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Efficient Immobilization and Patterning of Biomolecules on Poly(ethylene terephthalate) Films Functionalized by Ion Irradiation for Biosensor Applications

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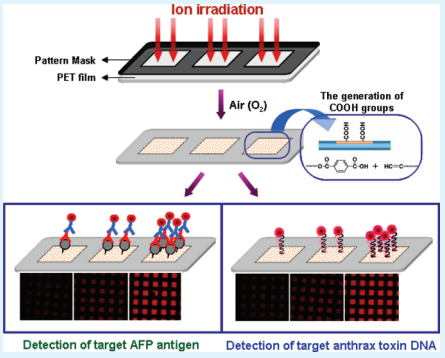
Supporting Information

ABSTRACT: The surface of a poly(ethylene terephthalate) (PET) film was selectively irradiated with proton beams at various fluences to generate carboxylic acid groups on the surface; the resulting functionalized PET surface was then characterized in terms of its wettability, chemical structure, and chemical composition. The results revealed that (i) carboxylic acid groups were successfully generated in the irradiated regions of the PET surface, and (ii) their relative amounts were dependent on the fluence. A capture biomolecule, anthrax toxin probe DNA, was selectively immobilized on the irradiated regions on the PET surface. Cy3-labeled DNA as a target biomolecule was then hybridized with the probe DNA immobilized on the PET surface. Liver-cancer-specific α-fetoprotein (AFP) antigen, as a target biomolecule, was also selectively immobilized on the irradiated regions on the PET surface. Texas Red-labeled secondary antibody was then reacted with an AFP-specific primary antibody prebound to the AFP antigen on the PET surface for the detection of the target antigen, using an indirect immunoassay method. The results revealed that (i) well-defined micropatterns of biomolecules were successfully formed on the functionalized PET surfaces and (ii) the fluorescence intensity of the micropatterns was dependent mainly on the concentrations of the target DNA hybridized to the probe DNA and the target AFP antigen immobilized on the PET films. The lowest detectable concentrations of the target DNA and target AFP antigen in this study were determined to be 4 and 16 ng/mL, respectively, with the PET film prepared at a fluence of 5×10^{14} ions/cm^2.



KEYWORDS: ion irradiation, patterning, biosensor, surface modification

INTRODUCTION

A biosensor is a device that detects target molecules with high selectivity on the basis of molecular recognition. Biosensors can be classified according to their biorecognition systems, based on antibody/antigen, DNA−DNA, enzyme−substrate, and cellular interactions. Various types of biosensors have been developed for use in the fields of biotechnology, medicine, clinical diagnosis, and environmental monitoring, and in the food industry.[1−8]

For the fabrication of biosensors based on biological recognition, the immobilization of biomolecules on a platform surface is considered to be very important. Thus, diverse immobilization strategies based on covalent attachment, biotin−avidin specific binding, and passive adsorption have been developed so far. Among these methods, covalent immobilization of capture biomolecules on functionalized surfaces via aldehyde, epoxide, amine, or carboxyl groups have been most widely utilized, because of their high specificity and long-term stability.[9−13]

Polymers are now regarded as a useful alternative substrate for the fabrication of biosensors, because of their low cost and outstanding processability. However, their poor surface properties (such as their hydrophobicity, chemical inertness, and poor biocompatibility) limit their biological applications.[14] Thus, the polymer must be subjected to a surface functionalization process before use.[15,16] Surface functionalization of various polymers has been performed using various techniques, such as chemical treatment, plasma treatment, UV irradiation, ion irradiation, and so on.[17−22] Among these surface functionalization methods, ion irradiation is a promising method for functionalizing the surface of a polymer to overcome its chemical inertness and hydrophobicity. This method offers several advantages, including the direct generation of hydrophilic functional groups on the surface without the use of any harsh chemicals, high reliability and controllability, and temperature independence.[23−27] Furthermore, even if the other techniques, which are based on electron beam, ultraviolet (UV) light, γ-ray, and X-ray,

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can be utilized for the surface modification of polymers, the ion beam-based technique is more effective for the surface structural modification of polymers than other techniques because an ion beam has a greater linear energy transfer and its penetration trajectory is fairly straight in comparison to the other techniques.[28,29] Nevertheless, to the best of our knowledge, surface modification of polymers by ion irradiation for the fabrication of biosensors has not been studied previously.

