Special Feature: Organic Materials

Review

Photoinduced Immobilization of Molecules on the Surface of Azobenzene Polymers: Principles and Application

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ABSTRACT This review consists of 5 topics, "Background Study: Nanofabrication", "Principles of Photoinduced Immobilization", "Application for Immunochips", "Immobilization Depending on the Azobenzene Moiety", and "Two Dimensional Arrangement and Area-selective Immobilization of Microspheres". In the "Background Study: Nanofabrication" and the "Principles of Photoinduced Immobilization", we introduce the principles of a newly created photoimmobilization technology using photoresponsive azopolymers and its background technology. The photoinduced immobilization is based on the deformation process on the surface of azopolymer. In the "Application for Immunochips", we introduce the immunochip which is made of immunoglobulin immobilized on the azopolymer surface. In the "Immobilization Depending on the Azobenzene Moiety", we try to understand the immobilization mechanism using different azopolymers. In the "Two Dimensional Arrangement and Area-selective Immobilization of Microspheres", we introduce a novel method to attain area-selective and controlled arrangement for colloidal crystals using the azopolymers.

KEYWORDS Azopolymer, Photo-isomerization, Surface deformation, Immobilization, Biomolecules, Microspheres, Arrangement of microspheres

1. Introduction

A large number of investigations concerning the photoisomerization of azobenzene derivatives have been reported so far because of their potential applications in optical recording media, holographic technology and optical components.[1-16] In particular, the so-called azopolymers, in which azobenzene derivatives are included in a polymer chain, have attracted attention because of phenomena that they undergo involving molecular reorientation and shape variation by mass transportation, which are induced by combinational movement of the azobenzene derivatives and the polymer chain.

This review introduces the photoinduced immobilization of microobjects onto the surface of azopolymers as a newly developed photoresponsive phenomenon of azopolymers. The size of the microobjects can extend over a wide range, from a few nanometers to several micrometers, and can include biological molecules such as DNA, enzymes, immunoglobulin or cells; and microspheres made of polymeric, inorganic or metallic materials. **Figure 1** shows the principle of photoinduced immobilization, which represents a very simple technique.[17-19] First, the microobject (immunoglobulin in the case shown in Fig.1) is set on the surface of the azopolymers, which is then photoirradiated from above. The surface of the azopolymer deforms in the presence of the immunoglobulin because the viscoelastic properties of azopolymer surfaces change during photoirradiation.

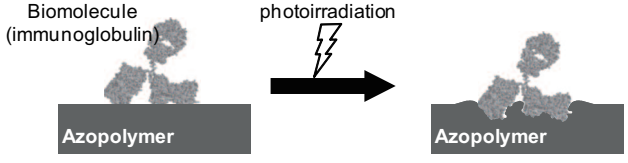

Fig. 1 Schematic illustration of the photoimmobilization of biomolecules (immunoglobulin) on the surface of an azopolymer. The surface of the azopolymer is deformed to the shape of the immunoglobulin after photoirradiation.[18]

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The deformation occurs such that it enfolds the immunoglobulin, and so the contact area between the surfaces of the immunoglobulin and the azopolymer increases. This deformation mainly occurs through the photoplastization of the azopolymer matrix owing to a trans-cis-trans isomerization cycle of the azobenzene moiety, as shown in **Fig. 2**. The surface of the azopolymer glaciates again and maintains the deformed shape after ceasing the irradiation, as shown in Fig.1 (right-hand side). As a result, the immunoglobulin is effectively immobilized on the surface of the azopolymer without chemical modification.

This novel method is useful for the immobilization of a variety of small particles such as charged proteins, negatively charged DNA, and hydrophobic polystyrene microspheres on azopolymer surfaces, and it has been shown that the immobilized biomolecules can maintain their higher order structure without damage to their functionality. This versatility in terms of immobilization is a significant advantage of this technique.

The photoinduced immobilization technique is closely related to the formation process used for surface relief gratings (SRG) because both phenomena are based on mass transportation of the azopolymer surface. The relief structure on the azopolymer surface is induced by the interference of the two coherent beams that are used for the irradiation, with the same periodic structure as the interference light, as shown in **Fig. 3**. Intensive studies for SRG formation have been reported since it was first developed in 1995.[2,8] Various deformed structures that can be induced by photoirradiation have been demonstrated in addition to SRG.[20,21] The surface deformation mechanism needs to be understood by considering not only photochemical phenomena involving the azobenzene moiety and the mobility of the polymer matrix but also by interactions of the irradiation light with electric fields. Therefore, a large number of researchers are making continued efforts to clarify the complex deformation mechanism.[22-25]

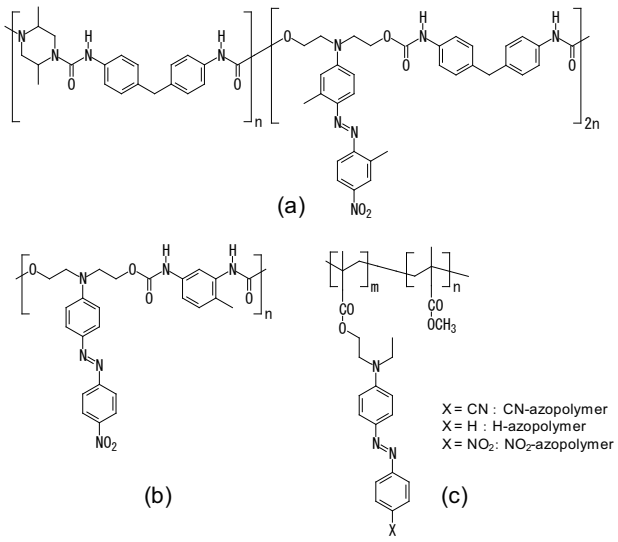

Fig. 2 The chemical structures of the azopolymers described in this article.

