Damage-free Fabrication of Perfluoropolymer Microaperture Array Device for Single-molecule Imaging

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Optical microdevices have attracted much attention as promising tools for advanced bioimaging and/or biosensing at the single-molecule level. Various technologies developed by the semiconductor industry are applied effectively to fabricate their microstructures. We studied fabrication process of a new single-molecule imaging device consisting of a microaperture array in a transparent perfluoropolymer film coated on a glass plate, with special attention to process-induced optical damage. Highly anisotropic etching using argon/oxygen mixed plasmas was firstly examined for engraving the aperture array, but it was found that the UV emission from the excited argon in the plasma causes optical damage to the polymer and that the degree of damage was not negligible for the purpose of using the device in single-molecule imaging. Then, an alternative process that involves thermal nanoimprinting and oxygen-plasma removal of thin residual layers was adopted to enable damage-free fabrication process of the polymeric microaperture array device for single-molecule imaging.

Keywords: Single-molecule imaging, Microaperture array, Perfluoropolymer, Microfabrication, Plasma-induced damage

1. INTRODUCTION
The last two decades have seen remarkable advances in the imaging technology of biomolecules with high sensitivity, down to the single-molecule level. Single-molecule imaging is currently recognized as a promising approach for elucidating biomolecular functions and biochemical reaction mechanisms that cannot be revealed by the traditional approach wherein statistically averaged behaviors of massive molecules are observed.1-3 In most single-molecule fluorescence microscopy method, specially designed optics systems are used to localize the illumination volume, which is very effective to reduce background fluorescent noise and to obtain a high signal-to-noise (S/N) ratio. A confocal microscope and a total internal reflection fluorescent microscope (TIRFM) are representative ones.4, 5 TIRFM utilizes a light beam in the substrate that is obliquely incident upon the substrate/liquid interface at an angle greater than the critical angle of refraction. Since fluorescent molecules just close enough to the substrate surface are excited by the evanescent wave, a very small illumination volume can be easily obtained using TIRFM.

On the other hand, an innovative approach has evolved from lab-on-a-chip technology where optical microdevices are used to reduce the observation volume significantly. A successful example is a zero-mode waveguide (ZMW) device, which is composed of a subwavelength aperture array in a thin aluminum film deposited on a glass substrate. It can realize an observation volume of as small as 10 zeptoliters, more than four orders of magnitude smaller than the diffraction limit.6, 7 The ZMW device was used as a core element of the third-generation high-throughput genome sequencer.8 Thus, the microdevice technology is coming under focus in the field of bioimaging and bioanalysis applications. We are currently developing a new single-molecule imaging device consisting of a subwavelength aperture array in a transparent perfluoropolymer film coated on a glass plate. For this purpose, we started to develop its fabrication process on the basis of our recent study on anisotropic etching of the perfluoropolymer in argon/oxygen plasmas as well as surface hydrophilization treatment using argon plasmas.9-10 Although plasma etching is a mature technology for the microfabrication of semiconductor devices, plasma-induced damage during polymeric device fabrication still remains largely unexploited.11

In this paper, we firstly investigated the fabrication process of a polymeric microaperture array device including anisotropic plasma etching, but nonnegligible optical damage was found when the performance of the fabricated device was evaluated. Then, we determined the fluorescence characteristics of perfluoropolymer films after argon or oxygen plasma exposure to understand the extent and origin of the plasma-process-induced damage. On the basis of these results, an alternative damage-free fabrication process of the polymeric microaperture array device for single-molecule imaging by employing nanoimprint technology was designed and demonstrated successfully.
2. DESIGN OF PERFLUOROPOLYMER MICRO-APERTURE ARRAY DEVICE