Anthrax is an acute disease caused by the anthrax toxin, which is usually fatal to mammals.[30,31] Hapatocellular carcinoma (HCC) is also considered one of the most common and highly malignant cancers in the world.[32] Thus, the detection of the disease-related specific molecules permits the early diagnosis of these malignant diseases. To achieve the early diagnosis of these diseases, it is essential to choose a disease-related specific biomarker that can be used as a molecular probe, such as DNA, antigen, antibody, or enzyme.[33,34] Among other biomarkers, an anthrax toxin DNA for anthrax and an α-fetoprotein (AFP) antigen for liver cancer have been widely used, because of their specificity.[30−32]

In this study, a convenient and effective surface functionalization method for a flexible poly(ethylene terephthalate) (PET) substrate by ion irradiation was demonstrated for the fabrication of biosensors for anthrax toxin and liver cancer disease. The surface of a PET substrate was selectively functionalized by ion irradiation to generate carboxylic acid groups in the irradiated regions of the PET surface, on which the typical marker biomolecules of anthrax toxin probe DNA and liver-cancer-specific α-fetoprotein (AFP) antigen were immobilized. The quantitative molecular recognition capability of the fabricated DNA- and immuno-sensors for the detection of anthrax toxin and liver cancer disease was also investigated by fluorescence microscopy.

EXPERIMENTAL SECTION

Materials. PET films (100 μm thick, Toray Saehan Co., Ltd.) were ultrasonically washed in methanol for 20 min and dried in an oven at 60 °C before use. Silver nitrate ($AgNO_3$) was purchased from Tokyo Chemical Industry. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Aldrich and were used without additional purification. All of the oligonucleotides related to the anthrax toxin in this study were purchased from Genotech Company (Korea). An oligonucleotide containing an amino group at its 3′-position with the sequence 5′-GGATTATTGTTAAATAATGATAAGGAT-NH_2-3′ was used as the capture DNA and an oligonucleotide, which was labeled with Cy3 at the 5′-position with the sequence 5′-Cy3-ATCCTTATCATTATTTAACAATAATCC-3′ and an oligonucleotide with the sequence 5′-Cy3-ATCCTTATCAATATT-TAACAATAATCC-3′ were used as the target DNA having complementary and noncomplementary sequences with the capture DNA, respectively. The α-fetoprotein (AFP) antigen, mouse monoclonal AFP antibody, and Texas Red-labeled mouse IgG secondary antibody were purchased from AbCam.

Surface Functionalization by Ion Irradiation. The surface of the PET films was functionalized by ion irradiation. This process has been described in a previous publication by the authors.[35] Briefly, the surfaces of the PET films were irradiated with H^+ ions through a customized mask (SUS, 40-μm square spaces) at room temperature with 200 keV H^+ ions at fluences ranging from 1×10^{14} to 1×10^{15} ions/cm^2. The current density of the ion beams was kept at ∼0.5 μA/cm^2 to prevent a detrimental thermal effect on the PET films. The penetration depth of 200 keV H^+ ions into the PET film calculated by the SRIM98 program was ∼2.4 μm. After the irradiation, the samples were placed in the air for 24 h for oxidation.

Characterization of Ion-Irradiated PET Surface. The chemical structure of control and irradiated PET surfaces was investigated via attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), using a Bruker Model Tensor 37 system. The surface chemical composition of the control and irradiated PET films was investigated using an X-ray photoelectron spectrometer (XPS, MultiLab 2000, ThermoElectron Corporation, U.K.) with an Mg Kα X-ray source. The applied power was 14.5 keV at 20 mA, and the base pressure of the analysis chamber was $<10^{-9}$ mbar. To estimate the relative number of carboxylic acid groups that were generated on the irradiated PET surface, the control and irradiated PET films were immersed in a 5% aqueous solution of silver nitrate (TCI Chemicals) for 1 day under the conditions of darkness at room temperature for complete complexation between the carboxylic acids and Ag ions. Afterward, the films were thoroughly washed with deionized water and then dried in a vacuum oven at 45 °C. The resulting films were investigated by XPS analysis, and the silver contents on the control and irradiated PET surfaces were estimated from the Ag 3d peak in the XPS spectra.[36−38] The water contact angle on the control and irradiated PET surfaces was measured via the sessile drop method, using a contact-angle analyzer (Model Phoenix 300, Surface Electro Optics Company).

