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Chemiluminometric enzyme-linked immunosorbent assays (ELISA)-on-a-chip biosensor based on cross-flow chromatography

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ABSTRACT

A chemiluminometric biosensor system for point-of-care testing has been developed using an immunochromatographic assay combined with an enzyme (e.g., horseradish peroxidase) tracer that produces a light signal measurable on a simple detector. Cross-flow chromatography, a method previously investigated by our laboratory, was utilized in order to accomplish sequential antigen–antibody binding and signal generation. This enzyme-linked immunosorbent assay (ELISA) was effectively carried out on a plastic chip that was redesigned to simplify the fabrication process. To enhance the sensitivity, biotin–streptavidin capture technology was employed in preparing an immuno-strip that was then incorporated onto the chip in order to generate the ELISA-on-a-chip (EOC) biosensor. Samples containing cardiac troponin I (cTnI) were analyzed using the EOC. A chemiluminescent signal proportional to the analyte concentration was produced by adding a luminogenic substrate to the tracer enzyme complexed with the analyte on the chip. The luminescent signal was detected in a dark chamber mounted with a cooled charge-coupled device and the signal was converted to optical density for quantification. This EOC biosensor system was capable of detecting cTnI present in serum at concentrations as low as 0.027 ng mL^{-1}, 30 times lower than those measured using the conventional rapid test kit with colloidal gold as the tracer. In addition, the final data was acquired within 30 s after the addition of the enzyme substrate, which was faster than the detection time required when using a colorimetric substrate with the same tracer enzyme.

1. Introduction

Biomarkers such as hormones, proteins, and infectious microorganisms indicating symptoms or diseases of the human body are normally present at rather low concentrations in specimens (e.g., blood, urine, and saliva). These markers can be traced via immunoassays based on antigen–antibody binding [1–5]. In recent years, these assays have been increasingly carried out at sites of patient care, such as doctor's offices, emergency rooms, and even at home [6,7]. This trend allows for rapid estimates of the state or progress of an illness. Toward this end, an immuno-analytical system that can accomplish these analyses with both high sensitivity and ease of handling is in demand.

A device with such high analytical performance capability may be possible for point-of-care testing (POCT) through the principle of immuno-chromatography in which a porous membrane strip is utilized as the immunosorbent [7–9]. A lateral flow through the membrane strip expedites the reaction allowing it to be completed in a relatively short time and further enables the in situ separation of unreacted components, allowing for one-step analysis. This technology has been widely applied in the detection of various biomarkers to date [7,10]. The conventional rapid test kits using this concept, however, are available mostly for qualitative analyses, generating color signals from a colloidal gold tracer that are perceptible to the naked eye and exhibiting sensitivities lower than those associated with the traditional enzyme-linked immunosorbent assay (ELISA) [1]. This feature is definitely insufficient for the assessment of most biomarkers for which quantitative analyses for early diagnosis and progress monitoring are classically constrained.

As exemplified in ELISA, enzymes can be used as alternative types of tracers that can be applied to immunosensors for POCT [1]. An enzyme tracer generates an enhanced signal resulting from its catalytic action and also provides different signal

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types that are measurable with comparatively simple detectors (e.g., based on colorimetry, chemiluminometry, or electrochemistry [1,11,12]), depending on the substrate as well as the enzyme used. The enzyme reaction, a feature distinct from those of other tracers, should be conducted separately for signal generation following the completion of antigen–antibody binding reactions. Standard protocols for heterogeneous immunoassays require washing steps for the separation of the immune complexes formed on solid surfaces from the unreacted reagents [13]. In order to employ this procedure at the sites of care, a novel method of cross-flow immuno-chromatography has been developed in which immunological binding and the enzyme-based detection reaction are sequentially conducted [1]. The cross-flow procedure has been conveniently utilized on plastic chips, i.e., ELISA-on-a-chip (EOC), with fluidic channels engraved on the surfaces, and it has been demonstrated to allow sensitive detection of biomarkers from different analytical fields [14,15].

To advance high performance EOC biosensors for POCT one step further, in this study we adopted a novel signal generation system in conjunction with the development of a new chip design. For signal generation, the EOC previously employed a chromogenic enzyme substrate, producing a color signal that accumulated over a certain period of time. This allowed us to achieve a high sensitivity, but caused an unavoidable delay in analysis time. Furthermore, in the chip assembly, an adhesive had to be used to bond the two components of the chip together and, thus, the fabrication processes were rather complex. For the innovations described here, the substrate was replaced with a chemiluminogenic substrate to produce a light signal that resulted in high sensitivity detection without time integration. To improve the chip production process, a second-generation chip has been devised that can be assembled without using adhesive, making the assembly process simpler and, consequently, more reproducible. As a model analyte for this research, cardiac troponin I (cTnI), a specific biomarker of acute myocardial infarction (AMI) [16], has been used.