2. Background Study: Nanofabrication

We have recently switched the objectives of our research into SRG formation to now consider interactions between small objects and azopolymer surfaces, such as mass transportation and molecular reorientation, and have investigated photoinduced nanofabrication using a novel approach. We have discovered some interesting phenomena that are applicable to nanometer-scale fabrication by irradiating light onto microobjects set on azopolymer surfaces.[26-35] The resolution of recording or fabrication processes that is defined by light is determined by how narrowly the irradiating light can be focused, and, because of diffraction limits, in practice this equates to about half of the wavelength of the irradiating light. The use of the optical near field can overcome diffraction limits to reach nanometer-scale dimensions, and this has been expected to become a powerful tool for attaining nanometer-scale manufacturing capability.[36-38] This section

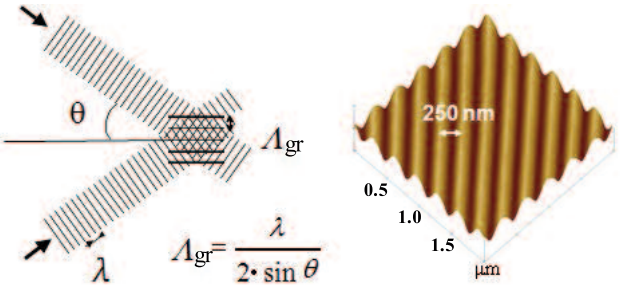

Fig. 3 Formation of SRG on an azopolymer, as generated by two-beam interference irradiation. The grating pitch, Λ_{gr} is determined by the wavelength and the incident angle of the irradiated light. The figure on the right exhibits a topographical image as measured by AFM.

demonstrates nanoscale deformation phenomena that can be induced by the optical near field when using microobjects set onto an azopolymer; these phenomena were investigated by our group and triggered our work into photoinduced immobilization.

Various sizes of microspheres (from tens of nanometers to several micrometers) made of various materials such as polystyrene or silica can be easily obtained, and it is possible to place these into an ordered arrangement because of the uniformity of their diameters. If a microsphere is irradiated with light, an optical near field is induced around the microspheres, as shown in **Fig. 4**. We selected polystyrene microspheres for use as the near-field light source and demonstrated a topographical nanostructure-patterning technique on the surface of an azopolymer. Nanostructured patterning was carried out as shown schematically in **Fig. 5** using the azopolymer shown in Fig. 2, which has a glass transition temperature of 145°C and a maximum absorption of 475 nm. A film of azopolymer with a thickness of 0.5 μm was spin coated onto a glass substrate from a pyridine solution, and the surface of the film before irradiation with light showed no regular structural periodicity. An aqueous solution containing polystyrene spheres was dropped onto the surface of the polymer films, and then the spheres were allowed to arrange themselves into a hexagonal-packed monolayer by a self-organization process. After drying the samples, they were irradiated from the side using a 488-nm Ar-ion laser with an intensity of tens of milliwatt per square centimeters to eliminate the influence of gravity, as shown in Fig. 5. After irradiation, the sample was washed with water and benzene to remove the microspheres and then the surface structure of the polymer film was observed using atomic force microscopy (AFM) and scanning electron microscopy (SEM). **Figure 6** shows AFM images of the resulting polymer surfaces, where hexagonal structures (500- and 100-nm microspheres) were directly transcribed onto the polymer surface as a series of indentations. A very fine indented structure was also observed in the case of 28-nm microspheres, although the arrayed structure was distorted. It can be concluded that these structures were induced by the optical near field around the polystyrene microspheres,

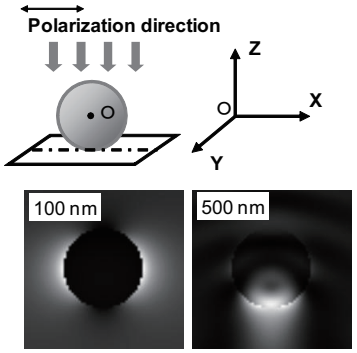

Fig. 4 Calculated distribution of the optical intensity on the X-Z plane of the polystyrene microspheres, 100 nm (left) and 500 nm (right). The bright region indicates a relatively strong intensity.

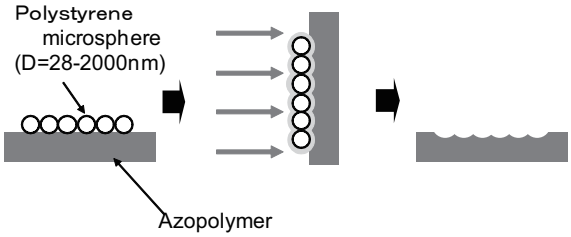

Fig. 5 Schematic representation of a nanopatterning process formed by using microspheres as the near-field source, showing the alignment of the microspheres (left), light irradiation (center), and the elimination of the microspheres (right).

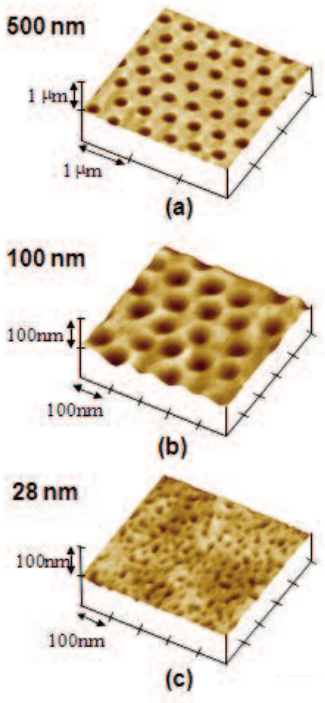

Fig. 6 AFM images of nanopatterned structures formed on an azopolymer surface using microspheres with diameters of (a) 500 nm, (b) 100 nm, and (c) 28 nm, respectively.[19]

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because the dimensions of the 100- and 28-nm diameter spheres are beyond the diffraction limit.

3. Principles of Photoinduced Immobilization

Figure 7 also shows SEM images of the resulting polymer surfaces (in addition to those shown in Fig. 6), including both the indented structures and the microspheres that remain after the process. These images confirm that the indentations are formed directly below the microspheres. Although the microspheres should obviously be removed for nanofabrication experiments, the removal of the microspheres from the azopolymer has been found to be difficult in the course of these studies. We looked at this problem from a different angle, which led them to proactively suggest that this phenomenon could be applied as an "immobilizing" technology. This section demonstrates a photoinduced immobilization technique for microobjects and introduces our recent experimental results.[17-19]

Polystyrene microspheres were therefore deliberately photoimmobilized onto an azopolymer surface for the first time. A monolayer of 1-μm microspheres that had been applied to the azopolymer surface was irradiated with a linear-shaped laser beam of 488-nm wavelength and 10- mW/cm^2 optical power density using a cylindrical lens for 5 min, as shown in **Fig. 8**a.

