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Amo, Hikaru
Kanki, Yusuke
Fujii, Miku
Morita, Kenta
Maruyama, Tatsuo

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Clickable Plastic Surfaces with Controllable Azide Surface Density

Hikaru Amo,[†] Yusuke Kanki,[†] Miku Fujii,[†] Kenta Morita,^{†,‡} Tatsuo Maruyama^{†,‡,}*

[†] Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe

University, 1-1 Rokkodaicho, Nada-ku, Kobe 657-8501, Japan

[‡] Research Center for Membrane and Film Technology, Kobe University, 1-1 Rokkodaicho, Nada,

Kobe 657-8501, Japan

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Corresponding Author

*tmarutcm@crystal.kobe-u.ac.jp

ORCID

Tatsuo Maruyama: 0000-0003-2428-1911

ABSTRACT

This study investigates the surface functionalization of plastic substrates through dip-coating in azide-functionalized polymer solutions, followed by a click reaction (i.e., strain-promoted azide-alkyne cycloaddition). Acrylic, poly(ethylene terephthalate) (PET), and nylon substrates are dip-coated with a series of polymers containing various azide groups grafted onto the poly(methyl methacrylate-*co*-hydroxyethyl methacrylate) backbone to examine structural effects on the surface density of clickable azide groups. X-ray photoelectron spectroscopy and fluorescence spectroscopy confirm the subsequent click-immobilization of cycloalkyne-tagged fluorescein, which is quantified to calculate the surface density of clickable azide groups. The further investigations demonstrate that the surface density of azide groups can be controlled by manipulating the polymer ratio during dip-coating, thus enabling the preparation of a linear surface gradient in terms of azide group density. Finally, the microcontact printing (μ CP) method is employed to pattern the functionalized surfaces and quantified the functional molecules immobilized on the surface by μ CP. This study highlights the adaptability of click chemistry and polymer coating techniques for the advanced functionalization of plastic surfaces for materials science and engineering applications.

1. INTRODUCTION

Plastics are among the most commonly used commercial materials owing to their low cost, light weight, overall versatility, and applicability in daily commodities, wearable electronics, medical apparatus, and vehicles. Surface functionalization of plastic materials is essential for enhancing their utility in these areas. Chemical approaches to surface modification can impart new properties, such as hydrophilicity,^{1,2} antimicrobial activity,³ improved adhesion properties,^{4,5} selective molecular recognition,⁶ catalytic abilities,⁷ and optical and electronic properties.⁸ Such functionalization expands the practical scope of plastics significantly.

Considering that commodity plastics are generally inert, it is necessary to introduce reactive sites on their surfaces to impart the desired functionality. To date, various methods have been developed to introduce reactive sites, including plasma treatments,⁹⁻¹¹ chemical grafting,¹² chemical vapor deposition,^{13,14} and coating with functional polymers.^{15,16} In particular, coating a plastic substrate with a functional polymer is simple and versatile approach that can be applied anywhere. Our group previously employed a dip-coating method using a solution of functional polymers to functionalize the surfaces of plastic substrates; the reactive functional groups in the polymer were effectively displayed on the outermost surface of the plastic according to surface segregation.¹⁷⁻¹⁹

Recent advancements in “click chemistry”²⁰⁻²³ have demonstrated its applicability outside of organic synthesis, i.e., for the surface functionalization of solid materials. Among several types of reactions in click chemistry, azide-alkyne cycloaddition is one of the most typical.^{24,25} The

cycloaddition reaction proceeds rapidly and selectively under ambient conditions (e.g., in water and air) with high yields and no byproducts. The simplicity and reliability of the reaction facilitate its application for diverse molecules and materials. For example, Lahann et al. reported a series of studies on chemical vapor deposition (CVD) with various substrates to introduce alkyne groups on their surfaces.^{14, 26-28} They also demonstrated that microcontact printing (μ CP) was effective for the alkyne-displayed surface. Additionally, Reinhoudt et al. described the preparation of azide-immobilized Si (or SiO₂) surfaces and demonstrated that μ CP using alkyne-containing ink enabled effective azide-alkyne cycloaddition without a catalyst in the confined environment of an azide-displaying substrate surface.²⁹ These studies indicated that this click reaction could serve as a useful chemical approach for surface functionalization. Although numerous studies have explored click reactions on solid substrates, there have been limited investigations regarding the surface densities of clickable sites and the factors that control such aspects.³⁰⁻³² To our knowledge, there have been no reports quantifying the conversion of clickable surface sites via μ CP.

Our group has developed a method for quantifying the surface densities of amino groups,^{17, 33} carboxy groups,¹⁸ and cycloalkyne groups¹⁹ on plastic substrates. In the present study, we aimed to decorate plastic surfaces with azide groups by dip-coating the substrates in functional polymer solutions to immobilize functional moieties on the surface via the SP-AAC (i.e., strain-promoted azide-alkyne cycloaddition). Quantification of the immobilized functional molecules enabled control over the surface density of azide groups. Their immobilization was evaluated quantitatively via μ CP.

2. Results and Discussion

Synthesis of polymers for coatings

Our group previously demonstrated that the surface segregation of polymer functional groups was highly influenced by the chemical structures of those functional groups.^{17, 18} To evaluate the effects of the molecular structures on the surface display of azide groups, we first synthesized the backbone polymer, PMH (Scheme S1), via free radical polymerization of MMA and HEMA (monomer ratio = 9:1) in ethyl acetate. The integrated intensity of signals in the ¹H-NMR spectrum suggested that MMA and HEMA were copolymerized in the ratio of 9:1 as intended (Figure S1a). Size-exclusion chromatography (SEC) measurements showed that the number-average and weight-average molecular weights of PMH were 27,000 and 32,000, respectively (Figure S2). Four different types of azide groups were then conjugated with the hydroxyl groups on the PMH side chains via condensation. We prepared four polymers with different azide groups (Figure 1a) to investigate the effects of their molecular structures on their surface densities. The ¹H-NMR spectra revealed that 97% of the hydroxyl groups in PMHPA, 98% in PMH(PEG)3Ah, 98% in PMHBA, and 96% in PMHFBA were conjugated with azide groups. Notably, the four different polymers exhibited the same degree of polymerization and had different azide groups in their side chains.