Immobilization and Hybridization of DNA for the Detection of Anthrax Toxin. To covalently immobilize biomolecules on the PET surface, we used an EDC/NHS coupling reaction between the amine groups of biomolecules and carboxylic acid groups on the irradiated PET surfaces. For the immobilization of the capture DNA for anthrax toxin onto the ion-irradiated regions, the irradiated PET films were immersed in a solution containing 15 mM NHS, 45 mM EDC, and 50 μg/mL of the amine-modified capture DNA. After incubation overnight, the resulting capture DNA-immobilized PET films were thoroughly washed with a copious amount of deionized water and used for hybridization with the target DNA. The capture DNA-immobilized PET films were incubated in Cy3-labeled target DNA solutions at various concentrations, ranging from 4 ng/mL to 50 μg/mL in a hybridization buffer (5× SSC, 0.4 mg/mL BSA, and 0.1% SDS) under a coverslip for 12 h at 37 °C. The films were then washed well with 3× SSC for 5 min, with 2× SSC for 5 min, and finally, with 1× SSC for 5 min. The final hybridized films were investigated using a fluorescence microscope (Olympus Model BX61, Japan). In order to obtain the fluorescence intensity, the obtained original fluorescent micrographs were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Each fluorescence intensity value was taken as an average value measured from three different positions on the fluorescent micropatterns.

Immobilization of Target AFP Antigen and Indirect Immunoassay. The immobilization of the AFP antigen onto the ion-irradiated PET surface was carried out using a procedure similar to that used for the immobilization of the capture DNA. Ten microliters (10 μL) of the target antigen solution with various concentrations ranging from 16 ng/mL to 50 μg/mL was applied over the irradiated PET films overnight at room temperature. To block the residual active NHS ester group, a 1% BSA

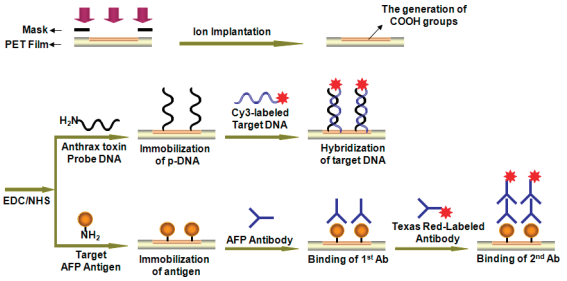

Figure 1. Schematic representation of the immobilization of biomolecules on PET films by ion-irradiation-induced surface functionalization.

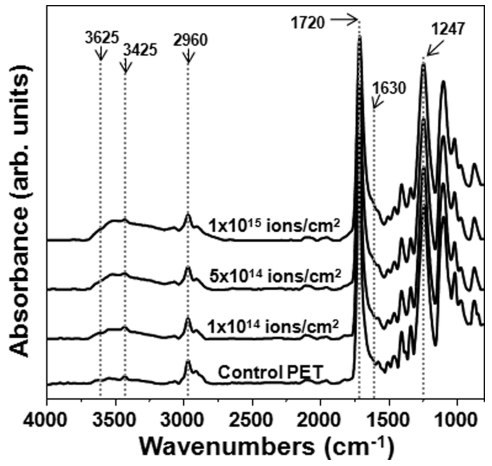

Figure 2. FTIR spectra of the control and irradiated PET films as a function of the fluence.

