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Poly(acrylic acid)-Grafted Fluoropolymer Films for Highly Sensitive Fluorescent Bioassays

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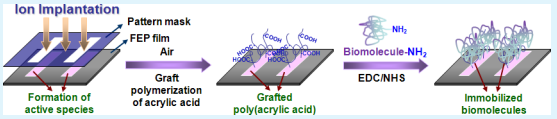
Supporting Information

ABSTRACT: In this study, a facile and effective method for the surface functionalization of inert fluoropolymer substrates using surface grafting was demonstrated for the preparation of a new platform for fluorescence-based bioassays. The surface of perfluorinated poly(ethylene-co-propylene) (FEP) films was functionalized using a 150 keV ion implantation, followed by the graft polymerization of acrylic acid, to generate a high density of carboxylic acid groups on the implanted surface. The resulting functionalized surface was investigated in terms of the surface density of carboxylic acid, wettability, chemical structure, surface morphology, and surface chemical composition. These results revealed that poly(acrylic acid) (PAA) was successfully grafted onto the implanted FEP surface and its relative amount depended on the fluence. To demonstrate the usefulness of this method for the fabrication of bioassays, the PAA-grafted FEP films were utilized for the immobilization of probe DNA for anthrax toxin, followed by hybridization with Cy3-labeled target DNA. Liver cancer-specific α-feto-protein (AFP) antigen was also immobilized on the PAA-grafted FEP films. Texas Red-labeled secondary antibody was reacted with AFP-specific primary antibody prebound to the AFP antigen using an immunoassay method. The results revealed that the fluorescence intensity clearly depended on the concentration of the target DNA hybridized to the probe DNA and the AFP antigen immobilized on the FEP films. The lowest detectable concentrations of the target DNA and the AFP antigen were 10 fg/mL and 10 pg/mL, respectively, with the FEP films prepared at a fluence of 3×10^{14} ions/cm^2.



KEYWORDS: *ion implantation, surface grafting, poly(acrylic acid), anthrax toxin, liver cancer, fluorescence*

INTRODUCTION

The fabrication of bioassays for various applications including biomedical diagnostics, biomolecule analysis, drug discovery, and environmental monitoring has attracted tremendous attention over the past decade because of their advantages such as fast and high-throughput detection of target molecules.[1−7] Most bioassays have generally been fabricated using inorganic substrates, such as silicon, metal, and glass.[8−10] However, the construction of bioassays on inorganic substrates has several drawbacks such as a time-consuming and expensive process in manipulation and fabrication, which can limit their diverse uses for practical applications.[11,12] In this respect, various polymers including poly(methyl methacrylate) (PMMA), polydimethylsiloxane (PDMS), polycarbonate (PC), polytetrafluoroethlyene (PTFE), fluorinated ethylene propylene copolymer (FEP), and polychlorotrifluoroethlyene (PCTFE) have been regarded as attractive alternative substrates for the fabrication of bioassays, biosensors, biochips, and microfluidic systems because of their several advantages over inorganic materials including their reduced cost, ease of fabrication, and feasibility of diverse functional groups amenable to modification.[13,14] Among these polymers, fluoropolymers have recently attracted a great deal of attention because of their excellent mechanical strength, thermal stability, and chemical inertness.[15,16] However, their strongly hydrophobic nature is not suitable for biological applications. Thus, these fluoropolymers should be subjected to surface functionalization to immobilize biological molecules.

Surface grafting is a promising methodology for the functionalization of polymeric substrates because of its unique merits, including the capability of providing highly dense functionalities for the covalent immobilization of biomolecules, chemical stability, and controllable introduction of grafted chains onto a surface without affecting the bulk properties.[17−19] Generally, since the total functional area in a whole surface is fixed, the sensitivity of biomedical devices is dependent on the total amount of biomolecules immobilized on the surface, which is directly connected with the performance of devices.

