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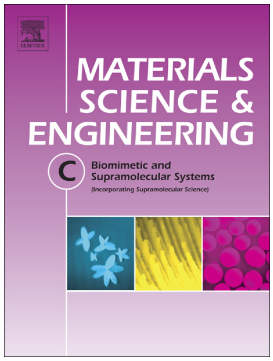

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Boronate-Functionalized Hydrogel as a Novel Biosensing Interface for the Glycated Hemoglobin A1c (HbA_{1c}) based on the Competitive Binding with Signaling Glycoprotein

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Abstract

According to recent increases in public healthcare costs associated with diabetes mellitus, the development of new glycemic monitoring techniques based on the biosensing of glycated hemoglobin A1c (HbA_{1c}), a promising long-term glycemic biomarker, has become a major challenge. In the development of HbA_{1c} biosensors for point-of-care applications, the selection of an effective biorecognition layer that provides a high reaction yield and specificity toward HbA_{1c} is regarded as the most significant issue. To address this, we developed a novel HbA_{1c} biosensing interfacial material by the integration of boronate hydrogel with glass fiber membrane. In the present study, a new boronate-functionalized hydrogel was designed and spatio-selectively photopolymerized on a hydrophilic glass fiber membrane by using N-hydroxyethyl acrylamide, 3-(acrylamido)phenylboronic acid, and bis(N,N'-methylene-bis-acrylamide). Using this approach, the boronic acid group, which specifically recognizes the cis-diol residue of glucose on the HbA_{1c} molecule, can be three-dimensionally coated on the surface of the glass fiber network with a high density. Because this network structure of boronate hydrogel-grafted fibers enables capillary-driven fluid control, facile HbA_{1c} biosensing in a lateral flow assay concept could be accomplished. On the proposed HbA_{1c} biosensing interface, various concentrations of HbA_{1c} (5–15%) in blood-originated samples were sensitively measured by a colorimetric assay using horseradish peroxidase, a glycoenzyme can generate chromogenic signal after the competitive binding against HbA_{1c} to the boronic acid residues. Based on the demonstrated advantages of boronate hydrogel-modified membrane including high analytical performance, easy operation, and cost-effectiveness, we expect that the proposed biorecognition interfacial material can be applied not only to point-of-care HbA_{1c} biosensors, but also to the quantitative analysis of other glycoprotein biomarkers.

Keywords: *biosensor; biorecognition layer; glycated hemoglobin; glycemic monitoring; boronic acid; hydrogel*

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1. Introduction

For the management of diabetes mellitus, glycemic monitoring is considered a cornerstone [1]. According to the recent increase in the diabetes patient population, social and clinical demands for more efficient and accurate glycemic monitoring technology have drastically increased [1, 2]. Therefore, glycated hemoglobin A1c (HbA_{1c}) has received attention in the clinical field as a new glycemic biomarker for the diagnosis of diabetes; it can overcome the drawbacks of conventional methods for blood glucose measurement, such as the requirement for periodical repetitive measurements with specific dietary conditions [3-11]. HbA_{1c}, representing a major fraction of glycated hemoglobin in the blood, is formed by the irreversible Amadori rearrangement reaction of blood glucose to the N-terminal valine residues of hemoglobin β-chains [4, 5]. Since the HbA_{1c} formation rate is proportional to the blood glucose level, the concentration of HbA_{1c} reflects the average glycemic profiles of an examinee over two months, corresponding to the lifespan of human erythrocytes. Thus, the measurement of HbA_{1c} allows long-term glycemic monitoring with minimal interference from sudden glycemic variation resulting from temporary physiological changes [5-7]. The concentration of HbA_{1c} is defined as the percentage of glycated hemoglobin against the total hemoglobin in the blood. Generally, a normal non-diabetic HbA_{1c} range is 5–6%, while the HbA_{1c} levels in diabetic patients are in excess of 7% [5-7]. An HbA_{1c} level of around 6.5% indicates prediabetes, requiring more aggressive glycemic control and management. Therefore, for the early diagnosis and active management of diabetes, the accurate monitoring of changes in HbA_{1c} concentration in the 6–7% range is highly recommended [5-7]. Conventional analytical methods, such as ion-exchange chromatography and affinity chromatography, are widely used for clinical HbA_{1c} analyses [7-11]. However, these methods

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can only be used in a centralized clinical laboratory owing to their inherent limitations, including high cost, bulk, and the need for a trained operator. Therefore, recently, the demands of diabetes patients for a user-friendly point-of-care (POC) HbA_{1c} analysis device have soared. Thus, the development of accurate HbA_{1c} biosensing technology has attracted attention in both academia and in vitro diagnostics industries [11-15]. Accordingly, numerous HbA_{1c} biosensors based on plasmonic, luminometric, and electrochemical methods have been developed [16-18]. Despite those academic studies for HbA_{1c} biosensing exhibit good analytical performance for the HbA_{1c} detection, their practical applications to clinical field are limited by several problems, such as comparably low sensitivity, insufficient reproducibility, low cost-effectiveness, and complicated equipment configurations [8, 11]. Since these problems are closely related to the type or property of the employed materials in HbA_{1c} biosensor interface, the development of novel HbA_{1c} biosensing interfacial material, which can overcome those limitations, is mandatory to materialize a clinically applicable HbA_{1c} biosensor.

Generally, for the development of an accurate biosensor, the construction of effective biorecognition layer providing a high reaction yield for target analytes is fundamental procedure. From this point of view, the biorecognition molecules, such as antibodies, enzymes, nucleic acids, and biochemical ligands, should exist on the biosensing interfacial layer at a high density [19-21]. In conventional biosensors, biorecognition molecules are generally immobilized on 2-dimensional (2D) planar sensing substrates (e.g., noble metal, silicon, glass, or polymeric surfaces) as a monolayer using surface modification methods, such as the self-assembled monolayer technique. In this case, the amount of biorecognition molecules on the interface is spatially limited by the 2D monolayer structure [19-21]. This could induce reductions in biorecognition efficiency for target analytes and assay sensitivity.

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Therefore, in order to implement more sensitive biosensing, the development of a new biorecognition interface, wherein biorecognition molecules can be arranged at a high density in a 3-dimensional (3D) configuration within a limited area, is required [20-22]. Accordingly, to develop a 3D HbA_{1c} biorecognition interfacial layer, we referred to membrane-based analytical methods [23, 24]. The membrane-assisted lateral flow immunoassay with a paper-based analytical device (PAD) has become a popular analytical method for POC diagnostics owing to its cost-effectiveness, ease of fabrication, and facile operation [23, 24]. The most noticeable feature with respect to the biorecognition interface is the inherent 3D structure (e.g., porous membrane and fibrous paper) allowing the immobilization of sufficient biorecognition molecules, a large surface-area-to-volume ratio, and facile capillary-driven fluidics control. We hypothesized that if certain biorecognition molecules for HbA_{1c} can be immobilized on the 3D microstructure of the membrane at a high density, we can efficiently construct a 3D HbA_{1c} biosensing interface that provides high sensitivity and operational convenience. There are two main types of biorecognition molecules utilized in HbA_{1c} biosensors, i.e., monoclonal HbA_{1c} antibodies and boronic acid derivatives that can specifically bind to the cis-diol residue of glucose on the HbA_{1c} structure [15, 25-27]. In this study, we employed boronic acid as a biorecognition molecule for the HbA_{1c} detection owing to its high chemical stability, inexpensiveness, ease of modification, and good reproducibility in HbA_{1c} assays. However, to the best of our knowledge, the application of a boronate-modified membrane for HbA_{1c} biosensing has not yet been reported. To address this, we introduced a boronate hydrogel (Fig. 1a) as a 3D HbA_{1c} biosensing interfacial material for implantation in the hydrophilic membrane.

