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Super-resolution fluorescence imaging of nanoimprinted polymer patterns by selective fluorophore adsorption combined with redox switching

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We applied a super-resolution fluorescence imaging based on selective adsorption and redox switching of the fluorescent dye molecules for studying polymer nanostructures. We demonstrate that nano-scale structures of polymer thin films can be visualized with the image resolution better than 80 nm. The method was applied to image 100 nm-wide polymer nanopatterns fabricated by thermal nanoimprinting. The results point to the applicability of the method for evaluating residual polymer thin films and dewetting defect of the polymer resist patterns which are important for the quality control of the fine nanoimprinted patterns. © 2013 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution 3.0 Unported License. [http://dx.doi.org/10.1063/1.4827155]

Recently, thermal nanoimprint lithography has received great attention due to its applicability in nano-fabrication technology.1–3 In contrast to photolithography which requires expensive light sources and lens systems, nano-scale resist patterns can be replicated by simply pressing a mold into a thermoplastic polymer thin layer in thermal nanoimprinting. Thus, thermal nanoimprinting enables the mass manufacturing of nanoscale patterns at a reasonably low cost.

In general, polymer thin films are used as a resist because of their high wettability and adhesion properties against a substrate as well as the high masking effect. It has been pointed out that physical properties of polymers change drastically when they are confined in a nanoscale space.4, 5 Obviously, nanoscale structural characterization of the polymer patterns is imperative for the further development of the thermal nanoimprinting technique.

Nanoscale patterns of polymer thin films are usually investigated by using scanning electron microscopy (SEM) and atomic force microscopy (AFM). Although AFM allows one to measure nanoscale structures in a non-destructive manner, an imaging speed of AFM is relatively slow. In addition, the image quality depends on a tip curvature which makes it difficult to measure the correct pattern widths. In a SEM measurement, polymer patterns often deform due to charging. Furthermore, SEM measurements often require a destructive coating of polymer patterns. The coating also causes contraction of the polymer patterns. In order to examine a large number of nanoimprinted samples accurately and rapidly, a convenient non-destructive method with nanometer scale resolution is desired.

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Optical microscopy is a powerful method for measuring microstructures in a non-invasive manner. In addition, applications of optical microscopy are not restricted to the sample surface. In spite of these advantages, the method was never considered as a tool to measure the images of nanometer scale objects since the spatial resolution of optical microscopy was limited by the diffraction of the light (∼300 nm in the visible wavelengths). However, recent development of super-resolution fluorescence imaging techniques, such as stimulated emission-depletion (STED) microscopy, saturated structured illumination microscopy (SSIM), and localization microscopy (e.g. PALM, STORM) has overcome the diffraction limit. The super-resolution fluorescence imaging techniques have become tools for the non-destructive nanoscale optical imaging. In particular, the localization microscopy has the potential to become a convenient tool for the nanoscale fluorescence imaging given that a conventional wide-field fluorescence microscope can be used for the measurements. The localization microscopy depends on spatio-temporal switching of individual fluorescent probe molecules between fluorescent and dark states. The switching between the fluorescent and dark states has been achieved by using photoswitchable fluorescent probe molecules, redox switchable fluorophores, and so on. Another strategy to achieve spatio-temporal switching of individual fluorescent probes is to use adsorption, desorption, and photobleaching of fluorophores on a specimen. The adsorption-based localization microscopy has been demonstrated to be a powerful means for nanoscale imaging of lipid bilayers as well as polymer thin film patterns fabricated by electron beam lithography. The contrast in the images in the latter case was achieved by fluorescence quenching of molecules adsorbed on a gold substrate.

Here, we report a super-resolution fluorescence imaging study on nanopatterns of polymer thin films by means of selective fluorophore adsorption combined with redox switching. Since the kinetics of spatio-temporal switching of individual fluorescent molecules can be controlled by both redox switching and adsorption, this method enables an efficient image reconstruction with high localization accuracy as compared with the super-resolution imaging based solely on the adsorption of the fluorophores to the surface. The principle is schematically drawn in Figure 1. We fabricated polymer nanopatterns on a silica surface of a glass cover slip. Fluorescent dye molecules in a buffer are adsorbed onto the surface randomly. However, due to the high affinity of the dyes to the hydrophobic polymer surface, the dyes are adsorbed preferably onto the polymer surface. Actually, the affinity of the dyes to the silica surface is very low so that fluorescence signals from the single dye molecules were not detected from the silica surface. Therefore, bright fluorescence images of individual molecules are detected only from the polymer surface. Furthermore, the fluorescent dyes show the reversible switching between fluorescent and dark states in the presence of appropriate reducing agents (Figure S7, see Supplementary Material).