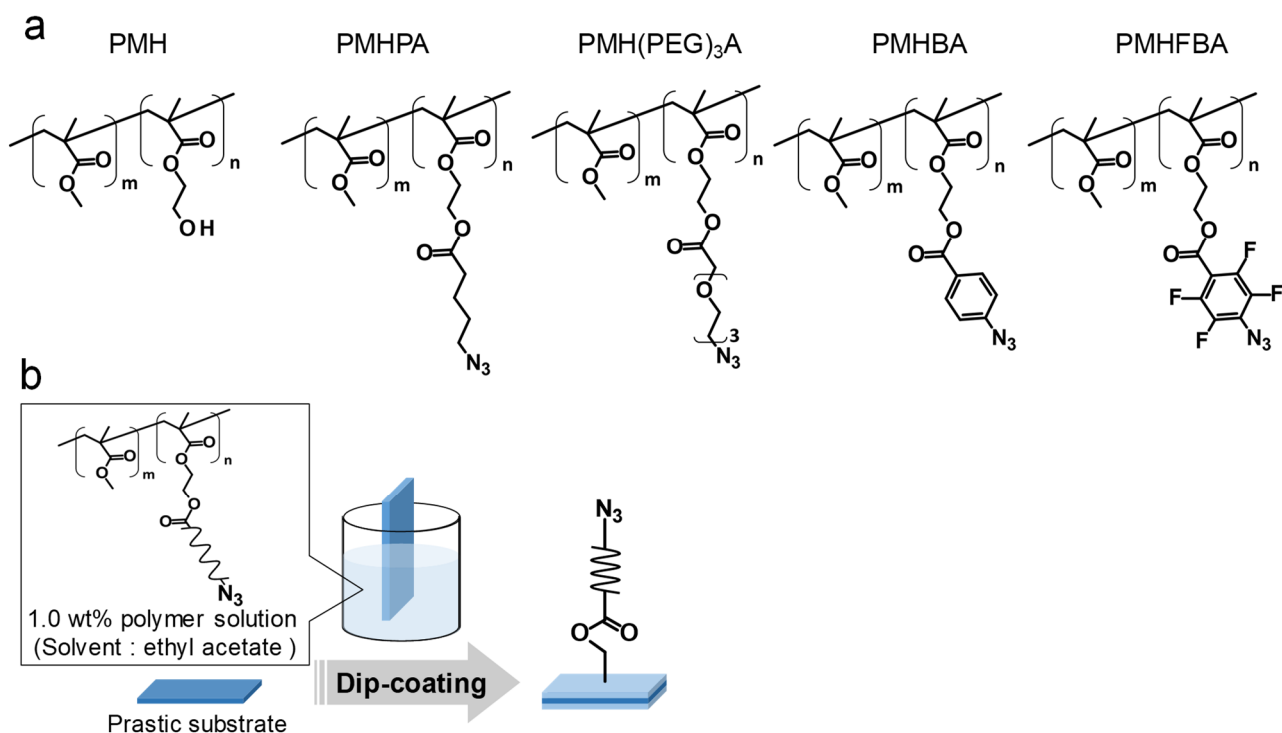


Figure 1. a) Molecular structures of the polymers synthesized for this work. b) Schematic illustration of the functionalization of plastic surfaces via dip-coating method.

Dip-coating substrates with polymers

The synthesized polymers were dissolved in ethyl acetate at 1 wt.%. Acrylic (i.e., poly(methyl methacrylate)) substrates were dip-coated in these polymer solutions (Figure 1b). The XPS spectra of all four polymer-coated substrates had peaks at 399 and 404 eV, which originated from the azide groups; these peaks were not observed in control sample spectra (a bare acrylic substrate and a PMH-coated substrate) (Figure 2). PET and nylon 6 films were also dip-coated with PMHBA using the same procedure, followed by XPS measurements (Figure S4). The characteristic N1s peak was observed around 400 eV for the PMHBA-coated PET substrate (Figure S4c). Because nylon has

nitrogen in its molecular structure, N1s peak was observed for a bare nylon substrate (Figure S4d).

After dip-coating with PMH, the N1s peak was still observed (Figure S4e) but smaller than that of a bare substrate, indicating portions of a nylon substrate being coated with PMH. The N1s peak observed for the dip-coating with PMHBA was larger than that of the PMH-coated nylon substrate (Figure S4f), indicating the presence of a PMHBA layer on the nylon substrate. It should be noted that an azide group is likely to be decomposed by X-ray.³⁴ The XPS measurements were conducted with a limited number of repetitions.

When the substrates dip-coated with the synthesized polymers were immersed in 0.1 M phosphate buffer (pH 8.0) and 5 mM NaOH aqueous solution, the polymer layers did not detach, thus indicating stable polymer coatings. The adhesion of the coated polymers to the substrates would be dipole–dipole interaction and van der Waals force.¹⁷ The thickness of the films was measured by weight measurements of the substrates before/after dip-coating using the density of azide-functionalized polymers. The density of the polymer was calculated using the flotation method. These measurements suggested that the film thickness on both sides of the substrates was calculated to be 0.5 μm for PMHPA, 0.6 μm for PMH(PEG)₃A, 0.7 μm for PMHBA, and 0.4 μm for PMHFBA.

Overall, these results indicate that the synthesized polymers were coated onto the substrates, with the azide groups displayed on their surfaces. Furthermore, FE-SEM and AFM observations revealed that there were no significant changes in the morphology of the plastic surface, except for nylon, before versus after dip-coating for all polymer and substrate combinations (Figure S5 and S6). While

a bare nylon substrate had a rolling surface, the coating with polymers seems to cover the rolling (Figure S5 and S6).