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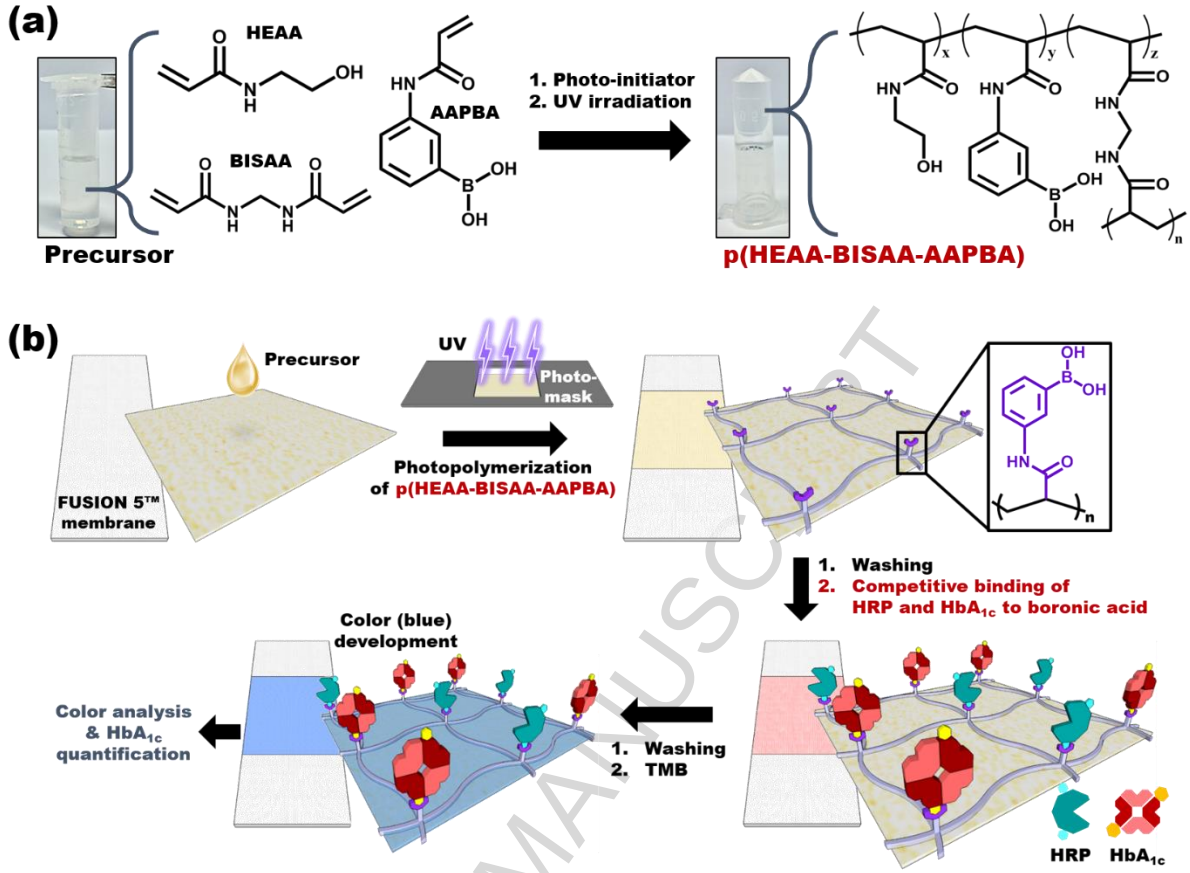

Figure 1. (a) Chemical structures and images of the boronate hydrogel precursor (left panel) and the synthesized p(HEAA-BISAA-AAPBA) boronate hydrogel (right panel). (b) Schematic illustration of fabrication procedures for the boronate hydrogel-patterned membrane strip and the colorimetric biosensing of HbA_{1c}.

A boronate hydrogel is 3-dimensionally crosslinked hydrophilic polymer network that is covalently modified with boronic acid residues [26-30]. Based on the sugar-recognition property of boronate groups in the hydrogel, the boronate hydrogel is used extensively for controlled drug release and the quantitative analysis of diol-containing molecules (e.g., sugar, nucleotides, and glycoproteins) [27-30]. We anticipated that abundant boronate residues, three-dimensionally positioned in the hydrophilic polymer backbone of the hydrogel, can be utilized as a 3D biorecognition layer for accurate and facile HbA_{1c} biosensing. To construct

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the boronate hydrogel pattern on the membrane structure, similar to a test line for a lateral flow immunoassay strip, the acrylamide compound-based in situ photopolymerization method was employed. In the presence of a photoinitiator and UV light, acrylamide-based compounds, such as N-hydroxyethyl acrylamide (HEAA) and 3-(acrylamido)phenylboronic acid (AAPBA), can be in situ polymerized on various substrates, including membranes or papers. Additionally, by controlling the UV-exposed area, the hydrogel can be spatio-selectively polymerized only on the desired region [31, 32]. Based on these features, a boronate hydrogel was spatio-selectively modified on the hydrophilic glass fiber membrane (Fusion5™) strip to construct a patterned HbA_{1c} biosensing interface, as shown in Fig. 1b.

In the present study, the chemical properties, composition, and mechanical structure of the boronate hydrogel-based HbA_{1c} biosensing interface were characterized using spectroscopic equipment and electron microscopy. Additionally, its selectivity and recognition capability as a biosensing interfacial layer toward HbA_{1c} were evaluated using a horseradish peroxidase (HRP)-based enzymatic colorimetry method. Finally, on the boronate hydrogel-patterned membrane, colorimetric competitive affinity biosensing of HbA_{1c} was conducted using human blood-derived samples, as depicted in Fig. S1. The details of the newly developed biosensing interface and its application to HbA_{1c} detection are summarized herein.

2. Experimental

2.1. Chemicals and equipment

N-Hydroxyethyl acrylamide (HEAA), 3-(acrylamido)phenylboronic acid (AAPBA), and 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure®2959) were acquired from

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Aldrich. Dimethyl sulfoxide (DMSO) was obtained from Sigma. Zinc chloride ($ZnCl_2$) was purchased from Sigma-Aldrich. HRP was acquired from Toyobo Enzymes. Polyethylene glycol (PEG, MW 3400) was purchased from Polysciences. Bis(N,N'-methylene-bis-acrylamide) (BISAA) was obtained from BioRad. Glycated hemoglobin A1c (HbA_{1c}) was purchased from Fluka and non-glycated normal adult hemoglobin (HbA_0) was purchased from Fitzgerald. The Fusion5™ glass fiber membrane was acquired from Whatman. Vivid™ 170 nitrocellulose (NC) membranes with 0.2-μm micro-pores were obtained from Pall Life Sciences. 3,3',5,5'-Tetramethylbenzidine (TMB) solution containing hydrogen peroxide (H_2O_2) was purchased from Pierce. Whole blood samples (in lithium-heparin tube) were purchased from Innovative Research. Phosphate-buffered saline solution containing 0.1 M sodium phosphate and 0.15 M NaCl (pH 7.2) was prepared in doubly distilled and deionized water (DDW) with a specific resistance of greater than 18 MΩ•cm. For membrane washing, a washing buffer solution (WBS, pH 8.0) containing 0.05 M TRIS, 0.1% Triton X-100, and 0.01% PEG (MW 3400) was prepared. For the dilution of HRP and D-sorbitol, a reaction buffer solution (RBS, pH 8.0) containing 0.05 M phosphate, 0.1% Triton X-100, and 0.01% PEG (m/w 3400) was prepared. All other materials and solvents were of the highest quality available and purchased from regular sources. Images of the color-developed test strips were recorded and documented using a digital camera. The HemoCue® Hb201+ (HemoCue) was utilized to quantify the concentration of total hemoglobin in the whole blood. Tosoh G8 HPLC analyzer (Tosoh Bioscience) was employed to evaluate the inherent HbA_{1c} concentration in the whole blood samples.