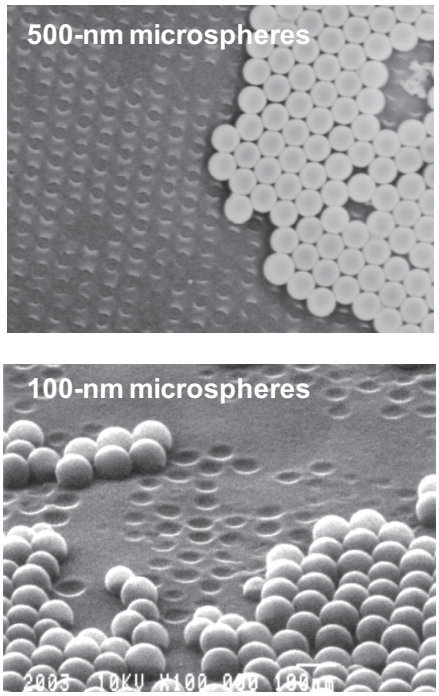

Fig. 7 SEM images of nanopatterned structures formed on an azopolymer surface formed by using microspheres with diameters of 500 nm (top) and 100 nm (bottom), respectively. The microspheres remain partially.[29]

The surface was washed in an ultrasonic cleaner and was then observed with an optical microscope, as shown in Fig. 8b. Only the microspheres in the linearly irradiated region were strongly immobilized, despite the ultrasonic washing and the relatively large size of the microspheres. DNA molecules were then selected as a potential target material for the immobilization of biological macromolecules. An aqueous solution of 1-mg/mL λ-DNA was spotted onto the surface of an azopolymer and covered with a cover glass, where the λ-DNA was stained with a fluorophore (YOYO-1 iodide, Molecular Probe) in advance, and the surface was then irradiated with the same linearly shaped laser beam for 5 min. The surface was washed for 5 min in an aqueous solution and was then observed using a conventional fluorescence microscope. Fig. 8 confirms that the labeled λ-DNA was only immobilized in the irradiated region. The same experiment using green fluorescent protein (GFP) also indicates the immobilization of protein in the irradiated region.

In this way, we could demonstrate that an azopolymer can capture micrometer- to nanometer-scaled microobjects, including synthetic polymers and biological molecules, on the photoirradiated area. The provision of an immobilization process is one of the

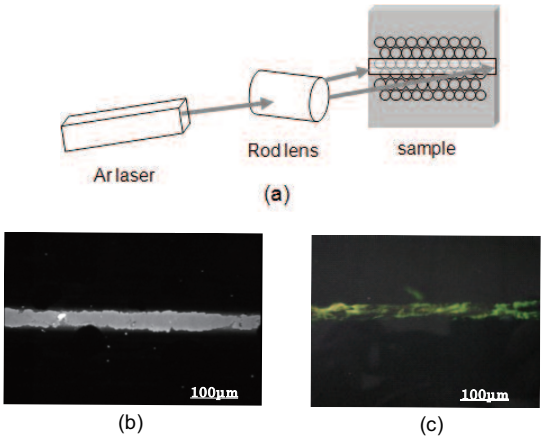

Fig. 8 (a) Experimental setup for patterned immobilization using a linearly shaped laser beam. (b) Dark field optical image of immobilized polystyrene microspheres with diameters of 1 μm on the surface of an azopolymer. (c) Fluorescence image of the immobilized λ-DNA molecules.[17]

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most essential processing steps that is required to obtain practical biomolecule carriers such as biosensors, bioreactors, or biochips. Therefore, a large number of immobilization techniques have already been developed for biological molecules, in which the molecules are immobilized on a carrier using covalent bonds,[39] ionic bonds,[40] physical adsorption,[41] cross-linkage of the biomolecules[42] or microencapsulation.[43] Chemically induced immobilization methods require optimized processes depending on the structures and properties of the individual biomolecules, which in turn require some complicated procedures;[44] yet these techniques are widely used. Azopolymers can immobilize microobjects that possess a variety of surface characteristics, including negatively charged DNA, charged proteins, and hydrophobic polystyrene. The characteristics of the azopolymer make it possible to immobilize a wide variety of biological molecules on the same substrate through a one-step photoirradiation process.

To take advantage of the functionality of these biomolecules, identifying an immobilization process that does not lead to deactivation of the molecules is important. In particular, biomolecules such as proteins show sensitive behavior in terms of changes in environment, as shown by the denaturing of proteins when the surrounding temperature increases even slightly. Since it is possible that damage to biomolecules following photoinduced immobilization could trigger functional degradation, we first examined the activity of an immobilized enzyme. An aqueous solution of 1-mg/mL bacterial protease (subtilisin; 27.5 kDa, Sigma) was spotted onto the surface of an azopolymer, and the surface was irradiated with a laser beam of 488-nm wavelength and 80-mW/cm^2 optical power density for 5 min to immobilize the enzyme. As a control experiment, a similar specimen was prepared without photoirradiation. The activity of the subtilisin was verified as the hydrolysis of the artificial substrate (*tert*-butoxycarbonyl-Gly-Gly-Leu-*p*-nitroanilide, Mw = 465.5, Merck). The artificial substrate solution was spotted onto the azopolymer surface in the same area where the subtilsin had been immobilized, and then the specimen was maintained at 37°C and 85% relative humidity for 1 h. The hydrolysis of the artificial substrate was determined spectroscopically by immediately measuring the absorbance of the reactant at a wavelength of 410 nm. The conversion ratio of the reaction was ~10% for the subtilsin-immobilized sample, whereas it was ~1% for the control sample (without photoirradiation). These results clearly show that biomolecules immobilized on an azopolymer surface can maintain their enzyme functionality during and after the immobilization process.