The AFM observation provided the root mean square (RMS) roughness of the surfaces (Figure S6). While the RMS roughness of a bare acrylic surface was 0.33 nm, those for the acrylic surfaces coated with PMH and with PMHBA were 0.95 nm, which were comparable with that of a bare surface. The RMS roughness of a bare PET surface was 1.01 nm, and those for the PET surfaces coated with PMH and with PMHBA were 0.50 nm for and 0.28 nm. The RMS roughness of a bare nylon surface was 5.13 nm, that for nylon coated with PMH was 6.28 nm, and that for the nylon surface coated with PMHBA was 3.39 nm. The differences in the roughness before and after dip-coating were less than few nanometers. We concluded no significant changes in surface roughness due to the dip-coating for acrylic and PET substrates (Figure S6). The dip-coating reduced the surface roughness of a bare nylon substrate.

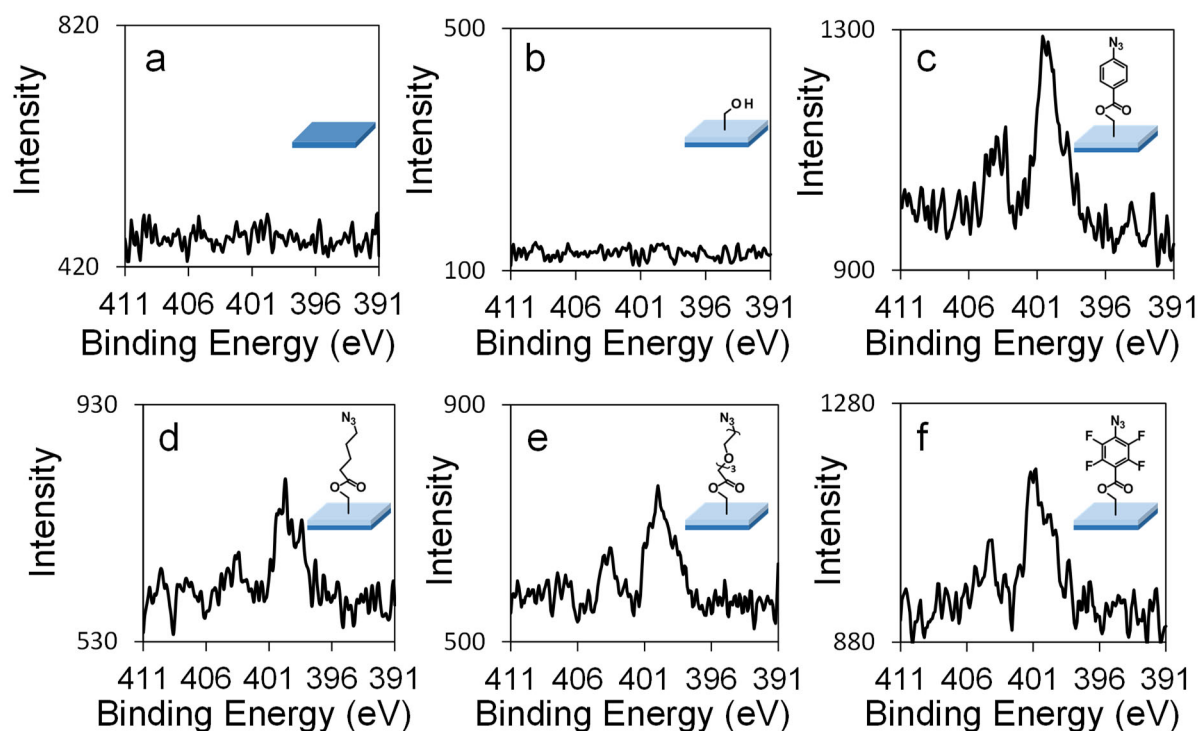


Figure 2. XPS spectra of acrylic substrate surfaces: (a) bare and coated with (b) PMH, (c) PMHBA, (d) PMHPA, (e) PMH(PEG)₃A, and (f) PMHFBA. N1s binding energies are observed at around 400 eV.

Quantification of the click-reactive azide group density after dip-coating

To quantify the density of clickable azide groups on the dip-coated surfaces, we synthesized *N*flu-DACN (Scheme 1). The diazacyclononyne derivative DACN contains a hydrophilic alkyne moiety, which reacts more selectively with azide groups than cyclooctyne without a catalyst; it has been used previously for Cu-free strain-promoted azide-alkyne cycloaddition.³⁵ The polymer-coated substrates were immersed in phosphate buffer containing *N*flu-DACN to allow for the immobilization of *N*flu-DACN on the substrate surface via the click reaction (Figure 3a). They were

then immersed in a 0.1 M NaOH aqueous solution to cleave the side chains of the dip-coated polymers via hydrolysis, thereby liberating the fluorophores. The free fluorophores were measured using a fluorescence spectrometer, and the results were analyzed to determine the surface density of azide groups that underwent the click reaction (Figure 3b).