2. Materials and methods

2.1. Materials

Cardiac troponin (cTn) I-T-C complex, cTnI, and a monoclonal antibody specific to cTnI (clone 19C7) were purchased from Hytest (Turku, Finland). Casein (sodium salt type, extracted from milk), Triton X-100, D(+)trehalose, ethylenediaminetetraacetic acid disodium salt (EDTA), sodium dodecyl sulfate (SDS), ascorbic acid, human serum, dithiothreitol (DTT), soluble 3,3′,5,5′-tetramethylbenzidine (TMB), protein G columns, and Sephadex G-15 were obtained from Sigma (St. Louis, MO). The SuperSignal West Femto Trial Kit as chemiluminogenic substrate for HRP, the Monomeric Avidin Kit, succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (SMCC), and 1-biotinamido-4-(4′-[maleimidoethylcyclohexane]-carboxamido)butane (biotin-BMCC) were supplied by Pierce (Rockford, IL). Streptavidin and horseradish peroxidase (HRP) were purchased from Calbiochem (San Diego, CA). HRP substrate solution containing insoluble TMB, human anti-mouse antibody (HAMA) blocker (mouse IgG fraction), and ficin protease were obtained from Moss (Pasadena, MD), Chemicon International (Temecula, CA), and Wako (Osaka, Japan), respectively. Nitrocellulose (NC) membranes (70 CNPH-N-SS40) and glass fiber membranes (PT-R5, GFB-R4) were supplied by MDI (Ambala Cantt, India). Cellulose membrane (17 CHR chromatography grade) was purchased from Whatman (Maidstone, England). Other reagents used in this study were of analytical grade.

2.2. Instrumentation

2.2.1. Construction of chemiluminometric detector

To analyze the chemiluminometric signals generated from the EOC (see below for details), a detector was devised using a cooled charge-coupled device (CCD) camera (ProgRes MF cool, JENOPTIK, Germany) installed on a detection chamber. The chamber was composed of two major parts, a bottom plate with an EOC holder and a cover in the shape of a hollow rectangular pillar. The camera was mounted on the top of the chamber and, when assembled, the signal was measured without interference from ambient light. The focus of the CCD camera was adjusted to clearly capture the image of the signal produced from the chip at the time of analysis.

2.3. Procedures

2.3.1. Preparation of biotinylated F(ab′)$_2$

2.3.1.1. Enzymatic digestion. A monoclonal antibody specific to cTnI (BD clone 12) was produced by following a standard protocol [17] as described elsewhere [14]. To prepare a F(ab′)$_2$ antibody fragment, this antibody (30-fold molar excess based on the protease) was enzymatically digested by mixing with ficin dissolved in 50 mM Tris–HCl, pH 7.0, containing 2 mM EDTA and 1 mM cysteine, and incubating at 37 °C for 2 h. The reaction mixture was then fractionated on a protein G column (1 mL gel volume) pre-equilibrated with 10 mM phosphate buffer, pH 7.4, containing 140 mM NaCl (PBS). After loading the mixture, the unbound fractions were washed with PBS and the bound components were sequentially eluted by switching the buffer to 0.2 M glycine, pH 3.0. Each eluted fraction was neutralized using a basic buffer and analyzed by means of the Bradford assay [18]. The antibody fragments in each peak were characterized using polyacrylamide gel electrophoresis according to a standard protocol [19]. The bound fractions were pooled, dialyzed against PBS, and stored at −20 °C after snap-freezing as aliquots before further use.

2.3.1.2. Biotinylation of F(ab′)$_2$. The F(ab′)$_2$ fragment produced was biotinylated for use as the capture antibody in immunoassays after immobilization on a streptavidin layer. For site-directed biotinylation, the fragment was reduced and then biotinylated by following the procedure described elsewhere [20].