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Thus, surface grafting is one of the most powerful approaches for the high yield of immobilized biomolecules on account of its capability of highly dense functionalization. Several methods, including UV radiation, the use of a chemical initiator, plasma treatment, and high-energy radiation (γ-rays, ion beams, and electron beams), have been developed to introduce graft chains onto the surface of a polymer.[20−22] Among them, surface graft polymerization by ion implantation is an attractive method to functionalize the surface of a polymer for biological applications. This method offers several advantages because of the greater liner energy transfer and more straight penetration trajectory of an ion beam in comparison to the other techniques:[23,24] a surface-specific modification without detrimentally affecting the bulk properties, outstanding reliability and controllability, biocompatible processing, and many others.[25−28] Despite these benefits, to the best of our knowledge, the surface functionalization of inert fluoropolymers using ion implantation-induced graft polymerization has not yet been studied for the fabrication of fluorescent bioassays.

In this study, an efficient and facile functionalization method for the highly dense surface functionalization of fluoropolymers is presented to improve the sensitivity of fluorescent bioassays, which can offer high sensitivity and selectivity and does not require any chemicals or additional amplification steps. FEP films were surface-functionalized using the ion implantation-induced graft polymerization of acrylic acid. Biomolecules, such as anthrax toxin probe DNA and liver cancer-specific α-feto protein (AFP) antigen, were then immobilized on the functionalized surface. The quantitative molecular recognition capability of the fabricated bioassays for the detection of anthrax toxin and liver cancer was evaluated using a confocal laser scanner.

EXPERIMENTAL SECTION

Materials. Perfluorinated poly(ethylene-co-propylene) (FEP) films (100 μm thickness, Asahi Glass Co., Ltd.) were washed by ultrasonication in acetone for 20 min and dried in a vacuum oven at room temperature overnight prior to use. Acrylic acid (AA), Mohr's salt ($(NH_4)_2Fe(SO_4)_2$), 1,1-dipheyl-2-picryhydrazyl (DPPH), toluidine blue O (TBO), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and *N*-hydroxysuccinimide (NHS) were purchased from Aldrich Company and used without further purification. For the immobilization of biomolecules, other chemicals were reagent grade and used without further purification. All the oligonucleotides related to anthrax toxin used in this study were synthesized by Genotech Company (Korea). An oligonucleotide coding for the lethal factor (LF) subunit containing an amino group at its 3′ position with the sequence 5′-GGATTATTGTTAAATATT-GATAAGGAT-NH_2-3′, an oligonucleotide labeled with Cy3 at the 5′ position with the sequence 5′-Cy3-ATCCTTATCAATATTTAACAA-TAATCC-3′, and an oligonucleotide with the sequence 5′-Cy3-CAATACTCATCGGAGCAGTTCACCGAT-3′ were used as a probe-DNA (p-DNA), a complementary target DNA (c-DNA), and a noncomplementary DNA (nc-DNA), respectively. α-Feto-protein (AFP) antigen, mouse monoclonal AFP antibody, and Texas Red-labeled mouse IgG secondary antibody were purchased from Abcam Company (Cambridge, U.K.).

Surface Functionalization of FEP Films by Surface Grafting. FEP films were surface-functionalized by surface grafting according to the following procedure as previously described.[25,27] For surface activation, FEP films were implanted through a customized mask (steel use stainless (SUS), 1 × 2 mm rectangular space/6 mm pitch, dimension = 2.5 cm (width) × 7.5 cm (length) × 0.1 mm (height)) at room temperature with 150 keV Ne^+ ions at fluences ranging from 3×10^{14} to 9×10^{14} ions/cm^2. After implantation, the implanted FEP films were stored in air for 24 h for further oxidation. For surface graft polymerization, the implanted FEP films were placed in sealed glass vessels containing an aqueous solution of 20 vol % acrylic acid, 0.2 M H_2SO_4, and 0.1 wt % Mohr's salt. After it was purged with nitrogen gas for 30 min to remove oxygen, the grafting reaction was performed in a constant temperature water bath at 65 °C for 12 h. The resulting FEP films were extracted with water to eliminate the physisorbed monomer and polymer. The poly(acrylic acid) (PAA)-grafted FEP (FEP-g-PAA) films were then dried in a vacuum oven at 50 °C.