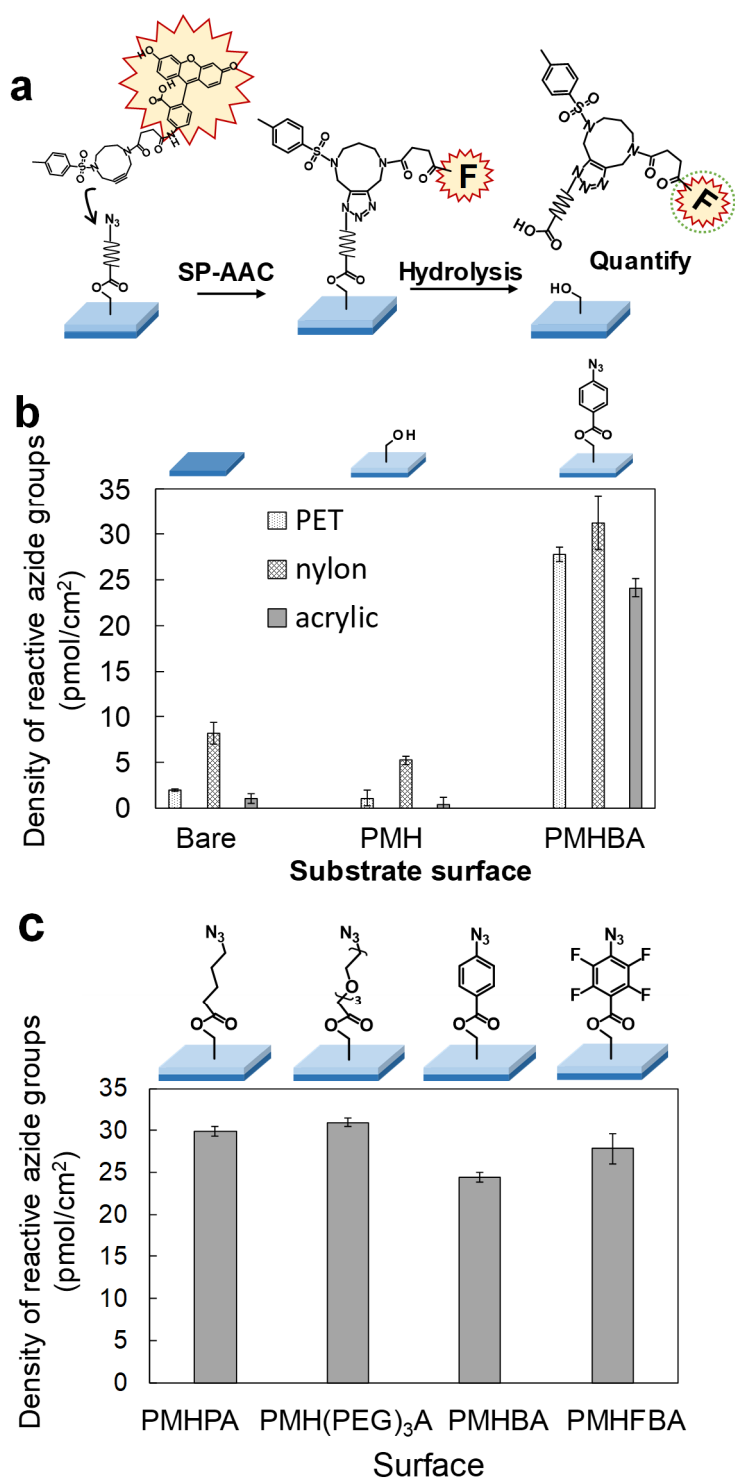


Figure 3. (a) Schematic image of the quantification of reactive azide group surface density. (b)

Density of clickable azide groups on plastic substrate surfaces coated with PMEBA (after dip-coating

in a 1 wt.% solution). (c) Densities of reactive azide groups on acrylic substrate surfaces dip-coated

with PMHA, PMHPA, PMH(PEG)₃A, PMHBA, and PMEFBA after dip-coating in a 1 wt.% polymer solution.

The N1s XPS spectra were recorded to investigate the *N*flu-DACN-immobilized acrylic surfaces before and after hydrolysis by NaOH_{aq} (Figure S7). The spectrum obtained before *N*flu-DACN immobilization contained a relatively sharp peak at 404 eV (Figure S7a), and the spectrum obtained after the click reaction had a broadened peak from 401 to 405 eV (Figure S7b). This change in peak shape indicated that azide groups were converted to triazoles during the reaction. After the hydrolysis of polymer side chains, the N1s peaks derived from azide and *N*flu-DACN disappeared (Figure S7c), suggesting that most of the *N*flu-DACN immobilized on the substrate surface were liberated through hydrolysis.

Figure 3b shows the surface densities of azide groups that underwent the click reaction on various substrate surfaces. A negligible amount of fluorescence was observed on bare and PMH-coated PET and acrylic substrates, which was attributed to nonspecific adsorption of *N*flu-DACN. There observed a certain amount of *N*flu-DACN adsorption on bare and PMH-coated nylon substrates. These results supported that portions of a nylon substrate were not coated with PMH as discussed above. In contrast, PET, nylon, and acrylic substrates coated with PMHBA had remarkable azide surface densities of 28, 31, and 24 pmol/cm², respectively. The higher fluorescence intensity on PET and nylon compared with acrylic might be attributed to increased nonspecific adsorption because

PET and nylon substrates were more flexible and could have cracks on their surfaces. These findings confirmed the azide presentation on the outermost surfaces of the plastic substrates following polymer dip-coating. The acrylic substrate was selected for further studies because of the low nonspecific adsorption of *N*flu-DACN.

Next, the surface densities of clickable azide groups were quantified for acrylic substrates coated with the four different polymers. Figure 3c shows the azide surface densities after dip-coating in PMHPA (30 pmol/cm²), PMH(PEG)₃A (31 pmol/cm²), PMHBA (24 pmol/cm²), and PMHFBA (28 pmol/cm²). All surfaces displayed azide groups at >24 pmol/cm², and the azide groups were reactive (i.e., clickable) with DACN in an aqueous environment. We did not find remarkable differences in the surface density among these azide groups.

We calculated the surface occupancy of the azide groups based on their surface densities in each case. For example, the PMHBA-coated substrate had the lowest azide surface density among the prepared samples, corresponding to an average distance between azide groups of approximately 2.6 nm. Assuming free rotation of *N*flu-DACN with a 1.4 nm radius of rotation when centered on the alkyne group (Figure S8), the surface occupancy of *N*flu-DACN was calculated to be approximately 96%. This result suggests that the surface was almost entirely covered by *N*flu-DACN.