2.3.1.3. Purification of biotinylated F(ab′)$_2$. The biotinylated F(ab′)$_2$ fragment was separated from the free antibody component using the monomeric avidin kit according to the manufacturer's instructions. Briefly, after pre-equilibration of the monomeric avidin column (2 mL gel volume) with PBS, the antibody fragments were applied to the column and incubated at room temperature for 1 h. The unbound portion was washed with PBS and the bound components were then eluted, based on competition for the avidin binding sites, by adding 2 mM D-biotin in PBS. The elution fractions were analyzed by the Bradford assay and solid-phase binding assays with anti-mouse goat IgG or streptavidin immobilized on the inner surfaces of a microtiter plate. For the binding assays, the anti-mouse antibody (1 μg mL^{-1}) and streptavidin (10 μg mL^{-1}) dissolved in PBS were incubated within separate microwells at 37 °C for 1 h. After washing with deionized water, the residual surfaces were blocked with PBS containing 0.5% casein (Casein-PBS) under the same conditions. After washing again, aliquots of each eluted fraction from the monomeric avidin column diluted 100 times with Casein-PBS containing 0.1% Tween 20 (Casein-PBS-TW) were added to the wells and incubated as in the previous step. The wells were then washed and anti-mouse goat IgG coupled to HRP diluted 5,000 times with Casein-PBS-TW was added and allowed to bind. After washing, a HRP substrate solution containing soluble TMB (200 μL)

[20] was dispensed into each well and reacted at room temperature for 15 min. Sulfuric acid (2 M, 50 μL) was subsequently added, and the absorbance at 450 nm was measured using a microtiter plate reader (VERSAmax, Molecular Device, Chicago, IL).

2.3.2. Synthesis of labeled antibodies

2.3.2.1. Labeling antibody with HRP. The monoclonal cTnI antibody, clone 19C7, was coupled with HRP via a chemical cross-linker basically following the protocol described elsewhere [14]. Briefly, the antibody was first reduced using 10 mM DTT at 37 °C for 1 h, and the excess reagent was removed on a Sephadex G-15 gel filtration column (10 mL volume). HRP was also activated with a 50-fold molar excess of SMCC, and the excess reagent was immediately removed by separation on a gel filtration column. The antibody was then combined with a 5-fold molar excess of the activated HRP and the conjugation was carried out overnight at 4 °C. The synthesized conjugates were stored as aliquots after snap freezing in liquid nitrogen.

2.3.2.2. Labeling antibody with colloidal gold. The monoclonal cTnI antibody, BD clone 12, was labeled with colloidal gold (mean diameter of 30 nm) synthesized using the sodium citrate method [21,22] as reported previously [1].

2.3.3. Construction of EOC sensor system

2.3.3.1. Preparation of immuno-strip. An immuno-strip to be installed within the EOC was prepared basically according to a previously reported protocol [14] except that the introduction of pads arranged for supplying biotin and streptavidin components was omitted. The strip consisted of five different types of functional membrane pads consecutively connected by partial superimposition. They were, from the bottom, a glass fiber membrane (4 mm × 15 mm, GFB-R4) for sample application, two glass membranes (4 mm × 6 mm, PT-R5) for the release of the detection antibody labeled with HRP and the biotinylated capture antibody, respectively, a NC membrane (4 mm × 25 mm) for signal generation, and a cellulose membrane (4 mm × 15 mm, 17 CHR) for absorption.

Since optimization experiments were carried out in this study, the fabrication conditions presented below represent the optimized conditions determined from these analyses. The release pad for the capture antibody was made by adding the biotinylated $F(ab')_2$ (5.45 pmol per assay) in PBS containing 5% casein and 40% trehalose. The pad for the detection antibody was prepared using conditions previously reported [14] except that the level of HRP-labeled antibody was increased 2-fold (0.87 pmol per assay) over the previously reported level. The signal generation pad was made by dispensing (1.5 μL cm^{-1}) a streptavidin solution (7 mg mL^{-1}) in PBS onto a site 5.5 mm from the bottom of the NC membrane using a micro-dispenser (Biojet 3000, Biodot, Irvine, CA). Goat anti-mouse IgG antibody (0.1 mg mL^{-1}) in PBS was also dispensed onto a site on the same membrane 11.5 mm from the bottom. The remaining parameters were identical to those presented in an earlier report [14].