Polymer nanopatterns were fabricated using hydrophobic thermoplastic polystyrene by thermal nanoimprinting (Figure S2, see Supplementary Material). The silica surface of the cleaned cover slip was functionalized with 4-[(3-trimethoxysilyl)propyl]oxybenzophenone (TMPBP) (Figure S1, see Supplementary Material) to carry out thermal nanoimprinting. A toluene solution of polystyrene (PS, $M_w = 12,500, 6$ wt%, $T_g = 373$ K) was spin-coated on the TMPBP-coated surface. The resulting film had a thickness of 0.13 μm. The PS thin film and the TMPBP layer were cross linked by UV...
irradiation followed by annealing at 463 K for 10 minutes. In thermal nanoimprinting, a mold is pressed into a polymer thin film at a temperature that is higher than \( T_g \) of the polymer to fabricate nanopatterns on the surface of the polymer. Since \( T_g \) of PS is 373 K, we pressed fluorinated silicon molds into the sample at 423 K with 30 MPa (Table S-I, see Supplementary Material).\textsuperscript{20–23} The molds have 1:1 – 1:10 line-and-space patterns with 200 nm height and 100 – 300 nm line widths. The fabricated patterns (Figure S3, see Supplementary Material)\textsuperscript{20} were used for the measurements without further treatment. Drop-casted PS nanopatterns were prepared on a clean cover slip. A few microliters of a toluene solution containing PS (4 wt\%) was dropped on the cover slip (Figure S4, see Supplementary Material)\textsuperscript{20} and dried under vacuum for 12 hours.

Fluorescence images of individual fluorescent dye molecules were recorded by using a homemade single-molecule fluorescence imaging setup.\textsuperscript{24} The samples were set on an inverted microscope, and a sodium bicarbonate buffer (pH = 8.2) containing a fluorescent dye Atto655 (1 – 5 nM) and a reducing agent \( \beta \)-mercaptoethylamine (MEA, 0 – 100 mM) was dropped onto the fabricated patterns.\textsuperscript{25} The Atto655 dye was previously reported as an excellent fluorophore for the redox switching (Figure S7 and S8, see Supplementary Material).\textsuperscript{14, 20} A 647-nm line of an Ar-Kr ion laser was introduced into an inverted optical microscope. A focusing lens positioned at the backside port of the microscope focuses the excitation laser beam at a back focal plane of the objective lens (\( \times 100 \), NA = 1.3). A circularly polarized excitation light at the sample plane was obtained by using a Glan-laser prism and Berek compensator. The excitation power was in the range of 0.03 – 3.14 kW cm\(^{-2}\). The fluorescence signal was collected by the same objective, passed through a dichroic mirror, an emission filter. Fluorescence images were focused on an EM-CCD camera (7.5 – 30 ms exposure time). We recorded 10,000 – 25,000 fluorescence images for each sample.

Fluorescence images were analyzed by using routines written in Matlab.\textsuperscript{26, 27} The positions of individual Atto655 dyes in each image were localized by using 2D Gaussian fitting,

\[
z = z_0 + A \exp \left( -\frac{(x - \mu_x)^2}{2\sigma_x^2} \right) \cdot \exp \left( -\frac{(y - \mu_y)^2}{2\sigma_y^2} \right)
\]

where \( \mu_x \) and \( \mu_y \) are the centroid positions, respectively. \( A \) and \( z_0 \) are the Gaussian height and offset, and \( \sigma_x \) and \( \sigma_y \) are the standard deviation. Peaks in the fluorescence images were detected by setting a threshold, and the detected peaks were fitted by the 2D Gaussian. The peaks in the images which can be attributed to the noise were removed before the 2D Gaussian fitting. Super-resolution images were reconstructed based on the localized fluorescence spots. The images were reconstructed by selectively using the fluorescence spots which were fitted by appropriate fitting parameters (Table S-II, see Supplementary Material).\textsuperscript{20} The fluorescence spots which did not satisfy the conditions were removed from the analysis. The reconstructed images were plotted with 1 nm pixel size.

The experimental conditions were carefully optimized so that the duration of the fluorescence on-state is comparable to the frame rate of the imaging, which maximize the signal-to-noise ratio of the image and therefore can provide highest localization precision (Figure S11, see Supplementary Material).\textsuperscript{20} In our experiments, the highest localization precision and the peak detection efficiency were achieved with a 7.5 ms exposure time, 0.18 W cm\(^{-2}\) excitation power, and 0.1 M MEA (Figure S9 and S11, Table S-III, see Supplementary Material).\textsuperscript{20} We also optimized the concentration of Atto655 (5 nM) and MEA to achieve an efficient detection of the localized peaks while maintaining the background fluorescence at very low level. The localization precision was in the range of 24 to 35 nm for the nanoimprinted samples with those conditions.