We also investigated the effect of the one-week storage on the surface density of azide groups. The PMHBA-coated acrylic substrates were kept in dark at room temperature for 1 week after polymer coating, followed by the immobilization of *N*flu-DACN. Figure S9 shows that there was

negligible difference in the amounts of the fluorophore liberated from the substrates. The surface displaying azide groups were stable at least for 1 week.

Control of the surface density of azide groups by dip-coating

It is valuable to be able to control the density of clickable azide groups on the surfaces of substrates. Thus, dip-coating solutions comprising various polymer concentrations were prepared by dissolving PMH and PMHBA, such that the sum of PMH and PMHBA concentrations was maintained at 1 wt.% (Figure 4a). Acrylic substrates were coated with this series of solutions, and the surface densities of the clickable azide groups were quantified. The higher the weight ratio of PMHBA, the higher the surface density of clickable azide groups. These results indicated that the density of clickable azide groups on a surface can be controlled by varying the weight ratio of PMHBA in the dip-coating solution.

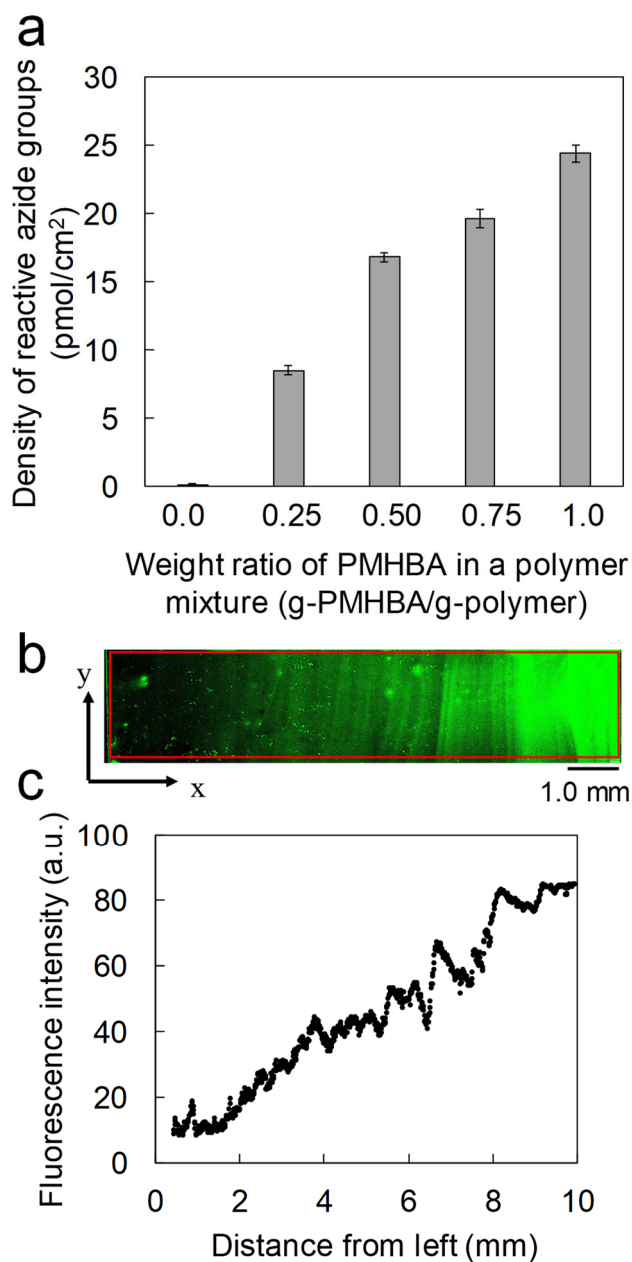


Figure 4. (a) Densities of reactive azide groups on acrylic surfaces after dip-coating with ethyl acetate solutions containing PMHBA and PMH in various weight ratios. The sum of polymer concentrations was maintained at 1 wt.%. The dip-coating procedure was the same with that in Fig. 3b. (b) CLSM image showing the chemical gradient on the surface using *N*flu-DACN (fluorescence

quantified using ImageJ software). (c) Linear gradient of fluorescence intensity on the dip-coated surface.

Preparation of a surface with a gradient in azide group density

We aimed to create a gradient in azide group density on the substrate surface by dynamically controlling the dip-coating conditions. Chemical concentration gradients on solid surfaces are important for a variety of biological processes, e.g., directed cell migration,^{36, 37} and as a result, methods for fabricating surface-gradient materials have gained significant attention.³⁸ To prepare a surface with a linear gradient in terms of azide group density, we continuously varied the ratio of PMH to PMHBA in the coating solution during the substrate withdrawal process. In the beginning of the process, the PMH and PMHBA concentrations in the coating solution were 1 wt.% and zero, respectively, and by the end of the withdrawal process, both concentrations were 0.5 wt.%. The obtained substrate was immersed in phosphate buffer containing *N*flu-DACN to allow for the immobilization of *N*flu-DACN on the surface via the click reaction. The CLSM results are shown in Figure 4b. The left side of the image corresponds to the dip-coating solution containing 1 wt.% PMH and no PMHBA. The proportion of PMHBA in the coating solution increased along the *x*-axis, reaching a 1:1 ratio of PMH to PMHBA at the right edge of the image. The plot in Figure 4c shows that the fluorescence intensity increases linearly as a function of the distance from left edge in Figure

4b. These results indicate that continuously varying the polymer concentrations in the dip-coating solution enables control over the gradient of azide groups on the substrate surface.

Surface patterning by microcontact printing using a click reaction on a dip-coated surface

Finally, we studied the surface patterning of *N*flu-DACN on the coated surface using the μ CP method based on the click reaction and quantified the immobilized molecules. A solution containing *N*flu-DACN was placed on a PDMS stamp and allowed to dry. Then, the stamp was brought into contact with acrylic substrates coated with the four polymers. After washing, the surface fluorescence of each sample was observed using CLSM (Figure 5), which revealed that the surface patterning of *N*flu-DACN corresponded to the geometry of the stamp.