Next, we investigated how deformation of an azopolymer surface can be induced by biomolecules as well as by microspheres. A phosphate–buffered saline (PBS) solution containing Cy-5-linked immunoglobulin, IgG, was spotted onto the surface of an azopolymer. After evaporating the solution, the surface was irradiated for 30 min with light of 470-nm wavelength and 10-mW/cm^2 optical power density from an array of blue light-emitting diodes (LEDs) and then the surface was washed for 30 min with PBS containing 0.01-wt% Tween 20 as a nonionic surfactant. The amount of immobilized IgG was confirmed by the fluorescence intensity of the spot, and the minimum detectable amount was 10 pg. Next, a surface image was obtained by tapping mode AFM (Digital Instruments, Dimension 3100) using a sharp silicon cantilever with a tip radius of < 5 nm. In **Fig. 9**a, the azopolymer surface is covered with a layer of small granulated particles of 10–30 nm in diameter and ~8nm height, where the height was estimated from the defects and the edge of the layer. The sizes of the particles were nearly equivalent to one subunit of IgG (~10 nm), considering that the image includes AFM tip convolution artifacts. The layer is so flat that the

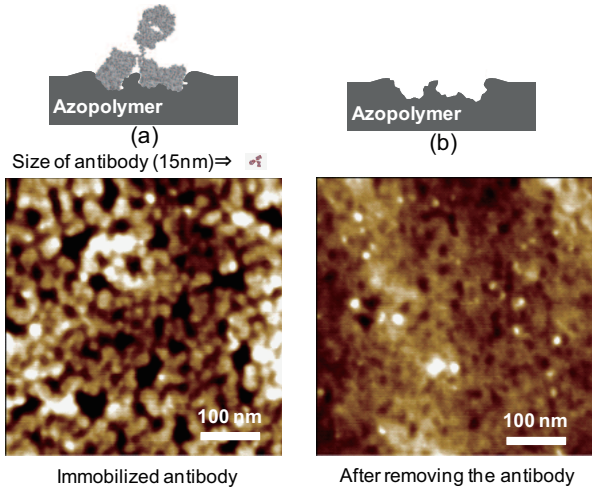

Fig. 9 AFM images obtained from the photoimmobilization process. (a) The surface was observed after the photoimmobilization of immunoglobulin. The real-size immunoglobulin is shown. (b) The surface was observed after an elimination process using 2-wt% sodium dodecyl sulfate (SDS) solution.

IgG monolayer is believed to be located on the azopolymer surface. The sample was subsequently washed with PBS containing 2-wt% sodium dodecyl sulfate to remove the IgG. After confirming that the fluorescence from the spot had disappeared, another AFM image was obtained, which is shown in Fig. 9b. Dents of ~20 nm diameter and 2-nm deep can be observed on the surface. In contrast, no dents were formed on the azopolymer surface where no IgG was deposited. Comparing these images, the dents formed on the surface in Fig. 9b are considered to mirror the surface shape of the IgG. These findings lead to the conclusion that the azopolymer surface "recognizes" each molecular shape and deforms along the contours of the biomolecules during photoirradiation, as shown in Fig. 9b. The results also suggest that the increase in contact area between the azopolymer and biomolecules after photoirradiation restrains desorption from the surface.

As described later, we also examined the possibility of antigen-antibody reactions on the surface of the azopolymers. PBS solutions of human serum albumin and bovine serum albumin with different concentrations were spotted onto azopolymer surfaces according to the layout shown in **Fig. 10**a. After evaporating the spotted solution, photoirradiation was performed over the entire surface using an array of blue LEDs and was then washed with PBS containing 0.01 wt% Tween 20 to remove the un-immobilized albumins. After drying the sample again, the obtained sample was reacted with anti-HSA monoclonal mouse antibodies, and then the washed sample was reacted with Cy-5-labeled antimouse polyclonal goat antibodies as a secondary antibody to detect albumins. Fluorescence emission was only observed from the spot on which the HSA had been immobilized, as shown in Fig.10b, which means that a reaction that was selective to the HSA had occurred on the antigen-immobilized surface of the azopolymer and then was detected. Next, the sample was treated with hydrochloric acid to separate the antibodies from the HSA. We confirmed that no fluorescence emission could be observed from the substrate after the HCl treatment. We then repeated the same immunoreaction on the treated sample, such that almost the same fluorescence image as that described earlier was again obtained, as shown in Fig.10b (center and right). This demonstrates that biochips fabricated on an azopolymer surface can be reused.

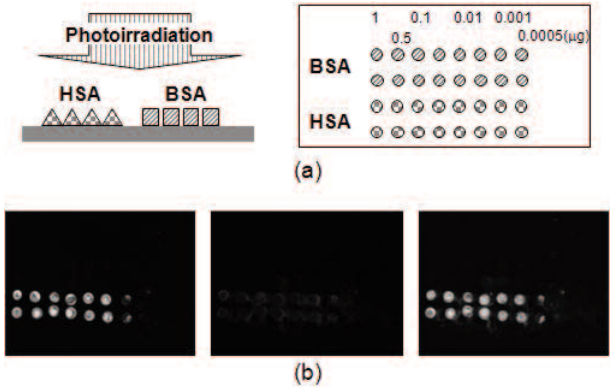

Fig. 10 (a) Layout of the spotting process using solutions of HSA and BSA with different concentrations on the surface of the azopolymer. (b) Fluorescence images observed after various treatments. The left-hand side image is as observed from the spotted sample after photoimmobilization, washing, and immunological reaction. Center image is as observed from a sample treated with hydrochloric acid following the first observation. The same immunological reaction was repeated after the acid treatment, when the right-hand side image was observed.

4. Application for Immunochips

In the next phase, we applied the photoimmobilization method to try to obtain protein chips, and more specifically, an immunochip. Enzyme-linked immunosorbent assay (ELISA) systems are commonly used [45,46] as a popular method for detecting small amounts of protein in sample solutions such as serums. Although the ELISA system is an excellent method for detection of proteins, it still has some problems; it is comparatively expensive, and it is difficult to detect proteins when small quantities of the sample solution are used. We might be able to realize an immunochip that could act as a micro-ELISA system with the capability to deal with small quantities of sample solutions if they could succeed in immobilizing the antibodies on the substrate. Such an immunochip could be used to measure multiple target proteins on the same substrate simultaneously, so it has the potential to become a novel method of replacing conventional ELISA systems in the fields of biochemical indexes, diagnostic agents, and clinical inspection.[47] One can say that the photoimmobilization method is one of the most promising prospects for immunochip applications because it can provide immobilization on the substrate surface irrespective of the surface states of the

biomolecules.