2.2. Synthesis of the boronate hydrogel

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As a monomer and a crosslinker, 40% (w/v) HEAA and 2% (w/v) BISAA were prepared, respectively. For photopolymerization, as a photoinitiator (PI), 0.5% (w/v) Irgacure®2959 was prepared. AAPBA (2.5% w/v) was prepared as a boronate compound. All precursors were dissolved in DMSO and prepared in amber vials. Prior to photopolymerization, 1 mL of HEAA, 1 mL of BISAA, 1 mL of PI, and 2 mL of AAPBA solution were mixed under darkroom conditions. The final concentrations of HEAA, BISAA, PI, and AAPBA in the mixed precursor solution were 8%, 0.4%, 0.1%, and 1% (w/v), respectively. Then, the mixture was transferred to transparent test tubes and exposed to UV light (6 W, λ = 365 nm) for 20 min. For the negative control, a boronate-absent hydrogel containing HEAA (8 % w/v), BIS (0.4% w/v), and PI (0.1% w/v) was prepared.

2.3. Characterization of the synthesized boronate hydrogel

To validate the functionalization of the boronate moiety in the synthesized hydrogel, physicochemical properties were analyzed by ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectroscopy and Fourier transform infrared (FT-IR) spectroscopy. To remove the unreacted precursor and DMSO solvent from the sample, the synthesized boronate hydrogel was mashed and dialyzed against 1 L of DDW using SnakeSkin™ dialysis tubing exhibiting a 3.5K molecular weight cut-off for 48 h. After dialysis, the resulting boronate hydrogels were lyophilized for 20 h. The lyophilized hydrogel sample was ground into a powder and stored at 4°C until use. As a negative sample, a hydrogel lacking boronate was prepared following the same methods and analyzed. For the FT-IR analysis, the prepared hydrogel powder samples were analyzed using a Magna-IR™ 550 spectrometer. To obtain the ^{1}H-NMR spectra, the prepared hydrogel powder samples were dissolved in deuterated water (D_2O) and analyzed

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using the Varian Mercury Plus 400 NMR instrument (400 MHz) at 25°C.

2.4. Construction and characterization of the boronate hydrogel-modified glass fiber membrane

To implant the boronate hydrogels onto the glass fiber membrane strip, a photopolymerization technique was employed. Prior to the photopolymerization of the boronate hydrogel, the Fusion5™ glass fiber membrane was cut into squares (2 cm × 2 cm in dimension) and washed using DDW. After the membrane was dried at room temperature, 160 μL of the prepared hydrogel precursor solution containing HEAA, BISAA, PI, and AAPBA was applied. Then, the precursor-applied membranes were exposed to UV light (6 W, λ = 365 nm) for 20 min. For the negative control, the AAPBA-absent hydrogel precursor was applied to the Fusion5™ membrane and photopolymerized in the same manner. After photopolymerization, the resulting membranes were washed with DMSO, ethanol, and DDW in sequence to remove excessive unreacted precursors. The washed hydrogel-modified membranes were dried at room temperature and stored at 4°C until use. To confirm whether the boronate groups were introduced to the hydrogel structure on the membrane, X-ray photoelectron spectroscopy (XPS) was carried out.

2.5. Analysis of the glycoprotein binding capacity of the boronate hydrogel-modified membrane

To demonstrate the glycoprotein binding capacity of the synthesized boronate hydrogel, an enzymatic colorimetric assay was carried out utilizing HRP, a glycoenzyme that generates a

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chromogenic signal. Prior to the analysis, samples with various concentrations of HRP (0, 1, 2, 4, and 8 μg/mL) were prepared in RBS. To the prepared boronate hydrogel-modified Fusion5™ glass fiber membranes, 10 μL of the HRP sample was applied and allowed to react for 5 min. After the rinsing step using WBS, 20 μL of TMB substrate solution containing hydrogen peroxide (H_2O_2) was treated for 5 min. When the reaction times of TMB and HRP were more than 5 min each, the chromogenic reaction product which were derived from the membrane-adsorbed minute concentrations of HRP through the nonspecific binding (NSB), were accumulated and appeared as a visible signal even in the boronate-absent membrane. Therefore, to minimize the effect of non-specific signals derived from NSB, each reaction time for HRP and TMB treatment were fixed as 5 min. Then, the images of color-developed membranes were obtained using a digital camera. As a negative control, Fusion5™ membranes modified with the AAPBA-absent hydrogel were utilized.

2.6. HbA_{1c} quantification on the boronate hydrogel-modified membrane

To certify whether the boronate hydrogel-modified membrane can be utilized in HbA_{1c} assays, an enzymatic colorimetric analysis of HbA_{1c} was conducted. As a sample, commercially available purified HbA_{1c} reagent were diluted with RBS and prepared in various concentrations (0, 0.1, 0.3, and 0.6 μg/mL). For the control sample, 4 μg/mL purified non-glycosylated hemoglobin (HbA_0) reagent was prepared in RBS. Each sample was mixed with 8 μg/mL HRP solution in a 1:1 volume ratio and applied to the prepared boronate hydrogel-modified Fusion5™ glass fiber membranes for 5 min. After rinsing with WSB, the TMB substrate solution was applied and allowed to react for 5 min. Images were obtained using a digital camera.

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2.7. Fabrication of a boronate hydrogel-patterned membrane strip for the HbA_{1c} analysis

To fabricate the HbA_{1c} biosensing interfacial layer on the membrane strip for a lateral flow assay, as shown in Scheme S1, the boronate hydrogel was locally patterned on the Fusion5™ glass fiber membrane using a photomask made with black aluminum foil. Prior to photopolymerization, the Fusion5™ glass fiber membrane was cut in rectangles (3 cm × 1 cm) and washed using DDW. After drying, 120 μL of the hydrogel precursor solution containing HEAA, BISAA, PI, and AAPBA was applied to the membrane strips and allowed to fully absorb in the membrane. For the patterned photopolymerization of the boronate hydrogel, a photomask containing a square window (1 cm × 1 cm) was placed on the center of the precursor-applied membrane strips. Then, UV light (6 W, λ = 365 nm) was applied to the photomask-staged membrane strip for 20 min. After photopolymerization, the resulting membranes were washed with DMSO, ethanol, and DDW in sequence to remove unreacted precursors. For the negative control, the AAPBA-absent hydrogel precursor was applied to the Fusion5™ membrane strip and locally photopolymerized in the same manner. The washed hydrogel-patterned membrane strips were dried at room temperature and stored at 4°C until use.

2.8. Characterization of the boronate hydrogel-patterned glass fiber membrane strip

To demonstrate the localized patterning of the boronate hydrogel on the membrane strip, a surface morphology analysis and a surface functionality analysis were carried out using scanning electron microscopy (SEM) and energy-dispersive spectroscopy (EDS), respectively.

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Using SEM and EDS, a photopolymerized region and an un-exposed region in a single boronate hydrogel-patterned membrane strip were analyzed and compared. As a control, an untreated pristine Fusion5™ membrane strip was examined.