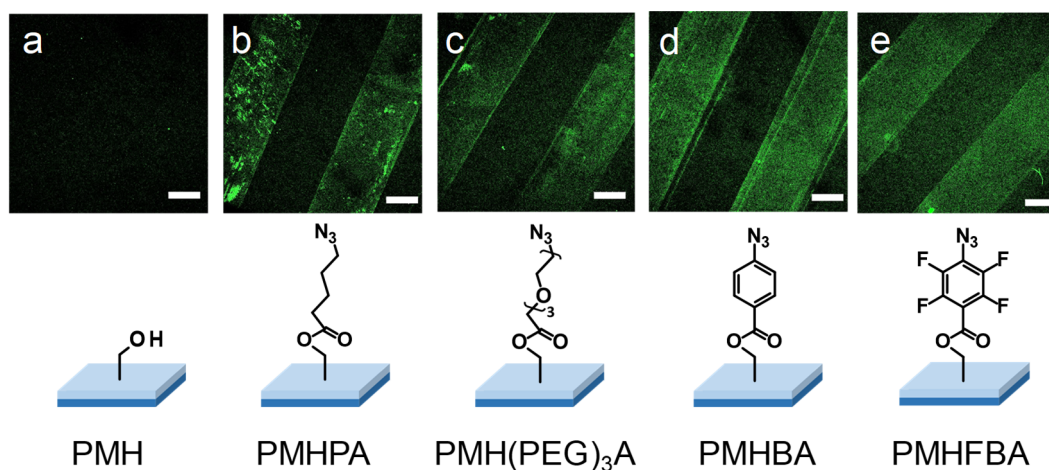


Figure 5. CLSM images and corresponding diagrams of (a) PMH-coated, (b) PMHPA-coated, (c) PMH(PEG)₃A-coated, (d) PMHBA-coated, and (e) PMHFBA-coated surfaces micropatterned using μ CP with *N*flu-DACN (scale bars = 500 μ m).

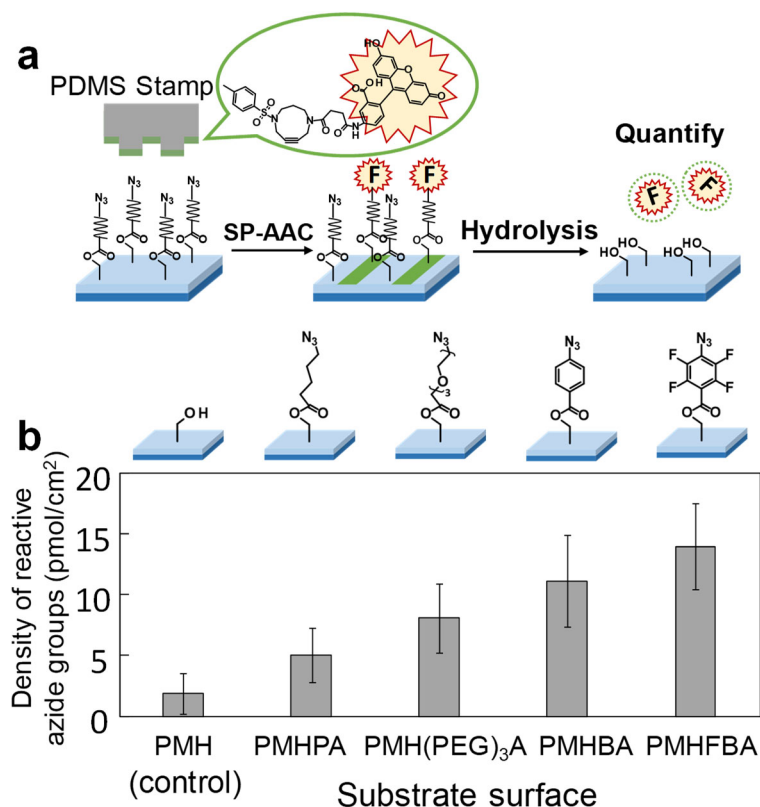


Figure 6. (a) Schematic illustration of the μ CP process via the click reaction on a polymer-coated surface and quantification of the immobilized molecules. (b) Surface densities of reactive azide groups on an acrylic substrate after μ CP-immobilization (polymer concentrations for dip-coating = 1 wt.%).

After patterning, the surfaces were treated with NaOH_{aq} to liberate fluorophores into the solution, and the fluorescence intensity was measured to quantify the surface density of *N*flu-DACN

immobilized by μ CP (Figure 6a). Figure 6b shows that significant amounts of *N*flu-DACN were immobilized on substrates coated with the four different polymers, whereas a negligible amount of *N*flu-DACN was immobilized on the PMH-coated control sample surface. The PMHFBA- and PMHPA-coated substrates had the most and least immobilized *N*flu-DACN, respectively, among the surfaces coated with azide-containing polymers. These results were in contrast to those presented in Figure 3c. This difference was attributed to the distinct reaction environments; one was in an aqueous environment and the other in air. In air, more hydrophobic azide groups would be segregated to the outermost surface.^{39, 40} Indeed, the side chain of PMHFBA was the most hydrophobic among the tested polymer side chains, and the PMHFBA-coated surface corresponded to the highest amount of immobilized *N*flu-DACN under air.

XPS measurements also revealed that the PMHFBA-coated substrate had the highest N1s to C1s peak area ratio in the dry state (Figure S10). The theoretical nitrogen-to-carbon (N/C) elemental composition ratios of the polymers are also presented in Figure S10, assuming that all hydroxyl groups in the polymers were fully conjugated with azide groups. The experimentally determined N/C ratios for surfaces coated with PMHPA, PMH(PEG)3A, and PMHBA were notably lower than the theoretical values. This suggests that the azide groups within these polymers tend to be embedded in the bulk of the polymer films when the polymers were dip-coated onto substrates to form polymer films. In contrast, the experimental N/C ratio for the PMHFBA-coated surface closely matched the theoretical value, indicating that the azide groups in PMHFBA remained exposed at the surface. The

high hydrophobicity of the tetrafluorobenzene moieties in PMHFBA likely facilitates the display of azide groups at the outermost surface. The hydrophobicity of the side chain of a polymer would affect the surface-display of azide groups.