Characterization of the Surface-Functionalized FEP. The amount of peroxide groups generated on the FEP surface after ion implantation was measured using a well-known DPPH method described in the literature.[29,30] Briefly, the implanted FEP films stored in air for 24 h were immersed in a glass tube containing a DPPH solution in toluene (10^{-4} M). After purging with nitrogen, the glass tube was heated to 70 °C and kept at that temperature for 5 h to decompose the peroxide groups formed on the surfaces. The numbers of DPPH molecules binding to the formed radicals were calculated from the difference in the absorbance at 520 nm between the original and residual DPPH solutions using an S-1100 UV−Vis spectrophotometer (Scinco Co., Ltd.).

The density of carboxylic acid (COOH) groups on the FEP-g-PAA surface was quantified using a TBO staining method.[25] The FEP-g-PAA films (30 mm × 30 mm) were incubated in a TBO solution (0.5 mM, pH 10) at room temperature for 6 h. Afterward, the stained films were thoroughly rinsed with an excess amount of sodium hydroxide solution (pH 10) and dried in a vacuum oven at 40 °C. Afterward, the TBO molecules were desorbed from the surface by incubating the stained films in 50% acetic acid solutions, and the optical density of the TBO-desorbed solutions was then measured at 633 nm using a UV−vis spectrophotometer (MQX 200 model, Bio-Tek Instruments). The density of COOH groups on the FEP-g-PAA surface was calculated from the calibration curve of absorbance versus the TBO concentration on the assumption of a 1:1 stoichiometric complexation between carboxylic acid group and TBO molecule.

The FTIR-ATR spectra of the control, implanted, and PAA-grafted FEP surfaces were recorded using a Bruker Tensor 27 Fourier transform infrared spectrometer (FTIR) equipped with an attenuated total reflection (ATR) prism. X-ray photoelectron spectroscopy (XPS) was performed using a Thermo MultiLab 2000 instrument with Mg Kα radiation. The static water contact angles were measured by a sessile drop method using a Phoenix 300 contact angle analyzer (Surface Electro Optics Co., Korea). Each reported value is the average of five independent measurements.

Immobilization and Detection of DNA for Anthrax Toxin. To covalently immobilize p-DNA on the PAA-grafted FEP surface, a solution containing 15 mM NHS, 45 mM EDC, and 50 μg/mL of NH_2-modified p-DNA in deionized water, was applied over the PAA-grafted FEP films. After incubation overnight, the p-DNA-immobilized FEP films were washed with deionized water and used for hybridization with c-DNA or nc-DNA. The p-DNA immobilized films were incubated with Cy3-labeled c-DNA with concentrations ranging from 10 fg/mL to 1 μg/mL in a SSC hybridization buffer (5× saline sodium citrate (pH 7.4, 0.4 mg/mL BSA, and 0.1% SDS)) under a coverslip for 12 h at 37 °C. The hybridization of Cy3-labeled nc-DNA (50 μg/mL) on the p-DNA-immobilized films was also performed as a control experiment. The films were then thoroughly washed with 3 × SSC for 5 min, 2 × SSC for 5 min, and finally, with 1 × SSC for 5 min.