We first examined specific reactions of photoimmobilized antibodies on azopolymer surfaces for the immunochip application. Solutions of anti-goat antibodies (left-hand side) and anti-rabbit antibodies (right-hand side) were spotted onto an azopolymer surface at different concentrations, the layout of which is shown in **Fig.11**a. After photoimmobilization and washing, the sample was reacted separately with Cy-5-labeled antigens (goat IgG and rabbit IgG). The anti-goat IgG antibody recognized goat IgG when Cy-5-labeled goat IgG was introduced onto the sample, whereas, anti-rabbit IgG recognized rabbit IgG when Cy-5-labeled rabbit IgG was introduced, as shown in Figs. 11b and 11c. The specific reactivity of the antibodies was realized by fixing photoimmobilized antibodies on the surface of the azopolymer. We also examined the preservation stability of the photoimmobilized antibodies. Although the reactivity of the antibodies dropped away over a period of 10 days when they were stored at room temperature, it was maintained for ~2 months when stored at 4°C. This result is acceptable in terms of commercial viability, though further increases in stability would be preferable.

We next examined the sensitivity for the immunochips. An immunochip usually has a two-dimensional (2-D) surface, so the detection limit for antigens can be estimated from the amount of immobilized antibodies that are present. It was difficult to increase the sensitivity of a detection system that uses a photoluminescence probe. However, we succeeded in obtaining higher sensitivity for an immunochip in which we adopted a chemiluminescence detection system using an enzyme reaction. We selected adiponectin, which is a biologically active agent that is excreted from adipose cells and which prevents arteriosclerosis, as the intended biological marker, and we tried to assay it using an enzyme sandwich immunoassay on the azopolymer surface. Anti-adiponectin antibodies were photoimmobilized on the azopolymer surface and then a solution including adiponectin was reacted on the fabricated immunochip. Subsequently, the sample that had captured the adiponectin on its surface using the immobilized antibodies was treated with biotin-labeled anti-adiponectin antibodies (first sandwich process) and then with alkaline phosphatase (ALP)-labeled streptoavidin (second sandwich process).[48] After introducing the chemiluminescent substrate onto the surface, the intensity of the chemiluminescence was measured to determine the concentration of adiponectin. We measured the intensity of the chemiluminescence against the concentration of adiponectin using samples with predetermined concentrations. **Figure 12** exhibits the calibration curve that was obtained in the region of low concentration, and it shows that a linear relationship exists between intensity and concentration. Adiponectin in a sample solution can be detected down to a concentration of at least 0.1 ng/mL, which is almost the same sensitivity as that obtained with ELISA. A conventional ELISA system and the IgG chip system were compared using mouse adiponectin

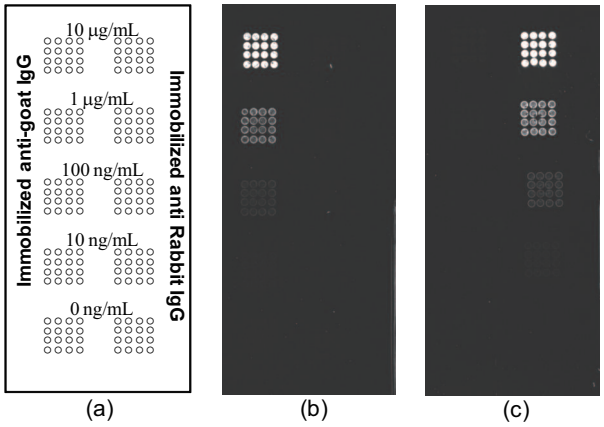

Fig. 11 (a) Layout of a slide that was spotted and immobilized using the anti-goat IgG rabbit antibody and the anti-rabbit IgG goat antibody. (b) Fluorescent image after incubation of a Cy-5-labeled goat IgG. (c) Fluorescent image after incubation of a Cy-5-labeled rabbit IgG.

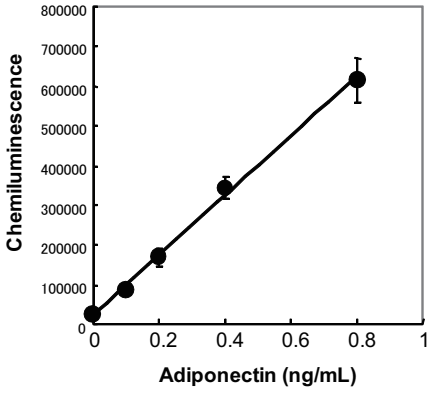

Fig. 12 Calibration curve for quantifying mouse adiponectin. Each error bar indicates the standard deviation for each data point.

of culture supernatant. **Figure 13** shows the correlation between the immunochip and conventional ELISA. A high degree of correlation exists ($r^2 = 0.97$) indicating that the use of an immunochip with an azopolymer film is a promising candidate for practical use.

5. Immobilization Depending on the Azobenzene Moiety

This section compares the photoinduced immobilization of IgG on two types of azopolymers (shown in Fig. 2) bearing various concentrations of 4-amino-4'-cyanoazobenzene (CN-azopolymer) or amino azobenzene (H-azopolymer). CN-azopolymer and H-azopolymer contain a push-pull-type azobenzene and an amino azobenzene, respectively.[49] These azobenzenes have different adsorption spectra, and they exhibit different deformation and immobilization features under photoirradiation. Therefore, information can be obtained about the photoimmobilization mechanisms by comparing these two types of azopolymers. First, we examined the relationship between immobilization efficiency and the indented depth with respect to the photoirradiation time and the specific azobenzene moiety. Second, we compared the relationship between immobilization efficiency and chemical structure, and elucidated how this correlated with the photoisomerization properties and the retention rate of immobilized antibodies.