These results demonstrate how the immobilization efficiency of the μ CP method varies according to the chemical structures of the reactive functional groups in the coating polymer. The μ CP was carried out at room temperature while the immobilization of *N*flu-DACN in water was at 40°C. The difference in reaction temperature would also affect the immobilization efficiency, which might account for the difference in the amount of reactive azide groups between Figure 3c and 6b.

Notably, not all azide groups displayed on the surface reacted with the alkyne groups brought by the μ CP method. For example, comparing the results in Figure 3c and Figure 6 indicates that approximately 56% of the azide groups displayed on the PMHFBA-coated substrate were conjugated with *N*flu-DACN following μ CP. The μ CP method has been widely used for micropatterning solid surfaces. However, there are few studies describing the quantification of molecules immobilized by μ CP. Herein, we successfully quantified the functional molecules immobilized on a solid surface via μ CP using strain-promoted azide-alkyne cycloaddition reactivity.

3. CONCLUSIONS

Surface functionalization of polymeric materials is crucial for enhancing the utility of plastic substrates for diverse applications. In this work, we modified plastic surfaces by immobilizing

functional molecules via dip-coating with azide-functionalized polymer solutions, followed by a click reaction (i.e., strain-promoted azide-alkyne cycloaddition). Dip-coating plastic substrates with azide-functionalized polymers decorated the outermost surfaces with clickable azide groups. The type of polymer, chemical properties of its side chains, and dip-coating conditions significantly influenced the surface density of azide groups. We also demonstrated that the surface density of azide groups could be controlled by varying the polymer composition of the dip-coating solution. Microcontact printing experiments illustrated the potential of this method for precise patterning of functional molecules on plastic surfaces. The quantification of immobilized molecules post- μ CP highlighted the effectiveness of the azide-alkyne cycloaddition reaction in a confined environment, which is valuable for advanced surface engineering applications. Creating a gradient in terms of azide group density across the substrate surface is particularly significant because it enables the preparation of surfaces with spatially-controlled chemical functionalities to support diverse applications (e.g., in biosensors, tissue engineering, and directed cell migration). For instance, the combination of a density-gradient surface and the μ CP technique will produce a surface model with gradually increasing azide density in a line pattern, which will lead to more complex cell culture platforms and a novel type of cell separation system. The results presented herein provide valuable insights for the design and implementation of plastic surface functionalization strategies by emphasizing the versatility of click chemistry combined with dip-coating and μ CP techniques.

Overall, this study advances our understanding of surface chemistry and offers new approaches to the functionalization of materials for various industrial and biomedical applications.

4. EXPERIMENTAL METHODS

Methyl methacrylate (MMA) and 2-hydroxyethyl methacrylate (HEMA) were used as monomers to prepare a backbone polymer. Details regarding the materials and synthesis of polymers (Figure 1a), i.e., poly(MMA-*r*-HEMA) (PMH), poly(MMA-*r*-HEMA(azidopentanoate)) (PMHPA), poly(MMA-*r*-HEMA(azidoPEG3acetate)) (PMH(PEG)₃A), poly(MMA-*r*-HEMA(azidobenzoate)) (PMHBA) and poly(MMA-*r*-HEMA(azidotetrafluorobenzoate)) (PMHFBA), are described in the Supporting Information (¹H-NMR spectra of the polymers are presented in Figure S1).

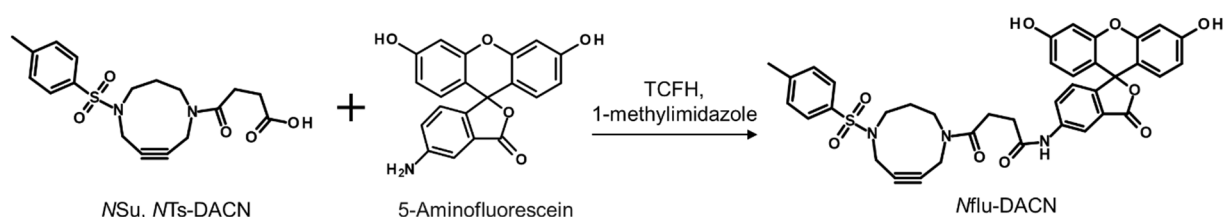
Synthesis of the fluorescent compound with a cyclic alkyne group (*N*flu-DACN)

N-Succinoyl-*N'*-*p*-toluenesulfonyl-4,8-diazacyclononyne (fluorescein conjugated with a diazacyclononyne derivative; *N*flu-DACN) (95.5 μmol), 5-aminofluorescein (467 μmol), chloro-*N,N,N',N'*-tetramethylformamidine hexafluorophosphate (157 μmol), and 1-methylimidazole (283 μmol) were dissolved in acetonitrile (8 mL) and stirred for 18 h at room temperature (Scheme 1).

The solvent was evaporated, and the resulting crude product was dissolved in 8 mL dichloromethane and filtered to remove insoluble impurities. The filtrate was washed twice with a 50 mM HCl aqueous solution (2 mL) and then twice with water. The dichloromethane layer was collected, and

the solvent was evaporated. The crude product was then purified by silica gel chromatography using ethyl acetate:*n*-hexane = 8:2 as the mobile phase, and the solvent was evaporated from the obtained solution to isolate the solid (yield = 25%). The product was characterized by ¹H-NMR and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) using an ultrafleXtreme mass spectrometer (Bruker, Billerica, MA, USA), and the results are presented in Figure S3.

Scheme 1. Synthesis of *N*flu-DACN.