2.3.3.2. Fabrication of EOC. The EOC for chemiluminometric assays was comprised of two plastic plates, top and bottom, made by injection molding of polycarbonate. The bottom plate of the chip (32 mm × 76 mm × 8 mm) consisted of fluidic channels arranged in the vertical and horizontal directions. The vertical channel was formed in the center of the width to hold the immuno-strip. The horizontal channel consisted of the supply channel for the enzyme substrate and the compartment for the horizontal flow absorption pad, each placed on the respective lateral side of the vertical channel to provide the flow path of the substrate across the signal generation pad of the immuno-strip. The supply channel was formed in the shape of a circular triangle expanding toward the vertical channel.

On the top plate of the chip, a window was installed for luminescent signal monitoring, as well as two ports for sample addition and enzyme substrate addition. The signal monitor window was furnished by slitting the top plate surface (2.2 mm × 18 mm) in the region corresponding to the ceiling of the signal generation pad of the immuno-strip located on the bottom plate. The sample application port, aligned with the sample application pad of the immuno-strip, was formed as an open-bottom well (5 mm × 10.5 mm × 4.5 mm) allowing a maximum sample load of 250 μL. The substrate supply port (5.3 mm in diameter) was provided at the far end of the supply channel structure as the inlet of the horizontal flow. The structure was in the shape of a circular triangle arranged to combine with the substrate channel in the bottom plate when assembled together, forming a closed flow passage with a thin-layer outlet. In addition, two air ventilation holes (1 mm in diameter) were also made at both ends of the projection areas near the outlet.

After placing the immuno-strip and a horizontal flow absorption pad (cellulose membrane; 14 mm × 12 mm) within the vertical channel and the absorption pad compartment of the bottom plate, respectively, the two plates were firmly combined using groove joints without introducing an adhesive. The assembled EOC was stored in a desiccator maintained at room temperature prior to use.

2.3.4. Optimization of analytical conditions

2.3.4.1. Analytical procedure. Using the EOC sensor system, cross-flow chromatographic analysis for the detection of cTnI was performed. A stock of cTnI (1 mg mL^{-1}; I-T-C complex form) was serially diluted with human serum determined to be negative for the analyte in order to prepare the sample at a pre-determined concentration. The sample (100 μL) was transferred into the application port of the EOC sensor. After 15 min, the horizontal flow absorption pad was connected to the lateral side of the signal generation pad, and a solution containing a chemiluminogenic substrate solution (200 μL) for HRP was immediately supplied into the corresponding port. The EOC was placed within the chemiluminometric detector, and 30 s after the substrate was added the light signals produced at the sites of analyte and control were captured as an image using the program provided by the manufacturer.

The light signals on the image were quantified using an image analysis program (Multianalyst version 1.1, Bio-Rad Laboratories, Hercules, CA) as optical densities and the data collected were stored using the Microsoft Excel program. The mean value of the background densities present between the analyte and control peaks was subtracted from the measured optical densities. The normalized optical densities under the analyte peak were then integrated so that a numerical signal value could be assigned.

2.3.4.2. Signal detection time. The chemiluminometric signal from the EOC was characterized over time to determine the minimal detection time required and was compared with the kinetic pattern of the color signal generated using a different substrate but with the same tracer enzyme. A standard sample of cTnI (0.2 ng mL^{-1}) was analyzed according to the analytical procedure except that the luminescent signal was monitored repeatedly over time. For the kinetic analysis of color signal development, the same steps were carried out as described above, except that a chromogenic substrate containing insoluble TMB was used as a substitute for the chemiluminogenic substrate as described previously [14].

2.3.4.3. Antibody concentrations. To enhance EOC performance, the concentrations of the two major components, biotinylated $F(ab')_2$ for capture and HRP-labeled antibody for detection, were optimized

for chemiluminometric EOC. To optimize the concentration of the capture antibody, the EOCs were fabricated using different release pads loaded with variable amounts of biotinylated $F(ab')_2$ in combination with pads containing a constant amount of the detection antibody (0.87 pmol per assay). The EOC performance levels were estimated by conducting sample analyses according to the procedure described above. Likewise, the concentration of the detection antibody was then optimized by varying the levels loaded onto the release pads while maintaining a constant concentration of the capture antibody (5.45 pmol per assay).

2.3.5. Analytical performance

2.3.5.1. Chemiluminometric EOC. To determine the detection capability of the EOC, a dose-response curve was obtained with cTnI by following the same analytical procedure described above. The analyses at each analyte dose were repeated three times, and the mean of the signal values was plotted against the analyte concentration. The detection limit was then determined as the concentration corresponding to the signal value that was calculated by multiplying the standard deviation of the signal at the zero dose by three [23,24].