Figure 1 shows the schematic of the perfluoropolymer microaperture array (PMA) device designed for single-molecule imaging. Small apertures are patterned in a transparent perfluoropolymer (Cytop™) film coated on a glass plate. The device is used with backside illumination at the oblique incident angle to satisfy the condition for total internal reflection at the glass/liquid interface. Since the refractive index of Cytop is almost the same as that of water, 1.34,12 the total reflection condition is satisfied at both glass/liquid and glass/Cytop interfaces and, consequently, the leakage of the laser beam is avoidable in principle. Moreover, Cytop has excellent transparency in the UV-visible light region, and shows very little autofluorescence. Therefore, the illumination volume of this device can be effectively localized in both horizontal and vertical directions by the combination of the subwavelength aperture and the evanescent wave on the glass surface. Since the aperture mask of the present device is made of a dielectric material instead of metal, it does not work as ZMW even when the aperture has the subdiffraction diameter. Namely, its observation volume cannot be as small as that of a ZMW device. However, one can expect other advantages in overcoming some issues of ZMW devices, i.e., the alteration of the single-molecule fluorescence lifetime or nonradiative damping of excited fluorescent molecules due to the electron leakage through a conductive device material13,14 or the temperature rise caused by light absorption. Moreover, the transparency of the whole device provides good compatibility with bright-field microscope imaging.

3. EXPERIMENTAL METHOD

3.1 Device fabrication

A glass plate (Matsumi 18 × 18 mm micro cover glass, thickness 0.12–0.17 mm) was cleaned in an oxygen plasma asher (Diener electronic FEMTO), followed by the spin-coating of a 700-nm-thick transparent perfluoropolymer (Asahi Glass Cytop CTX-809AP2) film on the surface. Then, an 8-μm-diameter aperture array was patterned in the perfluoropolymer layer by either of two different process flows as shown in Fig. 2.

A. Etching-based process

In the first step, photoresist masks were formed on a perfluoropolymer layer. Here, an epoxy-based thick photoresist (MicroChem SU-8 3005) was used because of the low etch rate selectivity of the perfluoropolymer over the photoresist. In addition, to overcome the poor adhesion of SU-8 on the perfluoropolymer, a fluorochemical surfactant (AGC Seimi Chemical Surflon™ S-381) was mixed in SU-8 3005 at 25ppm to adjust the interface free energy.15 Subsequently, the masked perfluoropolymer layer was etched in argon/oxygen inductively coupled plasma (ICP).9 A bell-jar-type plasma etcher with single-turn antenna and water-cooled sample stage was employed. ICP was generated by a 500 W (13.56 MHz) source power and the sample stage was biased by 8 W (13.56 MHz) RF power. The total pressure was 4 Pa and the partial pressures of argon and oxygen were 3.2 and 0.8 Pa, respectively. After etching, the photoresist was peeled off from the sample. Finally, the perfluoropolymer surface was hydrophilized in argon plasma.10

B. Nanoimprint-based process

Thermal nanoimprinting was applied to form microapertures of the PMA devices in the perfluoropolymer

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Fig. 1 Schematic of the PMA device. The evanescent field outside the aperture is substantially decayed within the 700-nm-thick polymer layer.

Fig. 2 Schematics of (a) etching-based process and (b) nanoimprint-based process.
layer. A silicon micromold with the micropillar array structure was fabricated using a cryogenic plasma etcher (Oxford Instruments, Plasmalab 80plus) using C4F8 and SF6 plasmas. The micromold was pressed against the perfluoropolymer-coated glass using a nanoimprinter (Nanonics, NanoimPro Type 210) by applying a force of 1.0 kN at 140ºC. Finally, thin residue layers that remained at the bottom were removed by oxygen plasma treatment, which simultaneously hydrophilized the device’s surface.

3.2 Fluorescence imaging of protein molecules and optical damage in perfluoropolymer

All the imaging experiments were conducted using objective-lens-type TIRFM comprised of an inverted fluorescent microscope (Olympus IX-71) with an oil-immersion objective (Olympus UApO 150-OTIRF) and an electron-multiplying CCD (EMCCD) camera (Hamamatsu Photonics C9100-13). In the device performance evaluation, a fluorescently labeled chaperonin protein, Cy5-GroEL, was suspended in HKM buffer (25 mM HEPES-KOH (pH 7.4), 100 mM KCl, and 5 mM MgCl2) at 370 pM and was spread over the device’s surface.