Dip-coating plastic substrates with synthesized polymers

Acrylic, poly(ethylene terephthalate) (PET), and nylon substrates (all 0.5 mm thick) were cut into 1 cm × 1 cm pieces. The four types of functional polymers and PMH were dissolved at 1 wt.% in ethyl acetate. Plastic substrates were immersed in the polymer solution for 5 seconds, withdrawn from the solution over 5 seconds, and dried under vacuum for 3 h at room temperature.

The XPS measurements were performed using a PHI X-tool instrument (ULVAC, Chigasaki, Japan) using an Al K α source (15 kV, 25 W). For a narrow spectrum, the photoelectron take-off angle

was 45°, and the spot size was 113 $\mu\text{m} \times 113 \mu\text{m}$. Survey scans were performed with a pass energy of 112 eV and a step size of 0.1 eV.

Specimens were coated with evaporated osmium tetroxide (Neoc-Pro, Meiwafoysis Co. Ltd., Tokyo, Japan). The FE-SEM observations were carried out using a field-emission scanning electron microscope (JSM-IT700HR, JEOL, Tokyo, Japan) operating at an accelerating voltage of 15 kV.

Quantification of the surface density of clickable azide groups

The dip-coated substrates were immersed in phosphate buffer (0.1 M, pH 8.0) containing 25 μM *N*-flu-DACN and shaken for 18 h at 40 °C. The substrates were then washed three times with 5 mM NaOH aqueous solution and once with ethanol. Next, the substrates were immersed in 2 mL phosphate buffer (0.1 M, pH 8.0), shaken for 1 h at 40 °C, and the same procedure was performed again with fresh phosphate buffer. Finally, the substrates were immersed in 2 mL phosphate buffer (0.1 M, pH 8.0) containing 0.1 M NaOH and shaken for 18 h at 60 °C to liberate the immobilized fluorophore into the aqueous phase. The pH of the aqueous solution was adjusted to 8 using 0.2 mL 1 M HCl aqueous solution, and the fluorescence intensity ($\lambda_{\text{ex}} = 495 \text{ nm}$, $\lambda_{\text{em}} = 515 \text{ nm}$) of the solution was measured using a fluorescence spectrometer (FP-8200, Jasco, Tokyo, Japan). The surface density of clickable azide groups was determined based on the standard curve (Figure S11). All measurements were conducted on three different samples to quantify the surface density, and the mean values obtained were used in the analysis.

Preparation of a gradient surface density of azide groups on an acrylic substrate

An acrylic substrate was immersed in a 1 wt.% PMH solution (i.e., a coating solution). The substrate was then lifted at a rate of 1.25 cm/min using a syringe pump. Simultaneously, a 1 wt.% PMHBA solution was added to the PMH solution at a rate of 1.33 mL/min. This addition continued until the substrate was completely lifted, resulting in a final concentration of 0.5 wt.% PMH and 0.5 wt.% PMHBA in the coating solution. The dip-coated substrate was dried under vacuum for 3 h at room temperature. Next, the substrate was immersed in phosphate buffer (0.1 M, pH 8.0) containing 25 μ M *N*flu-DACN and gently agitated for 18 h at 40 °C. The substrate was then washed three times with a 5 mM NaOH aqueous solution and once with ethanol. Then, the substrates were again immersed in phosphate buffer (0.1 M, pH 8.0) and gently agitated for 1 h at 40 °C. The surface of the substrate was observed using confocal laser scanning microscopy (CLSM; Fluoroview FL3000, Olympus, Tokyo, Japan). Four CLSM images were combined to create a single comprehensive image covering the entire substrate. The fluorescence intensity was quantified along the direction of lifting during dip-coating using the ImageJ image processing software (National Institutes of Health, Bethesda, MD, USA).

Microcontact printing of *N*flu-DACN on a dip-coated surface

A SYLGARD 184 Silicone Elastomer Kit (Dow Inc., Midland, MI, USA) was used to prepare a polydimethylsiloxane (PDMS) stamp. Base and curing agents were mixed in a volume ratio of 10:1 and poured into a brass stamp mold. After vacuum drying to remove air bubbles, the mixture and mold were heated for 4 h in a vacuum oven at 60 °C. After curing, the PDMS stamp was removed from the mold and cut into pieces (0.6 cm × 1.0 cm) with three lines (Figure S12). The surfaces of the PDMS stamps were hydrophilized via plasma treatment.⁴¹

To prepare the ink solution, *N*flu-DACN was dissolved in ethanol at 2 mM. Then, 1.5 µL ink solution was placed on each line of the stamp, and the system was dried under vacuum for 1 h at room temperature. The stamp was placed on the dip-coated substrate, and a 100 g weight was placed on top of the stamp for 18 h. The stamp was then removed, and the *N*flu-DACN remaining on the substrate was roughly removed by tape stripping. The substrates were then washed three times with a 5 mM NaOH aqueous solution and once with ethanol. Next, the substrates were immersed in phosphate buffer (0.1 M, pH 8.0) and agitated for 1 h at 40 °C, followed by immersion in a 1 wt.% sodium dodecyl sulfate aqueous solution and agitation for 1 h at 40 °C. Because the ink of *N*flu-DACN was pressed on a substrate surface, solid state *N*flu-DACN was deposited on the surface. The deposition was removed using a sodium dodecyl sulfate solution. The substrates were then immersed again in phosphate buffer (0.1 M, pH 8.0) and agitated for 1 h at 40 °C. Finally, the substrates were immersed in phosphate buffer (pH 8.0) and observed by CLSM. To quantify the amount of *N*flu-

DACN immobilized by μ CP, the immobilized fluorophore was liberated using the same method described above, and its fluorescence intensity was measured.

Atomic force microscope (AFM) observation

AFM was conducted using a SPA-400 (Hitachi, Tokyo, Japan). AFM images were obtained in tapping mode with cantilevers at 41 N m^{-1} .

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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