Immobilization of Detection of AFP Antigen and Antibody. To covalently immobilize antigen or antibody on the PAA-grafted FEP surface, we used EDC/NHS coupling reaction. A solution (10 μL) containing 15 mM NHS, 45 mM EDC, and target AFP antigen (10 pg to 10 μg) in deionized water was applied to the PAA-grafted FEP films overnight at room temperature. To block any residual active NHS ester groups, the antigen-immobilized FEP surface was treated with 1% BSA. For the detection of the immobilized AFP antigen, we carried out an immunoassay. A 10 μL primary antibody solution (10 μg/mL of anti-AFP primary antibody, 1% BSA, 0.5% triton X-100, and 0.05% sodium azide in pH 7.2 PBS) was applied to the antigen-immobilized FEP surface and allowed to interact with the immobilized antigen.

After a 6 h incubation period, the antigen/antibody complex was detected with a 10 μL solution of Texas Red-labeled secondary antibody (1 μg/mL of secondary antibody, and 1% BSA in pH 7.2 PBS) for 1 h. To confirm the selectivity of the antigen-immobilized surface, a solution of primary antibody-preblocked secondary antibody was prepared by adding a primary antibody solution (10 μg/mL) to a Texas Red-labeled secondary antibody solution (10 μg/mL). The prepared solution was allowed to interact with the primary antibody prebound to the antigen-immobilized surface for 6 h. Afterward the films were thoroughly washed with PBS and deionized water to remove the unbound antibody.

For the immobilization of antibody on the FEP surface, a solution (10 μL) containing 15 mM NHS, 45 mM EDC, and anti-AFP primary antibody (10 pg to 10 μg) was applied to the PAA-grafted FEP surface overnight at room temperature. The immobilized primary antibody was detected with a 1 μg/mL of Texas Red-labeled secondary antibody. After a 6 h incubation period with the secondary antibody solution, the films were thoroughly rinsed with PBS and water to remove free antibody.

Fluorescence Intensity Scanning and Data Analysis. A commercial confocal laser scanner, ArrayWoRx (Applied Precision Inc.), was used to investigate the fluorescence basal level of the implanted and PAA-grafted FEP films and to detect the target biomolecules. The fluorescence intensity was determined using ImageJ software (National Institute of Health, Bethesda, MD, U.S.A.) from the original images without further treatment.

RESULTS AND DISCUSSION

The efficient surface functionalization of inert hydrophobic FEP films based on radiation-induced surface grafting to generate abundant COOH groups is schematically illustrated in Figure 1.

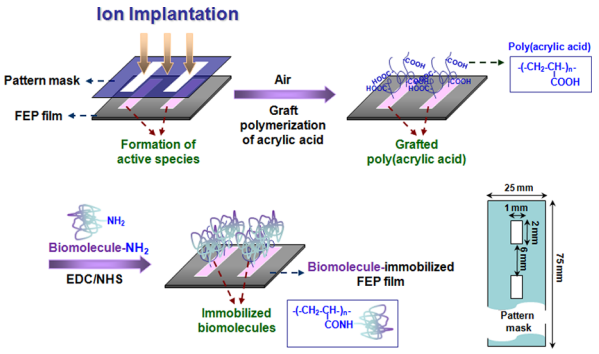

Figure 1. Schematic representation of biomolecular patterning by ion implantation.

The surface of FEP films was selectively activated by Ne^+ implantation to form active species such as peroxide groups, and acrylic acid was then thermally graft polymerized onto the activated surface. Finally, the resulting surface-functionalized FEP films were utilized for the immobilization and detection of biomolecules.