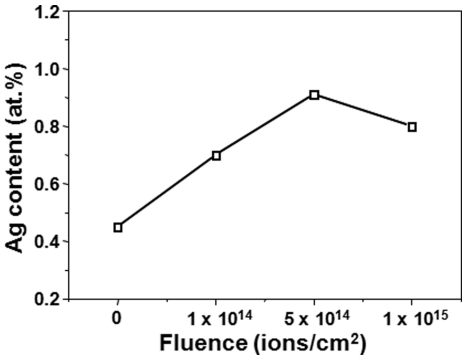

Figure 3. Ag atomic concentration on the control and ion-irradiated PET films after an exchange reaction with $AgNO_3$ obtained from an XPS analysis.

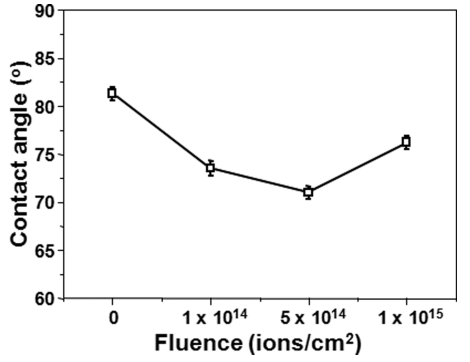

Figure 4. Water contact angles of the control and ion-irradiated PET films, as a function of the fluence.

solution was applied over the antigen-immobilized PET films. To detect the AFP antigen, we carried out an indirect immunoassay. A primary antibody solution containing 10 μg/mL of anti-AFP antibody was applied over the antigen-immobilized PET surfaces and was allowed to interact with the immobilized antigen. After incubation for 6 h, the prepared films were subsequently incubated with Texas Red-labeled secondary antibody (1 μg/mL). After 1 h, the films were rinsed well with PBS and deionized water to wash out the unbound secondary antibody. To confirm the selectivity of the antigen-immobilized PET surface, a solution of primary antibody-preblocked secondary antibody prepared by adding primary antibody (10 μg/mL) to Texas Red-labeled secondary antibody (10 μg/mL) was allowed to interact with the primary antibody prebound to the antigen-immobilized PET surface. After 6 h, the PET surface was thoroughly washed with PBS and deionized water to remove the unbound antibody.

RESULTS AND DISCUSSION

Surface Functionalization by Ion Irradiation. A schematic representation of the surface functionalization process used in this study is shown in Figure 1. The PET films were irradiated through a pattern mask with proton ions to generate the carboxylic acid groups selectively in the irradiated regions. These carboxylic acid-generated regions were utilized to immobilize biomolecules further for biosensor applications.

The changes in the chemical structure of the PET film surface after ion irradiation were investigated by ATR-FTIR. These results are shown in Figure 2. In comparison to the spectrum of the control PET, new peaks assigned to the OH, COOH, and C=C groups were clearly observed at 3625, 3425, and 1630 cm^{-1}, respectively, in the spectrum of the irradiated PET films. Moreover, their intensities were dependent on the fluence. This result confirmed that carboxylic acid groups were successfully generated on the surface of the PET films by ion irradiation-based surface functionalization.[26]

To confirm the generation of the carboxylic acid groups and to measure their relative amounts on the irradiated PET surfaces, the pristine and irradiated PET surfaces after a complexation reaction between the carboxylic acids and silver ions were investigated by XPS analysis.[27] As shown in Figure 3, the silver content on the control PET surface was ~0.45 at.%. However, in the case of the ion-irradiated PET surfaces, the silver content was increased to 0.91 at.% as the fluence increased to 5×10^{14} ions/cm^2; beyond this fluence value, the silver content gradually decreased to 0.59 at.%. This result indicates that ion irradiation at a fluence of $<5 \times 10^{14}$ ions/cm^2 can effectively generate the carboxylic acid groups on a PET surface, whereas ion irradiation at a fluence of 1×10^{15} ions/cm^2 can bring about considerable carbonization, resulting in a reduction of the carboxylic acid groups.[27] The changes in the water contact angle of the control and irradiated PET films, as a function of the fluence, are shown in Figure 4. Compared to the contact angle of the control film (81°), the angle of the irradiated PET films was gradually reduced to ~71° as the fluence increased to a fluence of 5×10^{14} ions/cm^2, above which the angle increased to 76°. This phenomenon can be ascribed to the fact that hydrophilic groups on the PET