2.9. Preparation of whole blood-derived HbA_{1c} samples

For the preparation of whole blood-based HbA_{1c} samples, the hemoglobin fraction was separated from whole blood samples using our previously reported Hb separation technique [33, 34]. Briefly, using a surfactant and zinc ions, the hemoglobin fraction was specifically precipitated in the human whole blood hemolysate. Then, using the nitrocellulose membrane and a hard-disk drive, the Hb fraction was re-collected from various blood components and transferred to phosphate-buffered saline. Since the tested human whole blood sample has an inherent HbA_{1c} concentration of 5.8% (as measured using the Tosoh G8 HPLC analyzer), the separated Hb fraction from whole blood sample was spiked with the purified HbA_0 and HbA_{1c} reagents at concentrations of 4.6, 7.7, 9.6, 11.5, 13.3 and 15.2%. Each sample was prepared to a total hemoglobin concentration of 8 μg/mL. This study was approved by the Institutional Review Board (IRB, 201309-HM-EX-001) at Ajou University.

2.10. Quantitative analysis of whole blood-derived HbA_{1c} samples

Using the developed boronate hydrogel-patterned membrane strip and HbA_{1c} biosensing strategy, a quantitative analysis of HbA_{1c} in spiked whole-blood lysate samples was performed, as shown in Scheme S3. Prior to the assay, samples with various concentrations of HbA_{1c} (4.6%, 5.8%, 7.7%, 9.6%, 11.5%, 13.3% and 15.2%) were mixed with 8 μg/mL HRP

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solution at a 1:1 volume ratio. Then, 40 μL of the solution was applied to the boronate hydrogel-patterned region on the HbA_{1c} test strips and allowed to react for 5 min. During this step, HbA_{1c} and HRP competitively bind to the boronate residues, while HbA_0 does not bind (Fig. S1). To remove the unreacted HbA_0 and excessive HRP in the boronate hydrogel interface, a capillary force-mediated washing procedure was used. As shown in Scheme S3, 3 mL of WBS was applied to the left side of the membrane and a water-absorption pad was applied to the right side of the membrane. In this procedure, the WBS migrated to the right-hand side via capillary force on the glass fiber membrane and the liquid-wicking property of the absorption pad. As a consequence, unreacted proteins in the hydrogel-modified region of the membrane are rinsed away by the WBS flow. After washing with WBS, the TMB substrate solution was added to the resulting membranes for 5 min and images of color-developed strips were obtained using a digital camera. For the quantification of color intensity in the resulting strips, the resulting images were analyzed by the Adobe Photoshop software. An area of 106 pixels in width and 106 pixels in height was selected in the color-developed region of the strip image. By using the color profiling function of the Photoshop software, allowing a quantitative analysis of red, green, and blue (RGB) values, the blue values of each pixel (total 11,236 pixels) in the selected area were collected and average. Based on the registered averaged values, a calibration curve for HbA_{1c} analysis were finally plotted [35]. As a negative control, whole blood based-HbA_{1c} samples were analyzed using Fusion5™ membrane strips modified with the p(HEAA-BISAA) hydrogel in the same manner.

3. Results and discussion

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3.1. Design of the boronate hydrogel

In this study, to establish an HbA_{1c} biosensing interface that can be integrated on commercialized glass fiber membranes for lateral flow assays, a new boronate hydrogel was designed and synthesized. For the facile polymerization of the hydrogel, acrylamide compounds were utilized as precursor materials. To construct a hydrophilic hydrogel polymer backbone with minimal non-specific binding (NSB) to biomolecules, HEAA containing hydroxyl residue in its structure was employed as a monomer precursor. As a crosslinker to bridge each polymer backbone chain, BISAA containing two acrylic double bonds in its structure was used. To introduce the boronic acid functional group to the hydrogel structure for the specific recognition of carbohydrate moieties of HbA_{1c} and HRP, AAPBA was utilized. As a polymerization initiator (PI), Irgacure®2959, a photoinitiator, was utilized. As shown in Fig. 1a (left panel), the precursor solution containing HEAA (8% w/v), BISAA (0.4% w/v), PI (0.1% w/v), and AAPBA (1% w/v) was not polymerized and remained in the liquid phase in the absence of UV light. When the precursor mixture was exposed to UV light, PI decomposed to radicals. By the generated radicals, the polymerization of acrylic double bonds in the precursor compounds proceeded as shown in Fig. 1a (right panel), and the p(HEAA-BISAA-AAPBA) hydrogel polymer formed. The synthesized hydrogel after polymerization exhibited transparency and a pale yellow color induced by the solvent (DMSO). This color disappeared after the dialysis procedure. To confirm whether the boronate compounds were functionalized, the synthesized boronate hydrogel was characterized by FT-IR and ^{1}H-NMR.

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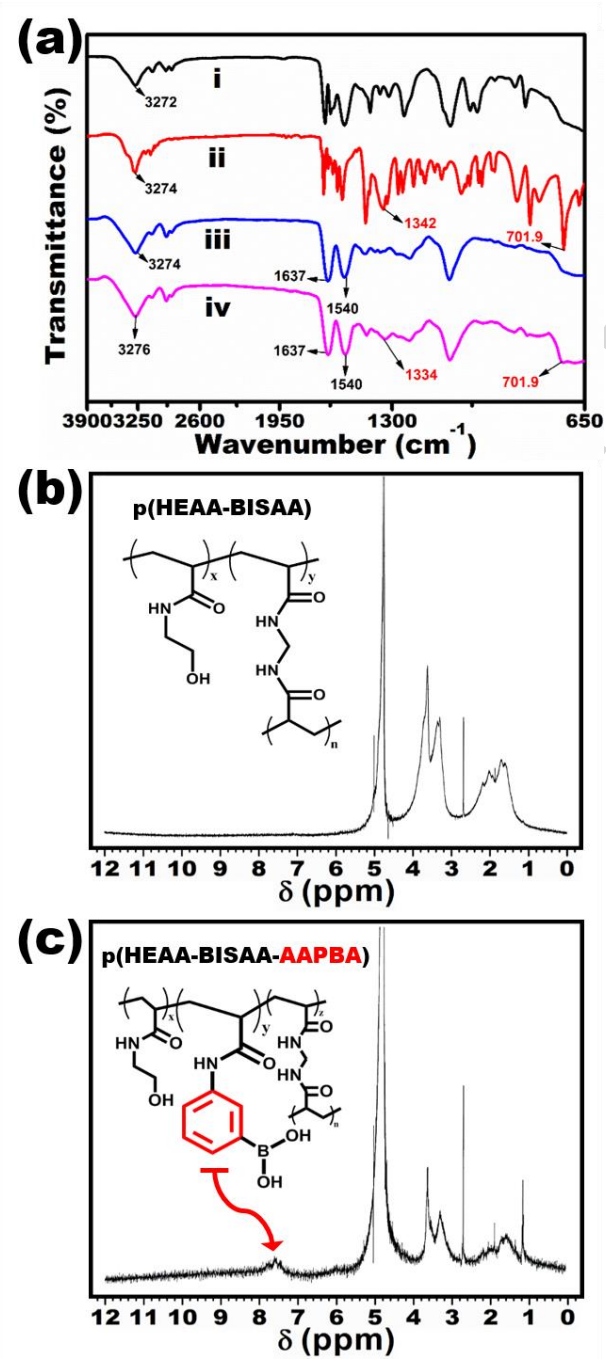

Figure 2. (a) FT-IR spectra for i) HEAA, ii) AAPBA, iii) p(HEAA-BISAA), and iv) p(HEAA-BISAA-AAPBA). (b) ^{1}H-NMR spectrum for p(HEAA-BISAA) in D_2O at 25°C. (c) ^{1}H-NMR spectrum for p(HEAA-BISAA-AAPBA) in D_2O at 25°C.