Surface Characterization. The concentration of peroxide groups generated on the implanted FEP surface at various fluences was quantified by a well-established DPPH method. The ion irradiation-induced generation of the peroxide groups on the FEP surface can be based on the fact that the free radicals on the surface generated by ion implantation under high vacuum reacted with oxygen in air after the implanted samples were taken out from the implanter's target chamber. The amount of the formed peroxides was dependent on the duration of the oxidation after the ion implantation, indicating the periods of storing in air as shown in Supporting Information Figure S1. After the optimized duration of storing in air, the peroxide groups were effectively generated on the implanted FEP surface and their concentration was increased with an increasing fluence as presented in Figure 2a.[29,30] This variation in the peroxide concentration with a fluence can be attributed to the fact that a higher number of radicals on the FEP surface formed by ion implantation at a higher fluence can give a rise to more oxidation via a reaction with oxygen in air, resulting in the higher formation of peroxide groups on the surface. Moreover, the concentration of the formed peroxide groups considerably influences the surface graft polymerization of acrylic acid because the formed peroxide groups act as an initiator for the graft polymerization. Thus, as shown in Figure 2b, the surface density of carboxylic acid groups on the PAA-grafted FEP surface increased with an increasing fluence and the highest surface density, 0.24 μmol/cm^2 (11.2 μg/cm^2), was achieved when the acrylic acid was graft polymerized on the implanted surface at a fluence of 9×10^{14} ions/cm^2.

The FTIR-ATR spectra of the control, implanted, and PAA-grafted FEP films are presented in Figure 3. The control FEP spectrum in Figure 3a shows characteristic bands corresponding to the stretching vibration of CF in the CF_2 group at 1201 and 1145 cm^{-1} and the stretching vibration of CF in the CF_3 at 985 cm^{-1}. There was no significant difference between the control and implanted FEP spectra as shown in Figure 3b. When the FEP surface was implanted with 150 keV Ne^+ ions to generate the peroxides, the penetration depth of Ne^+ ions into the FEP estimated using the TRIM98 simulation program was around 500 nm. The peroxides can be generated within the 500 nm

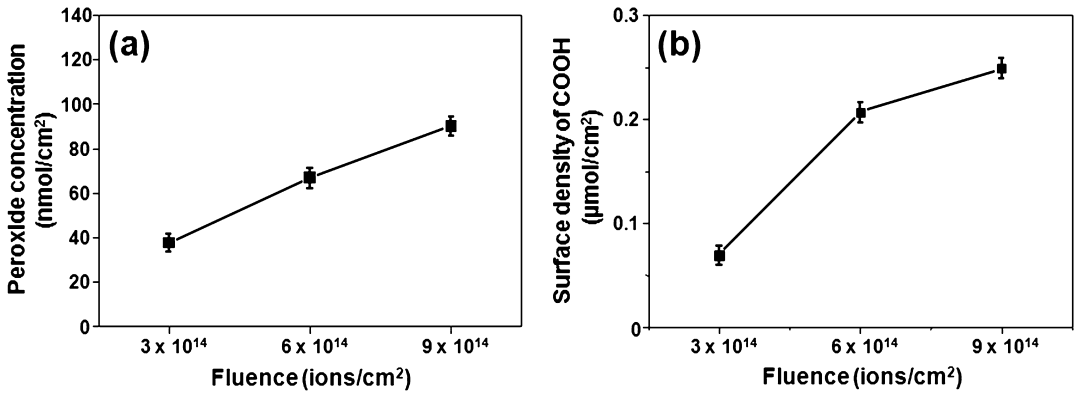

Figure 2. Peroxide concentration (a) and the surface density of carboxylic acid groups (b) as a function of the fluence.

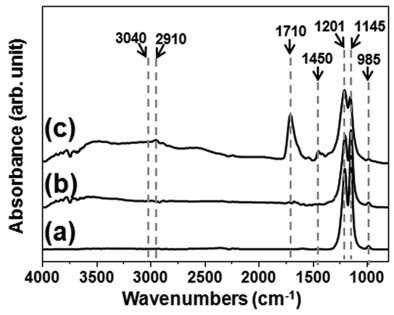

Figure 3. FTIR-ATR spectra of the control (a), implanted (b), and PAA-grafted FEP (c) films.

depth from the ultimate surface of the FEP. Thus, the shallow formation depth and the small amount of peroxides on the implanted FEP surface could not be detected by ATR-FTIR due to its limited resolution. On the other hand, in the PAA-grafted FEP spectrum (Figure 3c), new bands were observed at 3420 (the broad band of hydrogen-bonded OH in COOH), 2910 (the stretching vibration of C−H in CH_2), 1710 (the stretching vibration of C═O in COOH), and 1450 cm^{-1}(the bending vibration of C−H in CH_2, which could be assigned to the grafted PAA chemical structure. As a whole, these results indicate that the PAA was grafted onto the implanted FEP surface.