We tested if our experimental principle works properly by measuring fluorescence images of individual Atto655 dyes deposited on PS surfaces prepared on the cover slip using drop-cast method (Figure S4, see Supplementary Material).\textsuperscript{20} The conventional fluorescence image, which is reconstructed by summing all the recorded raw images, exhibits bright fluorescence from the PS surface with relatively high background fluorescence from the silica surface (Figure 2(a)). In contrast, the reconstructed super-resolution image shows bright fluorescence from the PS surface with very low fluorescence background signal from the silica surface (Figure 2(b)). These results demonstrated that the super-resolution image is reconstructed exclusively by the bright fluorescence...
signals from the individual Atto655 dyes adsorbed on the PS surface (i.e. the dim fluorescence signals from the dye molecules on the glass surface in each image do not contribute to the image reconstruction). A comparison between the conventional fluorescence image (Figure 2(c)) and the reconstructed super-resolution image (Figure 2(d)) further demonstrate that the image resolution of the reconstructed image is better than 80 nm. It should be noted that the labeling density of the sample with the fluorophores is often the factor which limit the image resolution in the localization microscopy.28 In our experimental scheme, the image resolution is not limited by the spatial density of the fluorescent probe molecules since we detect the fluorescence signals from randomly adsorbed fluorescent molecules on the PS surface. In our experiments, $12 \times 12 \mu m^2$ reconstructed images with the image resolution better than 80 nm were obtained within 75 s imaging time. The image acquisition time to reconstruct super-resolution image is much faster than that based only on the adsorption kinetics of the dye (10 hours).19 The improvement is due to the optimum spatial density of the fluorescent dyes and their switching kinetics in our experiment by adjusting the concentration of Atto655 dye and MEA in the solution.

Next, we recorded fluorescence images of the nanoimprinted patterns. Figure 3 shows reconstructed super-resolution images of the imprinted PS nanopatterns with 100 nm line width with different line pitches. As shown in Figure 3(b) and 3(d), 1:2 and 1:10 concave line-and-space patterns with 100 nm line width can be clearly resolved in the reconstructed super-resolution images. The results demonstrated that the polymer line patterns smaller than the diffraction limit of the light can be measured by the super-resolution imaging based on the combination of selective adsorption and redox switching. The fluorescence intensity profile of the image in Figure 3(b) is depicted in Figure 3(g). The image resolution was estimated to be approximately 70 nm from the edge sharpness of the intensity profile. The intensity profile shows that the bright and dark lines have widths of roughly 200 nm and 100 nm, respectively (Figure 3(g)). Since the pattern was prepared using the mold with 100 nm-width convex line and 200 nm-width space, the result demonstrated that the mold pattern was accurately imprinted on the PS thin film. Figure 3(f) shows a reconstructed super-resolution image of the imprinted PS nanopatterns fabricated by a mold which has a 100 nm line width with 1:1 line pitch. Although the image contrast in the 1:1 line-and-space sample is not as
FIG. 3. Conventional fluorescence images (a, c, e) and super-resolution images (b, d, f) of the thermally nanoimprinted polystyrene patterns fabricated using the molds which have (a, b) 100 nm line width with 1:2 line-and-space, (c, d) 100 nm line width with 1:10 line-and-space, and (e, f) 100 nm line width with 1:1 line-and-space. (g and h) Fluorescence intensity profiles obtained from the super-resolution images depicted in Figure 4(d) and 4(f). The red lines represent the line pitches of the molds used for the sample fabrication.

high as that in the 1:2 line-and-space, the fluorescence intensity profile provides clear evidence that the 100-nm line and space fabricated by thermal nanoimprinting can be visualized (Figure 3(h)).

A thin polymer residual layer remains in the gap region of polymer nanopatterns fabricated by thermal nanoimprinting. This implies that the image contrast in our experiments on the imprinted polymer patterns arises from lower affinity of the Atto655 dye molecules to the residual PS layers in the gap region of the patterns. While the origin of the lower affinity remains elusive, chemical properties of PS could be modified in the extremely thin residual layer. The results point to the potential applicability of the method for evaluating the residual thin layers in the nanopatterns fabricated by thermal nanoimprinting.

In addition to the surface characterization, our experimental scheme also provides a convenient tool to analyze structural defect of the patterns. Dewetting which causes structural defects is one of the problems in fabricating polymer nanopatterns using the thermal nanoimprinting technique. Figure 4 shows a reconstructed super-resolution image of a PS nanopattern fabricated by a mold which has a 200 nm line width and 1:1 line-and-space. Several dark regions on the PS pattern are evident in the image. These dark areas correspond to the regions where dewetting of the PS thin film occurs. The result demonstrates that our experimental scheme provides a convenient non-invasive tool to examine the quality of the polymer nanopatterns fabricated by the thermal nanoimprinting technique. Such the tool would be particularly useful for the examination of mass manufactured thermal imprinted polymer nanopatterns.

In conclusion, we demonstrated that the super-resolution fluorescence localization microscopy based on the combination of the selective adsorption and redox switching of the fluorescent probe molecules provides a convenient tool to visualize nanostructures of polymer thin films with image resolution better than 80 nm. The method was proven to be a powerful tool to examine the polymer nanopatterns fabricated by thermal nanoimprinting. Since the experiment is based on single-molecule fluorescence imaging, the method can easily be combined with other single-molecule techniques,
FIG. 4. (a) Reconstructed super-resolution fluorescence microscopy image of the thermally imprinted polystyrene nanopattern fabricated by using a mold which has 200 nm line width and 1:1 line-and-space. (b) Schematic drawing of the nanopattern with structural defects which was measured in Figure 4(a).

such as three-dimensional orientation imaging.29 Such the experiment will further provide insight into the nanoscale anisotropic conformation of the polymer chains as well as an unusual polymer dynamics (e.g. glass transition behaviors) of the nanoimprinted patterns fabricated by thermal nanoimprinting.

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