The photodeformation capabilities of the CN-azopolymer and the H-azopolymer were examined by determining the depth of the indents formed by polystyrene microspheres under LED irradiation. After photoirradiation and removal of the microspheres, regularly arranged indented patterns formed by the microspheres were observed on the surfaces of the azopolymers. The depths of the indents were plotted as a function of irradiation time for several kinds of azopolymers, as shown in **Fig. 14**. The indent depths in the azopolymer increased with increasing irradiation time. The depths of the indents saturated and reached a maximum after 30 min of photoirradiation for each of the azopolymers. The saturated depths were lowest in those azopolymers with the lowest content of azobenzene moieties. These results indicate that the photoresponsive moiety plays an important role in inducing photodeformation and that the indent depth is related to the content of the azobenzene in the azopolymers. There were no differences in photodeformation capabilities between the CN- and H-azopolymers, even though they contained different types of azobenzene. These results show that H-azopolymers could exhibit immobilization capabilities similar to CN-azopolymers, despite the differences in their chemical structures.

Photoimmobilization of IgGs was achieved by performing photoirradiation for 30 min to examine the efficiency of the immobilization process. The relative efficiencies of the photoimmobilization processes on the different azopolymers were plotted as a function of their azobenzene contents, as shown in **Fig. 15**. The immobilization efficiencies of both the CN- and H-

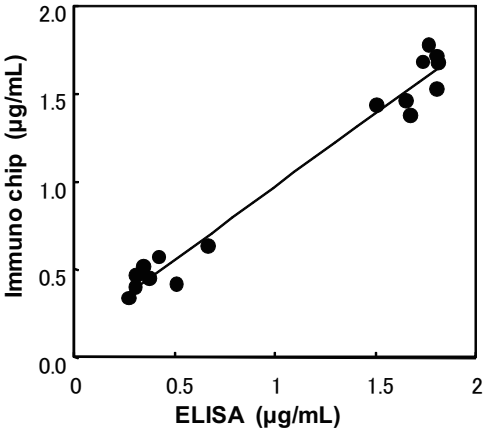

Fig. 13 Correlation between the two methods, ELISA and immunochip, for quantifying mouse adiponectin at a level of 16 culture supernatant.

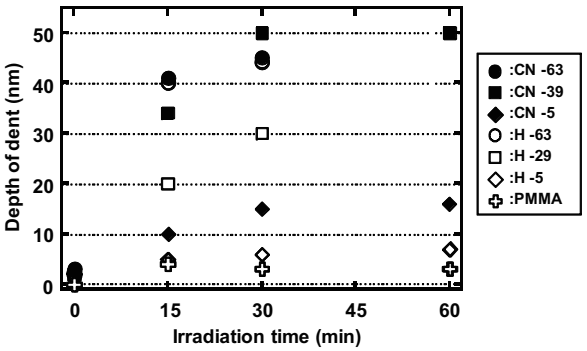

Fig. 14 Changes in the depth of dent as a function of photoirradiation time for CN-azopolymers (solid figures) and H-azopolymers (open figures). The numbers show the weight content of the azo moieties. The open crosses show the control experiment using PMMA.[18]

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azopolymers increased with azobenzene content up to ~30 wt% and then became saturated, although the saturated values were different between the CN- and H-azopolymers. This result indicates that an azobenzene moiety that exhibits photoisomerization is essential to immobilize IgGs on azopolymers when using a photoimmobilization process.

Next, we confirmed the relationship between the immobilization of IgG and the deformation efficiency of the azopolymer. The relative immobilization efficiencies were plotted as a function of indent depth, as shown in **Fig. 16**. Incremental changes in the immobilization efficiency were observed by increasing the depth of the indents. However, the immobilization efficiency of the H-azopolymers was higher than that of the CN-azopolymers across the whole range, and this is also shown in Fig. 15. This difference shows that the degree of photoimmobilization is not only affected by the deformation capability but is also a property of the surface of the azopolymer and is related to the chemical structure of the azobenzene that is incorporated in the azopolymer. Whitesides and coworkers have also reported that immobilization is influenced by the properties of the surface.[50]

Therefore, we attempted to examine the efficiency of the adsorption of antibodies onto the surfaces of the azopolymers. The adsorption efficiency for antibodies was determined by the efficiency of the immobilization process without photoirradiation. The value that was obtained for the CN-39 azopolymer was used as a baseline for the relative efficiency of the adsorption of antibodies. The relative adsorption efficiency of Cy-5-IgG on the azopolymers is shown in **Fig. 17**. The relative adsorption efficiency was lower than the relative photoimmobilization efficiency, which also demonstrates that photoirradiation is an important process if one wishes to firmly immobilize most of the antibodies. The relationship between the relative adsorption efficiency and the relative immobilization efficiency of Cy-5-IgG on each of the azopolymers showed that they were almost the same. Although the adsorption efficiency of the H-azopolymer was slightly

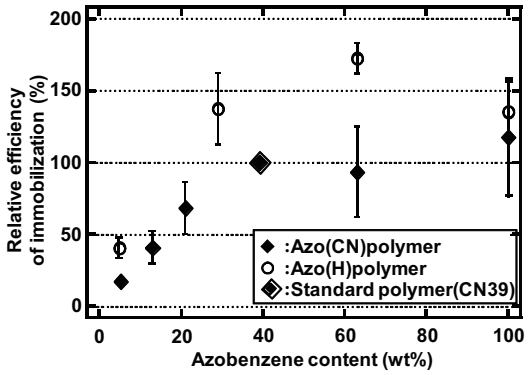

Fig. 15 Dependence of the relative immobilization efficiency of Cy-5-labeled antibodies on azopolymer content. The solid diamonds and open circles represent CN- and H-azopolymers respectively. CN-39 was used as the standard polymer, as shown by the solid diamond in an open diamond.[18]

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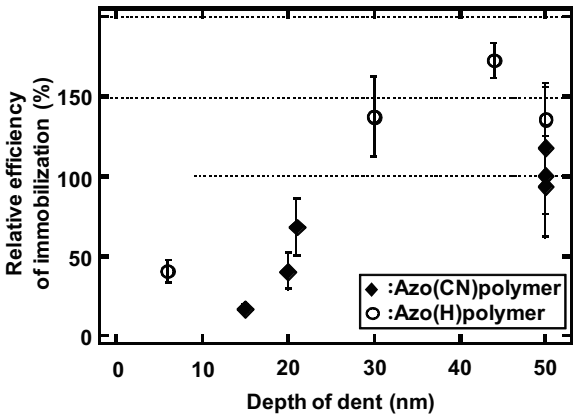