4. RESULTS AND DISCUSSION

4.1 PMA devices fabricated by etching-based process

The microapertures of the PMA devices fabricated by etching-based process had high fidelity to the designed diameter and slightly tapered sidewalls at approximately 75 degrees. A fluorescence imaging experiment was carried out by setting the device on TIRFM and using Cy5-GroEL as a sample. As shown in Fig. 3, undesirable fluorescent rings were observed around the etched apertures aside from the bright spots from Cy5-GroEL. This fluorescent ring was speculated to originate from the optical damage induced during the polymer microfabrication. The penetration depth of the evanescent light from the glass surface is estimated to be less than 200 nm when a 635 nm laser was used for excitation, whereas plasma-induced damage is produced in the near-surface region. Generally, the damage profile induced by energetic (< several hundreds eV) ions is limited within a few nm, but that by UV light can extend deep into the polymer. By considering all these factors together, the fluorescent ring is attributed to the UV-induced damage, and the activated ring area corresponds to the locally thinned part of the perfluoropolymer layer. Such optical damage becomes a serious problem particularly when the aperture diameter is shrunk to below the diffraction limit.

4.2 Evaluation of plasma-induced damage on perfluoropolymer

To further understand the plasma-induced damage on perfluoropolymer, we examined the optical damage of a 700-nm thick film after the 37 W argon or 150 W oxygen plasma exposure. Here, the input power was chosen to adjust the same ion current density between argon and oxygen plasmas. Figure 4 shows the fluorescence intensity excited by three different wavelengths and plotted against the plasma treatment time. The films exposed to argon plasma showed strong fluorescence, particularly when illuminated with light of relatively short wavelength, 488 and 532 nm. In contrast, the films exposed to oxygen plasma showed little fluorescence. Therefore, it is considered that the high-energy UV light emitted from excited argons causes the bond breakage of perfluoropolymer, and the subsequent recombination results in forming π-
conjugated systems which decreases the excitation wavelength of the polymer to the visible region. The fluorescence intensity tended to increase monotonically with the plasma exposure time, while early saturation was observed for long-wavelength excitation at 635 nm. Although it may be caused by concentration quenching, the reason is not clear at present.

4.3 PMA devices fabricated by nanoimprint-based process

On the basis of findings in the previous sections, an alternative damage-free process was developed by eliminating the plasma process that uses argon. Thermal nanoimprinting was applied to form apertures instead of anisotropic etching in argon/oxygen mixed plasmas. Figure 5 shows a TIRFM image of Cy5-GroEL on the PMA device fabricated by the nanoimprint-based process. The fluorescent ring as seen in Fig. 3 is no longer observed, and single-molecule fluorescence from Cy5-GroEL is clearly observed inside the aperture.

Fig. 5 Single-molecule fluorescence of Cy5-GroEL (370 pM) on the PMA device fabricated by nanoimprint-based process. The aperture (dotted circle) diameter was 8 μm.

5. CONCLUSION

The microfabrication of a microaperture array in transparent perfluoropolymer films was studied for a new single-molecule imaging device. Highly anisotropic etching in argon/oxygen mixed plasmas was firstly applied to fabricate microapertures of the PMA devices in the polymer layer. However, it was found that UV emission from the excited argon caused optical damage to the polymer and that was not negligible for the use of the device in single-molecule imaging. Therefore, the nanoimprint-based process was investigated as an alternative to the etching-based process, and the fabrication of the autofluorescence-free PMA device was successfully demonstrated. Although the aperture diameter of the PMA device fabricated in the present study is still larger than the diffraction limit, the nanoimprint technology is applicable to pattern structures down to several tens of nanometers. Hence, the developed process is expected to be applicable to the fabrication of PMA devices with the aperture diameter below the diffraction limit, which are useful for single-molecule imaging at high concentration.

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References


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