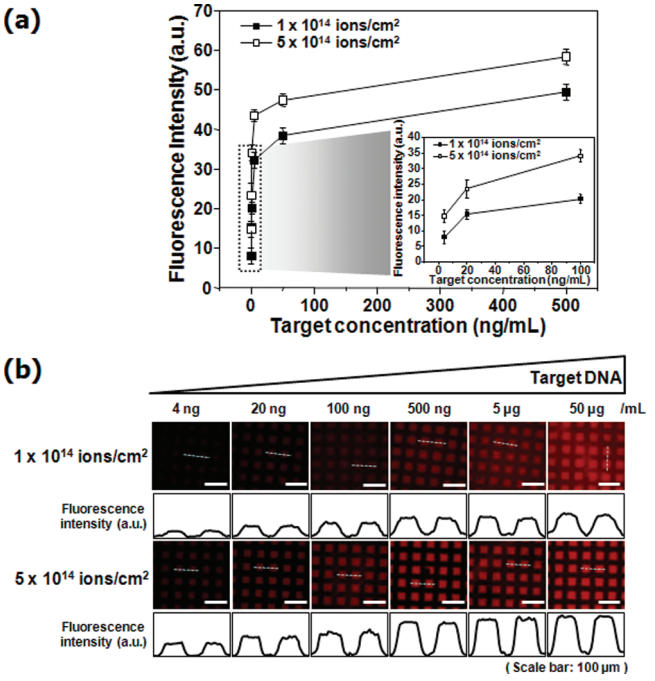

Figure 5. (a) Fluorescence intensity of Cy3-labeled target DNA hybridized to immobilized probe DNA as a function of the fluence at various concentrations of the target DNA and (b) fluorescence images.

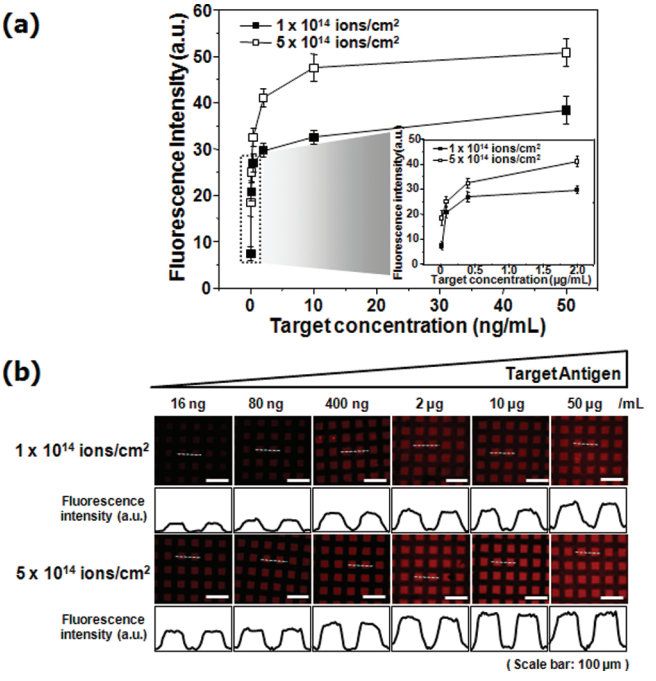

Figure 6. (a) Fluorescence intensity of Texas Red-labeled secondary antibody as a function of the fluence at various concentrations of the target AFP antigen and (b) fluorescence images.

surface were properly formed by ion irradiation at a fluence of $<5 \times 10^{14}$ ions/cm^2, while the carbonization prevailed at a higher fluence.