3.2. Characterization of the synthesized boronate hydrogel

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To confirm the chemical structure and functionality of the synthesized boronate hydrogel, FT-IR and ^{1}H-NMR studies were performed using the lyophilized hydrogel samples. Fig. 2a shows FI-IR spectra for i) HEAA, ii) AAPBA, iii) p(HEAA-BISAA), and iv) p(HEAA-BISAA-AAPBA). In the FT-IR spectra of all samples, a broad absorption band at around 3270 cm^{-1} was attributed to the formation of the hydrogen bond between the O–H and N–H stretching of the amide group [36]. For p(HEAA-BISAA) and p(HEAA-BISAA-AAPBA), peaks at 1637 cm^{-1} were assigned to the C=O stretching of amide groups (amide I band) and peaks at 1540 cm^{-1} were attributed to N–H in-plane bending vibration (amide II band) [36-38]. These results showed that the polymerization of acrylamide compounds was accomplished by the photopolymerization procedure. In the FT-IR spectra of the AAPBA compound and p(HEAA-BISAA-AAPBA) hydrogel, peaks at 1342 cm^{-1} and 1334 cm^{-1} were assigned to the B–O stretching vibration of the boroxol bond in the phenylboronic acid moieties [37-39]. In addition, the typical absorption peaks of the meta-substituted benzene ring of the phenylboronic acid residue were observed at 709.1 cm^{-1} in the FT-IR spectra of AAPBA and p(HEAA-BISAA-AAPBA) [40, 41]. These peaks indicated that AAPBA was functionalized in the synthesized hydrogel. This result was confirmed in the ^{1}H-NMR analysis, as shown in Fig. 2b and 2c. Compared with the ^{1}H-NMR spectra of p(HEAA-BISAA-AAPBA) with p(HEAA-BISAA), distinctive peaks between 7.1 and 7.9 ppm were detected only in the spectrum of p(HEAA-BISAA-AAPBA), and were assigned to the protons of the phenyl group [40, 42]. Based on these results, we concluded that the boronate hydrogel was successfully synthesized, as designed.

3.3. Design and construction of the boronate hydrogel-modified membrane

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To develop a facile and cost-effective HbA_{1c} analysis based on the concept of lateral flow assay strips, the proposed boronate hydrogel should be integrated onto the external membrane structure. In this study, a commercialized Fusion5™ glass fiber membrane, which is utilized in lateral flow immunoassays as a test strip and/or a blood cell separator, was utilized owing to its high solvent-stability, intensive hydrophilicity, and low NSB capacity to proteins [43, 44]. To assess whether the in situ polymerization of the boronate hydrogel on the Fusion5™ membrane can be accomplished, the boronate hydrogel precursor solution was applied to the Fusion5™ membrane and UV light was applied to the precursor-absorbed membrane. Fig. S2a and S2b show images of Fusion5™ membranes before and after hydrogel polymerization. As depicted in Fig. S2b, after photopolymerization, a polymeric film formed in the membrane, as intended. To clearly show the surface differences between the pristine glass fiber membrane and hydrogel-modified membrane, 30 μL of DDW was applied to each membrane. Compared to the water droplet was immediately absorbed by the pristine membrane (Fig. S2c), the water droplet was retained its structure for 30 seconds and was absorbed on the hydrogel-modified membrane. Fig. S2d shows an image of the retained water droplet on the hydrogel-formed membrane at 10 seconds after its application. These results indicate that the hydrogel was formed via in situ photopolymerization method and did not block the penetration of the solution.

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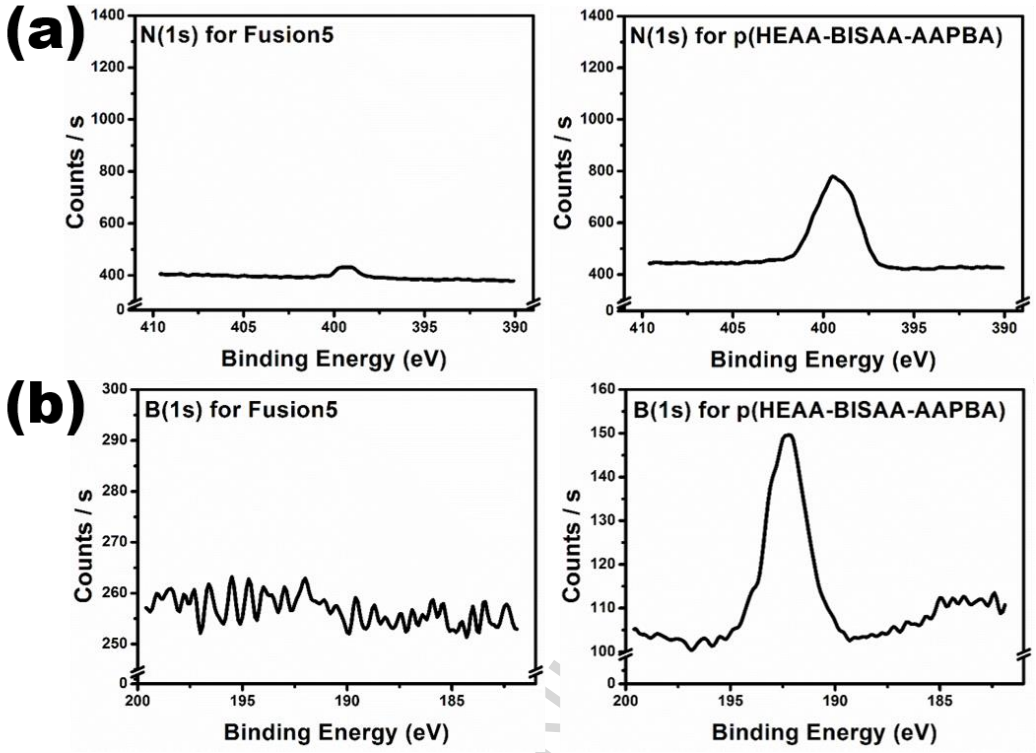

Figure 3. (a) N(1s) XPS spectra for the pristine Fusion5™ membrane and p(HEAA-BISAA-AAPBA)-modified membrane. (b) B(1s) XPS spectra for the pristine Fusion5™ membrane and p(HEAA-BISAA-AAPBA)-modified membrane.

For the analysis of the composition of in situ polymerized boronate hydrogels on the membrane, XPS was used. Fig. 3 shows the N(1s) and B(1s) XPS spectra from the pristine Fusion5™ membrane and the p(HEAA-BISAA-AAPBA)-modified membrane. As shown in Fig. 3a, the intensity of the N(1s) peak at 399 eV was substantially greater for the p(HEAA-BISAA-AAPBA)-modified membranes than the pristine Fusion5™ membrane, and this is considered an inherent effect of membrane additives for fiber binding. These results indicate that an increase in nitrogen atoms on the boronate hydrogel-modified membrane was induced by the polymerization of acrylamide compounds, including HEAA, BISAA, and AAPBA. In the XPS analysis of boron, a B(1s) peak at 193 eV was observed only on the p(HEAA-

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BISAA-AAPBA)-modified membrane, as depicted in Fig. 3b. These results indicate that the phenylboronic acid groups (AAPBA) were successfully introduced and covalently conjugated to the hydrogel structure via the photopolymerization process. Based on these results, we concluded that the boronate hydrogel interface can be implanted into the commercial glass fiber membrane.