The change in the wettability on the FEP surface brought about by implantation and graft polymerization was investigated by contact angle measurements (Figure S2 in the Supporting Information). The hydrophobic surface of the control FEP exhibited a water contact angle of 100°, while the contact angle was decreased to a minimum of 86° after implantation due to the formation of oxidized surfaces induced by implantation. The surface graft polymerization of the irradiated FEP with a hydrophilic AA further decreased the contact angle from 58° to 51° with an increase in the ion fluence and the contact angle was clearly dependent on the surface density of carboxylic acid groups on the PAA-grafted FEP. This result indicates that the hydrophobic FEP surface was converted into a more hydrophilic surface via the successful graft polymerization of hydrophilic AA onto the implanted surface.

To further investigate the changes in the surface chemical composition of FEP after the sequential surface treatment by implantation and graft polymerization, an XPS analysis was employed and the results are presented in Figure 4. In comparison to that of the control FEP, the [O]/[C] atomic ratio of the implanted FEP surface increased with an increasing fluence whereas the [F]/[C] atomic ratio decreased due to the occurrence of oxidation and defluorination by implantation

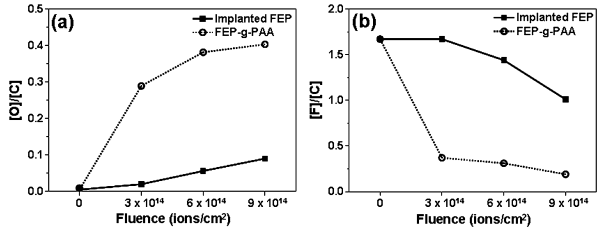

Figure 4. [O]/[C] and [F]/[C] ratios of the implanted (a) and PAA-grafted (b) FEP films obtained by XPS analysis as a function of the fluence.

(Figure S10 in the Supporting Information). After surface graft polymerization, the [O]/[C] atomic ratio further increased in comparison to that of the implanted FEP and, in reverse, the [F]/[C] atomic ratio significantly decreased. Accordingly, this result indicated that the PAA was successfully grafted onto the FEP surface.

Fluorescence Detection of Target DNA. Anthrax is an acute disease of mammals caused by the anthrax toxin, which is a potential biological warfare agent.[31,32] Hapatocellular carcinoma (HCC) is also considered one of the most highly malignant liver cancers throughout the world.[33] An oligonucleotide associated with anthrax and an alpha-fetoprotein (AFP) antigen associated with liver cancer have been generally used for the early diagnosis of these diseases due to their specific affinity.[31−36] On the basis of the results of the basal level of the fluorescence signal of the implanted FEP and PAA-grafted FEP films, the film prepared at a fluence of 3×10^{14} ions/cm^2 was selected for the fabrication of fluorescence-based bioassays (Figure S4 in the Supporting Information). To demonstrate the applicability of this surface functionalization method for the preparation of DNA-based bioassays, the PAA-grafted FEP surface was used for the detection of anthrax toxin target DNA. Capture p-DNA was first immobilized by amide bond formation between the amine groups of the capture DNA and the carboxylic acid groups on the functionalized FEP surface via EDC/NHS coupling chemistry as shown in Figure 5 and Supporting Information Figure S9. The presence of the p-DNA on the FEP surface was investigated by the hybridization of the target DNA. As shown in Figure 5, to determine the limit of detection, various concentrations of DNA solutions were applied to the p-DNA immobilized FEP surface. Figure 5 shows the relationship between the fluorescence intensity and the target DNA concentration. It can be clearly seen from Figure 5 that the fluorescence intensity decreased with decreasing concentration of the target DNA and the lowest detectable concentration was 10 fg/mL (1.15 fM), which is much lower than that on the ion beam-induced direct functionalization of a polymer substrate[36] and comparable to that on the surface-functionalized substrate by the surface treatment and immobilization of functional materials.[37−43] Moreover, hybridization with nc-DNA showed a negligible fluorescence signal (Figure S7 in the Supporting Information), which gives an additional proof for the selectivity of the p-DNA-immobilized surface. As shown in Supporting Information Figure S6, the fluorescence intensity increased with an increase in the concentration of the probe DNA. These results demonstrated that an FEP surface with a high density of functional groups can be beneficial for the fabrication of quantitatively measurable DNA-based bioassays.