Detection of Target DNA on the Functionalized PET Surface. To demonstrate the performance for DNA-based biosensors, surface-functionalized PET was used for the detection of anthrax toxin target DNA. The presence of capture DNA was investigated by the hybridization of target DNA as a target molecule which was complemented with capture DNA. As shown in Figure 5, to investigate the detection limit, a target DNA solution with concentrations ranging from 4 ng/mL to 50 μg/mL was applied onto the capture DNA-immobilized PET surfaces. As shown in Figure 5a, the fluorescence intensity was decreased as the concentration of the target DNA decreased; the lowest detectable concentration in this study was determined to be 4 ng/mL with the PET film prepared at a fluence of 5×10^{14} ions/cm^2. Moreover, at all of the given concentrations, the target DNA-hybridized PET films prepared at a fluence of 5×10^{14} ions/cm^2 showed higher fluorescence intensities, compared to films prepared at a fluence of 1×10^{14} ions/cm^2, as presented in Figure 5b. This result indicates that, with a higher amount of generated carboxylic acid groups on the PET surface, a greater amount of probe DNA became immobilized on the surface. In addition, the fluorescence intensity was gradually increased with an increasing concentration of the probe DNA, as seen in Figure S1 in the Supporting Information. This result demonstrates that the detectable fluorescence intensity was dependent on the concentration of the immobilized p-DNA.

Detection of the Target Antigen on the Functionalized PET Surface. For the detection of the AFP antigen as a target molecule, all of the experimental procedures were followed by an antigen−antibody immunoassay method. To examine the detection limit, a target AFP antigen solution with concentrations ranging from 16 ng/mL to 50 μg/mL was first immobilized on the functionalized PET surfaces. Figure 6 shows the fluorescence intensity, as a function of the target AFP antigen concentration. The fluorescence intensity showed a tendency to decrease as the concentration of the target AFP antigen that was immobilized on the PET surface decreased (see Figure 6a). Furthermore, similar to the detection of the target DNA, the fluorescent intensity of an immunoassay prepared at a fluence of 5×10^{14} ions/cm^2 was higher in comparison to that prepared at a fluence of 1×10^{14} ions/cm^2. The lowest detectable concentration of the target antigen in this study was 16 ng/mL with the PET film prepared at a fluence of 5×10^{14} ions/cm^2.

To confirm the covalent immobilization via EDC/NHS coupling reaction between the carboxylic acid group and amine group, the immobilization of biomolecules in the presence and absence of EDC/NHS, the hybridization with noncomplementary DNA, and an immunoassay using prebound antigens or antibodies, were carried out. As shown in Figure S2 in the Supporting Information, the absence of a fluorescence signal from the films in the absence of EDC/NHS indicates that biomolecules were not covalently immobilized on the irradiated PET films. These results demonstrate that the covalent immobilization of biomolecules onto the irradiated PET surface and the detection selectivity for target biomolecules were clearly proved.

CONCLUSIONS

The functionalization of a poly(ethylene terephthalate) (PET) film surface was successfully carried out by ion irradiation. The results of Fourier transform infrared (FTIR), X-ray photoelectron spectroscopy (XPS), and water contact-angle measurements revealed that carboxylic acid groups were successfully generated on the irradiated regions of the PET surface and that their relative amounts were dependent on the fluence. For the fabrication of a biosensor platform, anthrax toxin probe DNA and liver-cancer-specific target α-fetoprotein (AFP) antigen were

covalently immobilized on the functionalized PET surfaces. The results of the fluorescence microscopic analysis showed that well-established micropatterns of biomolecules were successfully formed on the PET surface. It was also found that the fluorescent intensity of the micropatterns was clearly dependent on the concentration of the target DNA and AFP antigen. The lowest detectable concentrations of the target DNA and target AFP antigen in this study were determined to be 4 and 16 ng/mL, respectively, with the PET film prepared at a fluence of 5×10^{14} ions/cm^2. This study shows that surface functionalization of PET by ion irradiation is a promising strategy for the fabrication of biosensors on flexible polymeric substrates.

■ ASSOCIATED CONTENT

Supporting Information. Dependence of the fluorescence intensity on the concentration of the immobilized p-DNA on the irradiated PET surfaces (Figure S1), and fluorescence images obtained after hybridization with Cy3-labeled target DNA in the absence and the presence of EDC/NHS (Figure S2). (PDF) This material is available free of charge via the Internet at http://pubs.acs.org.

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