2.3.5.2. Colorimetric EOC. The same procedure as stated above was repeated except the use of a chromogenic substrate, insoluble TMB, for colorimetric signal generation as described elsewhere [14,15].

2.3.5.3. Rapid test kit. As a reference, the conventional rapid test kit employing colloidal gold as a tracer was constructed using the same protocol as previously reported [14,15]. The capture antibody, clone 19C7, was immobilized directly onto the signal generation pad, and BD clone 12 labeled with colloidal gold as described above was deposited within the conjugate release pad for use as the detection antibody. The same samples as those used for the chemiluminometric EOC were loaded and allowed to migrate for 15 min. The colorimetric signals were quantified as described in the previous reports. The identical procedure was repeated three times with each sample, a dose-response curve was plotted, and the detection limit was calculated as mentioned.

3. Results and discussion

3.1. Chemiluminometric EOC system

The concept of cross-flow chromatography enables us to combine an enzyme tracer with the conventional rapid test kit such that high sensitivity detection can be achieved [1]. The analytical protocol in this study consists of a two-step procedure: initiation of sample flow to induce antigen–antibody binding coupled with biotin–streptavidin capture of the analyte and initiation of the flow of enzyme substrate for chemiluminometric signal generation (Fig. 1). First, the sample containing analyte is absorbed from the bottom of an immuno-strip (vertical flow), inducing immunocomplex formation at each of two pre-determined sites, indicated as analyte and control lines, on the signal generation pad. Second, two horizontally arranged pads are placed on each lateral side of the signal generation pad, and the substrate is then added onto the supply pad to initiate enzymatic signal generation (horizontal flow).

At the sites of complex formation, variable signals such as color, light, and current change, can be produced by supplying different enzyme substrates at the time of signal generation. For a rapid, accurate analysis, a chemiluminometric signal was generated in this study via catalytic oxidation of a luminogenic substrate, e.g., luminol (see Fig. 1) [25,26]. In this process, a light signal is emitted at a wavelength of 425 nm and can be measured using a detector equipped with a cooled CCD camera, which will be described below.

To perform assays using cross-flow chromatography, an EOC biosensor system was constructed by fabricating a plastic EOC and installing a detector with an image capture module (Fig. 2). The EOC was devised as an upgraded version of our previous design [14] such that no adhesive for assembling the top and bottom plates was