Fig. 16 Relationship between the depth of dent and the relative immobilization efficiency of Cy-5-labeled antibodies. The solid diamonds and the open circles represent CN- and H-azopolymers, respectively.[18]

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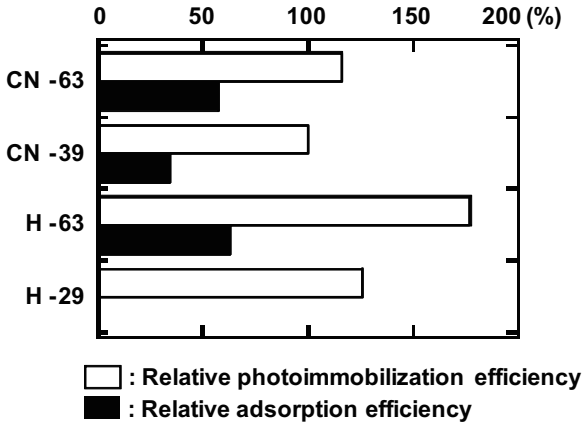

Fig. 17 The relative photoimmobilization efficiency and the relative adsorption properties of Cy-5-labeled antibodies on the azopolymers.[18]

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higher than that of the CN-azopolymer, the difference was not sufficient to explain the differences in the immobilization efficiencies.

We conjectured that the adsorption properties of the azopolymers may have originally been equal and that the differences in the photoimmobilization efficiencies could be generated by the photoirradiation process. To characterize these photoprocesses, the adsorption capabilities of the azopolymers after photoirradiation should be considered. Azopolymer films carrying Cy-5-IgG that had been immobilized by photoirradiation were held in stirred PBS to remove the antibodies from the surfaces of the azobenzene films. All of the H-azopolymers exhibited much better retention rates than the equivalent CN-azopolymers.

There are remarkable differences between the CN- and H-azopolymers in terms of the photoisomerization phenomena that occur on the azobenzene moieties. We also confirmed a stable cis state in films of the H-azopolymer by measuring the changes with time of the absorption capability of the films using a probe light during and after photoirradiation. In the case of the CN-azopolymer films, the cis state was almost totally backisomerized 30 min after the light was turned off. However, the H-azopolymer films showed a stable cis state and the relaxation time for the cis state was >160 h, which was the time calculated from the recovery curve of the absorbance of the trans state.

It was concluded that the photoimmobilization capability is not only controlled by photodeformation but also by the retention capability, depending on the chemical structure after photoirradiation. Changes in the adsorption properties after the photoimmobilization process are an interesting phenomenon in terms of dynamic changes in the surface properties for adsorption.

6. Two-dimensional Arrangement and Area-selective Immobilization of Microspheres

Photonic crystals exhibit interesting physical phenomena and enable the manufacture of novel optical devices.[51] Although a large number of studies directed towards fabricating photonic crystals for photonic applications have been reported, 2-D photonic crystals have attracted a great deal of attention because they provide a more suitable structure for integrated photonic circuit applications such as waveguides,[52] channel add/drop filters,[53] and directional couplers.[54] Several self-assembly approaches for obtaining 2-D colloidal crystals have been reported, such as processes that use capillary force,[55] electrophoretic migration,[56] and Langmuir-Blodgett films.[57] Although self-assembled 2-D colloidal crystals are of great interest, several problems remain to be solved before the technique can be applied for practical use; for example, there are the problems of polycrystalline domains, defects, and multilayers in crystals and difficulties associated with designing the arrangement and the intended defect structure. There have been several reports of crystallization on periodically patterned templates for self-assembled 2-D colloidal crystals.[21] Among these, relief structures fabricated on azobenzene-containing polymer films by photoirradiation with an interference light pattern are one of the most promising approaches for easily forming templates.[21] Additionally, to apply colloidal crystals to optical devices, it is important to have some form of selective arrangement. An area-selective arrangement of colloidal spheres has been achieved by skillfully managing the surface properties.[58] However, no simple method of simultaneously attaining an area-selective and controlled arrangement for immobilized 2-D colloidal crystals has yet been developed.

This section proposes a novel and simple method of solving these problems using two photoinduced phenomena of azobenzene-containing polymers. One is a well-known photodeformation process, which provides an intended template for arranging the microspheres. The other is the newly discovered photoinduced immobilization process described in the preceding sections. First, an indented template with a 2-D lattice structure is formed by repeatedly irradiating with a pattern generated from interfering light beams. Second, colloidal spheres are crystallized on the template structure. Finally, area-selective immobilization provides a 2-D photonic crystal slab that includes waveguides or defects for controlling the light waves.

First, the simple area-selective immobilization of colloidal spheres on an azobenzene-containing urethane polymer was demonstrated. An aqueous solution containing 1-μm diameter polystyrene microspheres (Duke Scientific Corp., 5100A) was dropped onto the flat surface of the polymer film, and the solution was then sucked up with a pipette to form self-organized colloidal crystals. Area-selective photoimmobilization was performed by moving the irradiation site using a confocal laser-scanning

microscope with a wavelength of 488 nm (Olympus, OLS1100). The sample was washed with ultrasonic cleaning to remove any un-immobilized and multilayered spheres, and was then examined with a microscope after drying. **Figure 18** shows a checkerboard design of 2-D colloidal spherical crystals immobilized on polymer films. The microspheres were immobilized only in the irradiated region. Photoinduced immobilization provides a simple method by which a patterned monolayer of spheres can be easily and selectively immobilized on the substrate.

Second, the implementation of designed arrays of 2D colloidal crystals immobilized on a polymer was demonstrated. Two sets of gratings were formed on a polymer film by irradiating with an interference pattern generated from an Ar-ion laser beam. Two kinds of cross-grating indented templates with 2-D tetragonal and hexagonal lattices were fabricated on the polymer, as shown in **Fig. 19** (left-hand side). The self-assembly of colloidal crystals on the polymer was carried out by using the dipping method. This template film was immersed in an aqueous solution of microspheres and was then drawn up at a rate of 1mm/min. The samples were irradiated with an Ar-ion laser (488 nm) to immobilize the microspheres and were then ultrasonically cleaned. Monolayered tetragonal and hexagonal arrangements of the microspheres were obtained from the corresponding templates, as shown in Fig. 19 (right-hand side), though there are a few defects present and also a multilayered area. The hexagonal arrangement of colloidal crystals on the template had an approximately unity structure without being multidomain, compared with the self-assembled arrangement on the flat film, as shown in the small image in Fig. 19b. The combination of both the arrangement on the template and the photoinduced immobilization provides an excellent method for fabricating large area 2-D colloidal crystals with controlled lattices and low defect densities.