3.4. Analysis of the glycoprotein binding property of the boronate hydrogel

To assess whether the boronate hydrogel recognizes glycoproteins, an enzymatic colorimetric assay was conducted using HRP as a model glycoprotein and a signal reporter. HRP is a well-known glycoenzyme containing nine N-linked glycosylation sites; thus, it can bind to the boronate residues on the boronate hydrogel (Fig. S1) [45, 46]. Furthermore, based on its biocatalytic activity toward chromogenic substrates, such as TMB, in the presence of hydrogen peroxide, HRP can be used as a signal reporter in colorimetric analyses. Accordingly, various concentrations of HRP were applied to the membrane samples. Then, for the visualization of HRP binding on the hydrogel-modified membrane, color development was induced by the addition of TMB, a chromogenic substrate of HRP [47]. As shown in Fig. 4a (upper row), color development for the p(HEAA-BISAA)-modified membranes was not observed in the HRP concentration range of 0 to 4 μg/mL. Weak color development was detected at 8 μg/mL HRP, and this can be interpreted as a noise signal, reflecting the increased NSB of HRP to the membrane induced by an excessive concentration of HRP. For the p(HEAA-BISAA-AAPBA)-modified membranes, the color intensity increased readily according to the increasing concentration of HRP, as shown in Fig. 4a (bottom row). These results indicate that the boronic acid residue in the synthesized boronate hydrogel can bind to

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the glycoprotein (HRP), as expected. The competitive binding of HbA_{1c} and HRP to the boronic acid residues in the boronate hydrogel can be used as a major strategy for the quantitative analysis of HbA_{1c}, as shown in Fig. S1. Therefore, the concentration of HRP in this competitive HbA_{1c} analysis is highly significant. A concentration of 4 μg/mL HRP resulted in the most intense color signal and minimal NSB and accordingly was used in subsequent experiments.

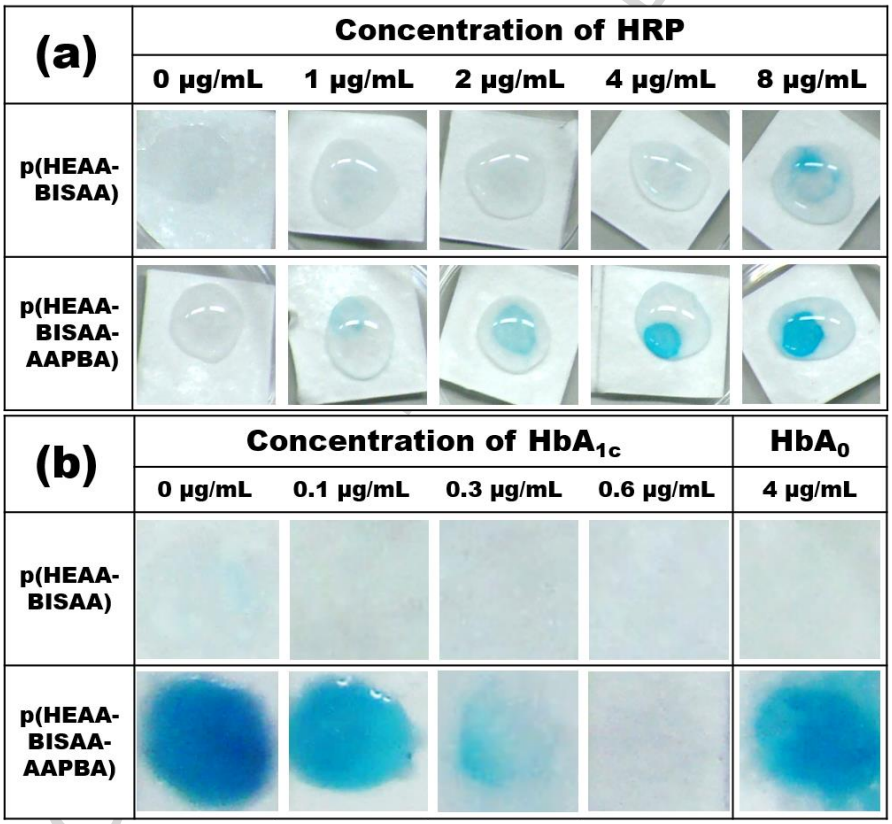

Figure 4. (a) Comparative analysis of the HRP-TMB chromogenic reaction to assess the glycoprotein binding properties of the synthesized boronate hydrogel. Assay results from the p(HEAA-BISAA)-modified membrane and from the p(HEAA-BISAA-AAPBA)-modified membrane were presented in upper row and bottom row, respectively. (b) Results of the colorimetric HRP-HbA_{1c} competition assay for the purified HbA_{1c} samples. For the control test, a purified non-glycated adult hemoglobin (HbA_0) sample was assayed. HbA_{1c} assay

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results for the p(HEAA-BISAA)-modified membrane and for the p(HEAA-BISAA-AAPBA)-modified membrane were depicted in upper row and bottom row, respectively.

3.5. HbA_{1c}-HRP competition assay on the developed HbA_{1c} biosensing interface

To demonstrate the feasibility of the application of the developed boronate hydrogel-modified membrane to HbA_{1c} biosensing, an HbA_{1c}-HRP competition assay was performed using commercial purified HbA_{1c} reagents. By regarding that 6.5% HbA_{1c} used as a certified diabetic index, we attempted to prepare the target samples with 2.5%, 6.5% and 15% of HbA_{1c} as model glycated hemoglobin samples, corresponding for non-diabetic, early diabetic, and diabetic states. Considering that the total hemoglobin (Hb) concentration in whole blood is commonly known as 150 mg/mL, 2.5, 6.5, and 15% of HbA_{1c} can be converted into 3.75, 9.75, and 22.5 mg/mL, respectively. In the blood sample analysis described later (*vide infra*, Section 2.9 and Section 3.7), the whole blood sample containing 150 mg/mL of Hb would be diluted to be containing 4 μg/mL of Hb. Therefore, by considering the converted HbA_{1c} concentrations and the dilution factor, 0.1, 0.3, and 0.6 μg/mL of HbA_{1c} samples were prepared for the HbA_{1c} quantification assay.

On the boronate hydrogel-modified membrane, the mixtures of HRP and HbA_{1c} reagent at various concentrations (0, 0.1, 0.3, and 0.6 μg/mL) were applied and allowed to react with the boronate groups. To assess the effect of non-glycated normal hemoglobin (HbA_0) on boronate-assisted binding of HRP, 4 μg/mL HbA_0 was mixed with HRP and assayed. Then, the HRP-TMB reaction was induced on the membranes for a colorimetric analysis. As shown in Fig. 4b (upper row), color development was not detected in the p(HEAA-BISAA)-modified membranes for all samples, reflecting the absence of boronic acid groups. For the

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p(HEAA-BISAA-AAPBA)-modified membranes, the color intensity decreased readily as the concentration of HbA_{1c} increased (Fig. 4b, bottom row). The signal intensity in the competitive assay was inversely proportional to the analyte concentration, indicating that HbA_{1c} and HRP competitively bound to the boronic acid moieties, as intended (Fig. S1). In addition, the intensive color development from the HbA_0-HRP competitive reaction shows that NSB of non-target proteins, such as HbA_0, to the hydrogel and membrane was prevented by the hydrophilic property of the materials. Based on the high target specificity to HbA_{1c}, we concluded that the application of the boronate hydrogel-modified membrane to HbA_{1c} biosensing as a biorecognition interface is promising.