Fluorescence Detection of Target Antigen and Antibody. The functionalized surface with carboxylic acid groups was used for the fabrication of immunoassays for liver cancer. Liver cancer specific AFP antigen with various concentrations was first immobilized on the PAA-grafted FEP surface through amide bond formation by EDC/NHS chemistry as shown in Figure 6a and Supporting Information Figure S9. For the detection of the immobilized AFP antigen, all experimental procedures followed an antigen−antibody immunoassay method. Figure 6a shows the fluorescence microscopic images of the Texas Red-labeled secondary antibody bound to the AFP antigen-primary antibody complex immobilized on the FEP surface. The fluorescence intensity decreased with decreasing concentration of the immobilized antigen. The lowest

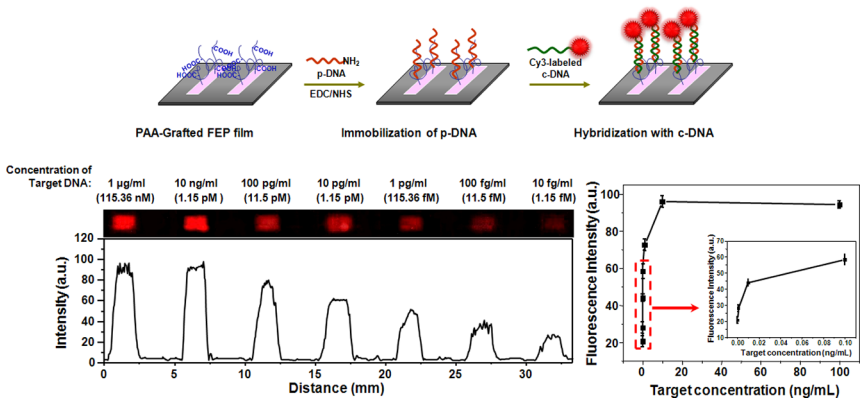

Figure 5. Schematic illustration and fluorescence intensity of the detected target anthrax toxin c-DNA on the PAA-grafted FEP films.