Finally, the area-selective photoimmobilization of 2-D arrays of colloidal spheres on templates formed in azobenzene-containing polymer films was demonstrated. Area-selective photoimmobilization was combined with the process whereby microspheres can be arranged on a template. Similar to the processes described earlier, area-selective photoimmobilization was performed by setting the template containing the

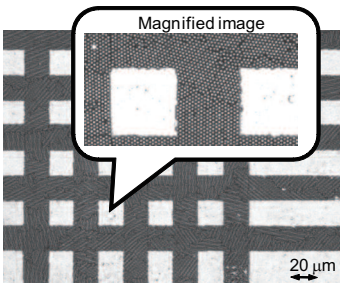

Fig. 18 Microscope image of area-selective immobilized 1-μm diameter polystyrene microspheres on the flat surface of an azobenzene-containing polymer film. The irradiated area was controlled by moving the sample stage.[35]

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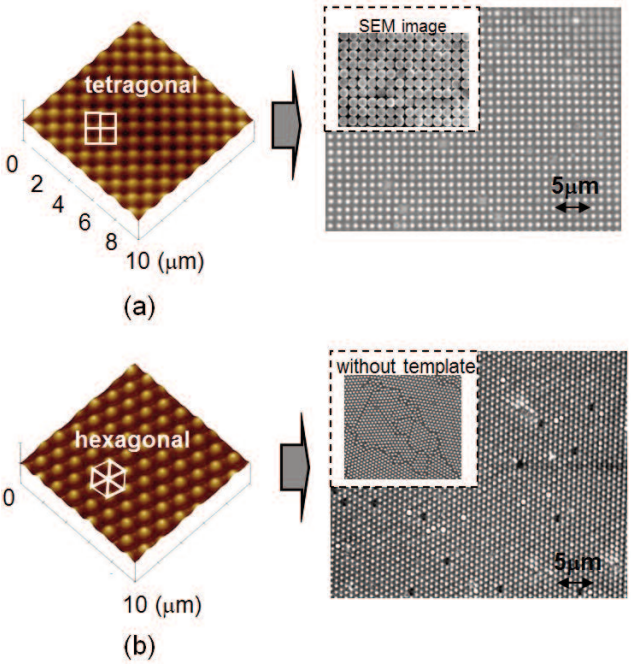

Fig. 19 AFM images of indented templates (left-hand side) fabricated by repeated irradiation of an azopolymer with an interference light pattern. The lattice structures are (a) tetragonal and (b) hexagonal. The structures were controlled by the incident angle of the interfering light pattern and the rotation angle in the plane before the second irradiation. Microscope images of photoimmobilized 2-D colloidal crystals (right-hand side) after arrangement on the templates. The small image in (a) shows SEM images of the photoimmobilized tetragonal arrangement, and the small microscope image in (b) shows self-assembled colloidal crystals with a multidomain structure on the flat surface.[35]

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array of spheres onto a moveable sample stage. After an ultrasonic wash, the 2-D colloidal crystals were examined. **Figure 20** shows waveguide-type 2-D crystals with a hexagonal arrangement. Several other types of patterns were also examined, such as bending waveguides and cross-type waveguides.

To conclude, we have succeeded in forming 2-D photonic crystal slabs that include deliberately introduced defects or waveguides. This was accomplished by a newly proposed method comprising two processes, the arrangement and the area-selective immobilization of microspheres. These processes were made possible by utilizing two different photoresponsive properties of azobenzene-containing polymers; namely, photodeformation and photoimmobilization.

7. Summary

Among a number of photochromic materials, azobenzene derivatives have a distinguished property that induces a spatially extended change in form because of their geometric isomerization. The SRG-related investigations inspired by Natansohn and Tripathy groups take an advantage of this characteristic property effectively. We have introduced the principles of a newly created photoimmobilization technology using photoresponsive azopolymers and reviewed its application to the fabrication of immunochips and the arrangement of microspheres. Although there have been a number of investigations into the photoinduced functionality of azopolymers, further interesting problems remain to be solved from the viewpoint of basic research.

We consider two important factors in our approach; the deformation process that is induced by interaction between the microobjects and the azopolymer surface and the immobilization process that resulted from the deformation process. In the first case, a novel aspect should be added, such as surface energy, radiation force, and intermolecular force as the moving force. The established knowledge accumulated by investigations concerning SRG, photoinduced orientation, and photoisomerization is obviously important to understand the mechanism. The photoresponse process would be controlled by the interaction, and this interaction would be affected by photoresponse conversely. In latter case, the research field could enlarge by considering how to use the immobilized surface such as biochip and bioreactor. In particular, the interaction from the position of adsorption in biological engineering should also be reconsidered. The interaction concerning the adsorption would involve the controlling the arrangement and orientation of microobjects. Not only the behavior and application of azopolymer by itself but also the relevant interaction with microobjects on the surface should be considered. We believe that there is further potential to develop this technique more widely; for instance, applications involving biological molecules are an intriguing field with high potential for future growth, including aspects of molecular orientation and the formation of organized structures. This novel approach would serve as a stepping stone to further development.

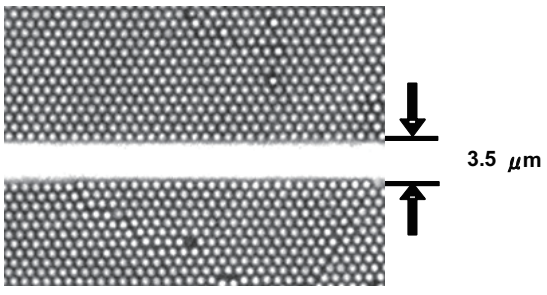

Fig. 20 Area-selective photoimmobilized 2-D colloidal crystals (waveguide-type pattern).

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