only in the irradiated microspots, indicating that these microspots became reactive to biotin hydrazide, while no fluorescence was detected from the unirradiated regions where the reactivity of the CHO groups was blocked by BSA adsorption. These results show that the CHO groups in the 172-nm VUV-irradiated microspots were photochemically converted to COOH groups, which were further converted to active N-hydroxysuccinimidyl esters by the activation treatment with NHS/EDC. Therefore, stable amide linkages were formed between the activated COOH groups and biotin hydrazide.

4. Conclusions
In this study, the COOH groups photochemically converted from the CHO groups of TESUD SAM were found to show excellent chemical reactivity to biotin hydrazide even in significantly small microspots (spot size: 2.5 × 2.5 μm²). Therefore, such patterned microspots with chemically active COOH groups arrayed in high density (8 × 10⁴ spots/cm²) as shown in this study are expected to serve as protein microarrays for high-throughput protein analysis.

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