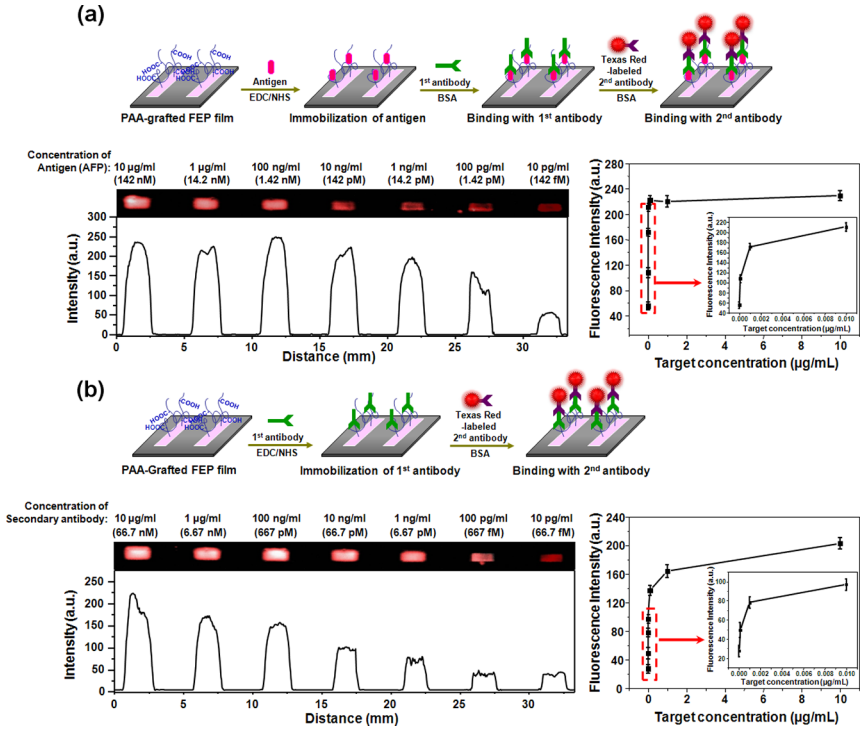

Figure 6. Schematic illustration and fluorescence intensity of the detected liver cancer-specific target AFP antigen (a) and the detected secondary antibody bound to the primary antibody (b) on the PAA-grafted FEP films.

detectable concentration of the antigen was found to be 10 pg/mL (142 fM) (Figure 6a). This detection limit in this system is comparable to the limits of around 5 pg/mL reported in the literatures based on various methodologies, including radioimmunoassay, chemiluminescence assay, fluorescence-based immunoassay, electrochemical immunoassay,[44−47] and enzyme-linked immunoassay, and satisfied the cutoff value of 25 ng/mL required for practical applications.[48,49] Moreover, the interaction between the secondary antibody preblocked with primary antibody and primary antibody prebound to the antigen-immobilized surface showed a negligible fluorescence signal, confirming the selectivity of the immobilized target antigen (Figure S8 in the Supporting Information).

The PAA-grafted FEP surface was also used to immobilize antibody as presented in Figure 6b. The immobilized antibody on the FEP surface was detected by secondary antibody with various concentrations. As in the previously mentioned results of the antigen immobilization, the fluorescence intensity showed a tendency to decrease with decreasing concentration of the anti-AFP antibody (Figure 6b). Therefore, these results demonstrate that an FEP surface with a high density of functional groups is effective for the immobilization of proteins, which is applicable to quantitative immunoassays used to detect target biomolecules such as antibodies, antigens, and other protein molecules (Figure S5 in the Supporting Information).

CONCLUSIONS

In this study, an effective surface functionalization of inert fluoropolymer substrates by ion beam-based surface grafting was successfully demonstrated to fabricate a new platform for fluorescence-based bioassays. Surface graft polymerization of acrylic acid by ion implantation generated a high density of carboxylic acid groups on an FEP surface. The results of the surface analysis revealed that the PAA-grafted FEP surface was successfully synthesized and that the surface density of the grafted PAA depended on the fluence. For practical use of the

resulting PAA-grafted FEP for the detection of disease-related target biomolecules, anthrax toxin p-DNA and a liver cancer-specific target AFP antigen were immobilized on the PAA-grafted regions of the FEP surface. The p-DNA-immobilized FEP surface allowed detection of the target DNA with a concentration of as low as 10 fg/mL (1.15 fM). The lowest detectable concentration of the target AFP antigen using this method was found to be 10 pg/mL (142 fM). Therefore, surface functionalization by ion implantation-induced graft polymerization is diversely applicable to various kinds of fluorescence-based bioassays, biosensors, biochips, and microfluidic devices for the detection of target biomolecules such as DNA, antibodies, antigens, and other biomolecules.

■ ASSOCIATED CONTENT

Supporting Information

Additional information and figures as specified in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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