3.6. Fabrication and characterization of a boronate hydrogel-patterned membrane strip

To develop a boronate-based HbA_{1c} biosensing interface for application to lateral flow assays, the boronate hydrogel was spatio-selectively patterned on the Fusion5™ membrane. For the morphological analysis and chemical functionality analysis of the hydrogel-patterned membrane, SEM and EDS analyses were conducted. Fig. 5a–5c shows SEM images of the pristine Fusion5™ membrane, area on the boronate hydrogel-patterned membrane not exposed to UV, and area on the boronate hydrogel-patterned membrane exposed to UV. As shown in Fig. 5a and 5b, the morphological differences between the pristine Fusion5™ membrane and unexposed region of the boronate hydrogel-patterned membrane were minor. This result indicates that polymerization of the boronate hydrogel on the region of the membrane not exposed to UV did not occur. In contrast, the thickness of glass fibers for the UV-exposed region in the boronate hydrogel-modified membrane increased, as shown in Fig. 5c. This result demonstrates that the boronate hydrogel was spatio-selectively polymerized in

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UV-irradiated membrane regions and was grafted to the glass fibers of membrane. Importantly, the overall microscale structure of the Fusion5™ membrane was not disrupted and clogged by the boronate hydrogel after photopolymerization. Even though the inside of the Fusion5™ membrane was sparsely covered by thin polymeric film fragments of boronate hydrogels, the general fibrous structure and the gap between glass fibers were not blocked by hydrogel polymers. The woven structure maintained at a microscale in the membrane allows the capillary-driven migration of liquid samples in the boronate hydrogel-patterned membrane.

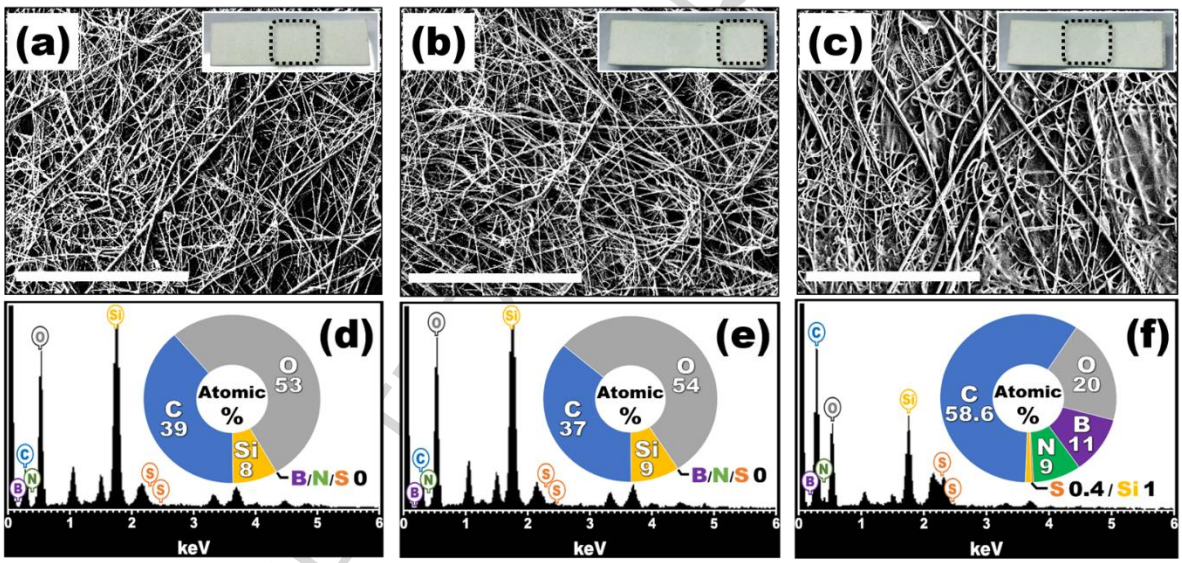

Figure 5. Results of SEM (a–c) and EDS analyses (d–f) for the untreated pristine Fusion5™ membrane, the region of the boronate hydrogel-applied membrane not exposed to UV, and the UV-exposed region of the boronate hydrogel-applied membrane. (a, d) SEM image and EDS spectrum of the pristine Fusion5™ membrane, respectively. (b, e) SEM image and EDS spectrum of the unexposed region of the boronate hydrogel-applied membrane, respectively. (c, f) SEM image and EDS spectrum of the UV-exposed region of the boronate hydrogel-applied membrane, respectively. Insets in SEM images show a membrane area analyzed by

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SEM (dashed rectangles). Scale bar indicates 400 μm. Inset donut charts in EDS spectra show the surface elemental compositions (% atomic concentration) for each membrane sample.

Fig. 5d–5f shows EDS spectra for the pristine Fusion5™ membrane, the area on the boronate hydrogel-patterned membrane not exposed to UV, and the area on the boronate hydrogel-patterned membrane exposed to UV, respectively. As depicted in Fig. 5d and 5e, highly similar elemental compositions were observed in the pristine Fusion5™ membrane and the UV-unexposed region of the boronate hydrogel-patterned membrane. This result reflects that the precursor compounds in the UV-unexposed region of membrane were washed away without polymerization. In contrast, as shown in Fig. 5f, the proportions of carbon, nitrogen, and boron in the UV-exposed region of the hydrogel-patterned membrane was increased than in the unexposed region, while the elemental ratios of silicon and oxygen decreased. The increased proportions of carbon and nitrogen confirm the spatio-selective polymerization of the boronate hydrogel, which is composed of acrylamide compounds, and the increase in boron indicates the covalent conjugation of boronic acid residues to the membrane-patterned hydrogel. These results suggest that the boronate hydrogel was successfully polymerized on the desired area of the membrane using the photopolymerization technique.

3.7. Quantitative analysis of HbA_{1c} in human blood samples

To demonstrate the applicability of the newly developed biosensing interface to the quantitative analysis of HbA_{1c} in human blood samples, HbA_{1c} biosensing was performed

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using spiked whole-blood hemolysate samples. In the application of HbA_{1c} biosensing to whole blood samples with boronate derivatives as bioreceptor, the most significant problem is signal interference from the undesired binding of various blood components containing carbohydrate residues (e.g., blood sugars, glycolipids, and glycoproteins, except HbA_{1c}) to the boronate-modified biorecognition interface. For the precise analysis of HbA_{1c} in the blood, those signal interferents should be removed. To address this, we revisited our previous work on the facile separation of the hemoglobin fraction from whole blood using zinc ion-mediated hemoglobin (Hb) precipitation (Scheme S2, see details in the Supporting Information) [33, 34]. In the present study, using this technique, samples with various concentrations (4.6%, 5.8%, 7.7%, 9.6%, 11.5%, 13.3% and 15.2%) of HbA_{1c} were prepared by spiking the purified HbA_{1c} and HbA_0 reagents in the Hb fraction that was separated and re-collected from the whole blood sample. Finally, a colorimetric HbA_{1c}-HRP competitive assay using the HbA_{1c} samples was performed on the boronate hydrogel-patterned Fusion5™ membrane. On the boronate hydrogel-patterned membrane strip, fluid flow for the HbA_{1c} assay (e.g., reagent treatment and washing steps) can be easily manipulated via capillary force, similar to the lateral flow assay strip, because the applied boronate hydrogel does not disrupt the fibrous network structure of the Fusion5™ membrane, generating strong capillary force and a high flow rate, as described in previous section. As depicted in Scheme S3, the unreacted and excessive proteins in the hydrogel-patterned region (in the middle of the strip) was removed by a capillary-driven washing process. In the washed boronate hydrogel-patterned region, TMB-mediated color development was induced to quantitatively analyze HbA_{1c}-HRP competitive binding.

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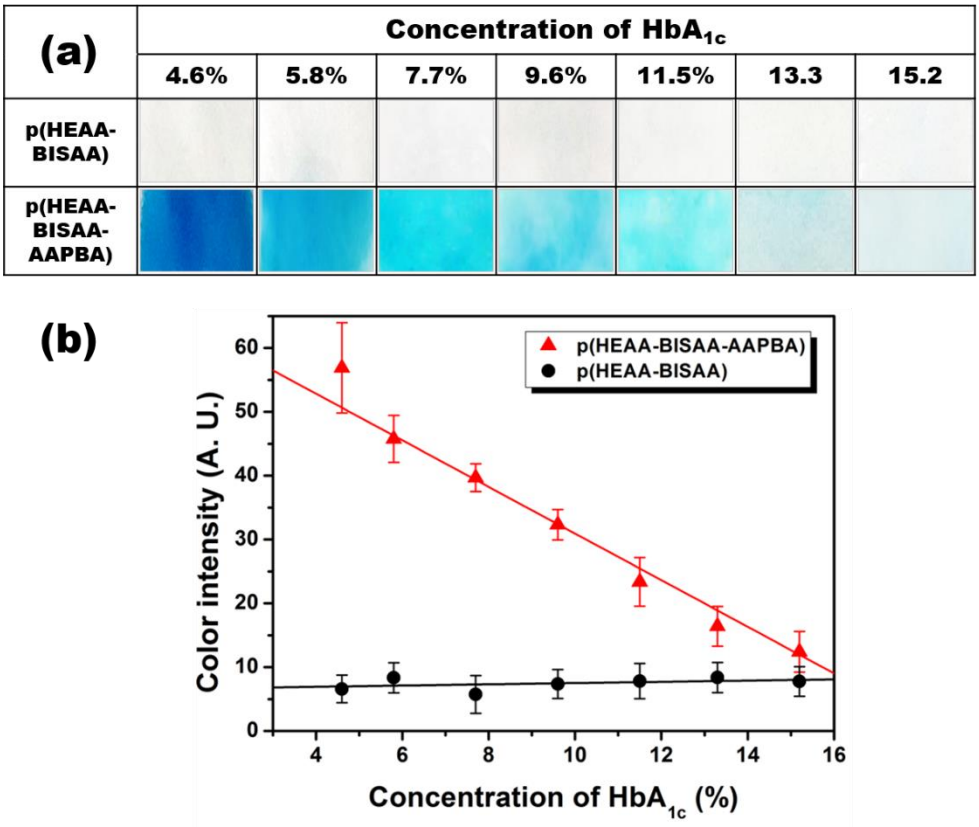

Figure 6. Colorimetric HRP-HbA_{1c} competition assay results using boronate hydrogel-patterned membrane strips. Various concentrations (4.6%, 5.8%, 7.7%, 9.6%, 11.5%, 13.3% and 15.2%) of HbA_{1c} samples were prepared in whole-blood sample after the removal of signal interferents (e.g., sugars and non-target proteins) via hemoglobin separation method. (a) Colorimetry results for the HbA_{1c} assay with the p(HEAA-BISAA)-patterned membrane (upper panel) and p(HEAA-BISAA-AAPBA)-patterned membrane (lower panel). (b) Dose-response curve for color intensity in the hydrogel-patterned membrane strips as a function of HbA_{1c} concentration (4.6%, 5.8%, 7.7%, 9.6%, 11.5%, 13.3% and 15.2%). Each data point represents the average and standard deviation of triplicate independent tests.

As shown in Fig. 6a, the intensity of the blue color on the boronate hydrogel-patterned membrane was inversely proportional to the concentration of HbA_{1c}, while no colorimetric

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signal was obtained for the membrane modified with the p(HEAA-BISAA) hydrogel. The obtained colorimetry results demonstrate that HbA_{1c} and HRP competitively bound to the boronate residues in the membrane, even in the presence of excessive normal hemoglobin. By measuring the color intensity (blue) in images of boronate hydrogel membrane strips using Adobe Photoshop software, a linear dose-response curve for HbA_{1c} was obtained is shown in Fig. 6b. Based on the guideline of Clinical and Laboratory Standards Institute, the minimum and maximum limit of detection values for the performed HbA_{1c} analysis were calculated as 3.8% and 14%, respectively [48]. This result showed that the practical HbA_{1c} detection range of the developed system ranged from 3.8% to 14%, which was consistent to the clinical HbA_{1c} reference range (4% to 15%) for the diagnosis of diabetes mellitus. Additionally, in the range of 5.8% to 9.6% HbA_{1c}, which is important for the diagnosis and management of prediabetes, the average interassay coefficient of variation for HbA_{1c} biosensing was as low as 6.9%, indicating a high reproducibility of the developed biosensing interface toward an HbA_{1c} detection. The acquired calibration results demonstrate that the application of the boronate hydrogel-patterned membrane as a biorecognition interface for the HbA_{1c} biosensing platform is promising.

4. Conclusions

In the areas of clinical diagnosis and in vitro diagnostics, the demand for an effective HbA_{1c} biosensing approach that enables the POC diagnosis of diabetes mellitus has increased over the last few decades. To address this, a p(HEAA-BISAA-AAPBA) boronate hydrogel that can be integrated with the glass membrane substrate was newly synthesized as a novel biosensing interfacial material for HbA_{1c} analysis. To develop an HbA_{1c} biosensing interface

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that facilitates efficient fluidics manipulation via capillary action, the designed p(HEAA-BISAA-AAPBA) boronate hydrogel was in situ polymerized and patterned in the commercial glass fiber membrane using a photopolymerization method. Even in the analysis of human blood-derived specimen, the proposed HbA_{1c} biosensing interface exhibited high detection sensitivity as well as operational convenience. Furthermore, by applying the robust HRP-mediated enzymatic colorimetry principle, HbA_{1c} biosensing results on the membrane strip could be easily visualized and quantified, without complicated optical instruments. Based on these advantages, including the strong analytical performance, ease of fabrication, facile operation, cost-effectiveness, and efficient signal detection procedure, we expect that the proposed biosensing material and interface can be utilized for the further development and commercialization of a POC device for analyses of glycated protein biomarkers as well as the detection of HbA_{1c} [49-51].

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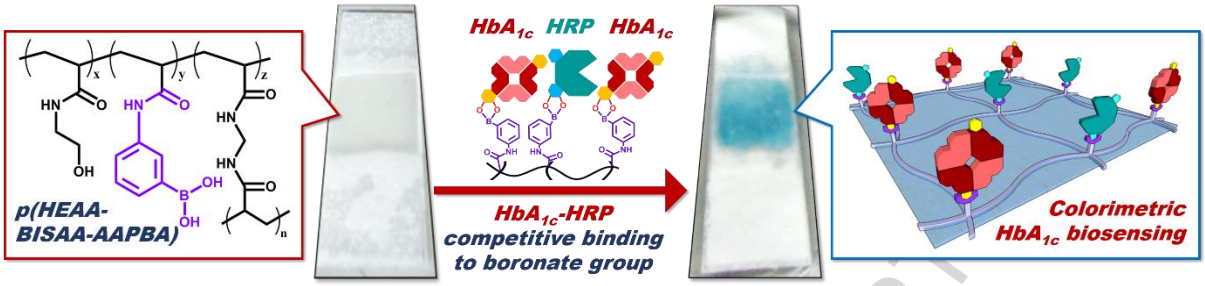

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Highlights

- Boronic acid (BA) hydrogel was synthesized for HbA_{1c} biosensing.
- BA hydrogel was patterned on the glass fiber membrane via photo-crosslinking.
- HbA_{1c} and peroxidase were competitively bound to BA hydrogel-modifed membrane.
- HbA_{1c} could be efficiently quantified by peroxidase-based chromogenic reaction.
- HbA_{1c} in the whole blood sample were determined within a range of 4.6–15.2%.

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