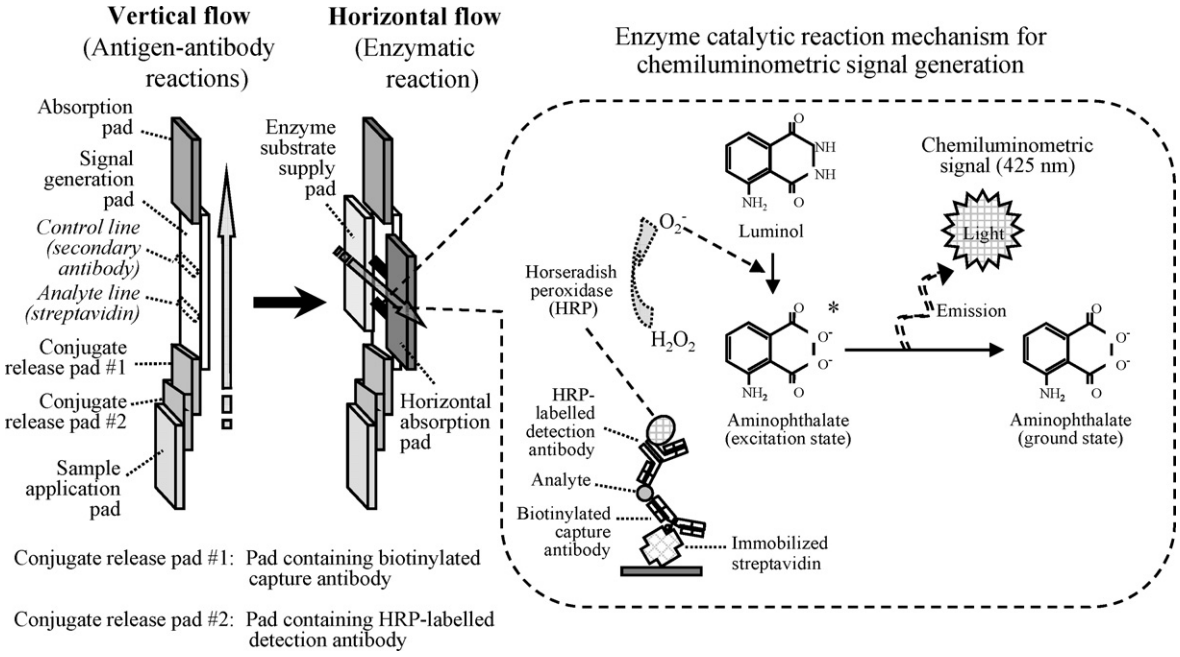

Fig. 1. Conceptual schematic of sequential cross-flow chromatography with chemiluminometric signal generation from HRP used as a tracer. Upon absorption of sample, it migrates upward along the immuno-strip (vertical flow) and antigen–antibody reactions occur with the binding proteins at each site. After completion, two horizontally arranged pads are placed on each lateral side of the signal generation pad and an enzyme substrate solution containing luminol is then added onto the supply pad. This solution flows across the signal pad (horizontal flow), which can initiate the production of a chemiluminometric signal from the enzyme contained in the immune complexes formed at the respective sites.

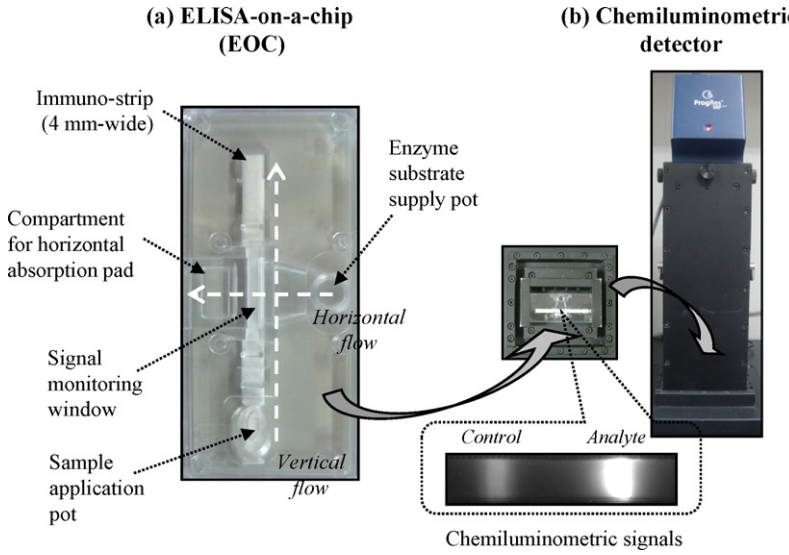

Fig. 2. Construction of an EOC biosensor system based on the concept of cross-flow chromatography. The EOC was fabricated by injection molding of plastic and assembly to form channels in the vertical and horizontal directions (a). The vertical channel held the immuno-strip as shown in Fig. 1. The horizontal channel consisted of a passage for supplying an enzyme substrate solution and a compartment for placing the horizontal absorption pad on the respective lateral side of the signal generation pad of the strip. For signal detection after antigen–antibody binding, the EOC was placed within a chemiluminometric detector mounted with a cooled CCD camera (b).

used, and it was constructed by injection molding of polycarbonate (Fig. 2a). Inside of the device, there were two channels: the vertical channel consisting of a 4 mm-wide immuno-strip as shown in Fig. 1, and the horizontal channel comprising compartments for an enzyme substrate supply and an absorption pad arranged on each lateral side of the vertical strip. The remaining ports and monitoring window were established on the surfaces of the chip, analogously to those in the old version.

Using the EOC, we have performed cross-flow chromatographic analysis using cTnI as the analyte. The sample was transferred into the application port of the chip and, after completion of the flow, a solution containing the chemiluminogenic substrate for HRP was added into the substrate supply port and, at the same time, the horizontal flow absorption pad was connected to the signal generation pad. Upon initiation of the substrate flow, a light signal at the site of complex formation was produced in proportion to the analyte concentration (see the light signals in Fig. 2b). A control was also run to monitor the consistency of the assay using a secondary antibody immobilized at a site above the analyte line.

3.1.1. Signal generation kinetics

For quantifying the light signal, we built a detector based on image capture using a commercially available cooled CCD camera. The luminescent chip was mounted in a dark chamber with the sensor module located above (Fig. 2b). The signals produced on the signal generation pad were captured by a program provided by the manufacturer, and the image was digitized in the vertical direction of the pad using software. When the signal corresponding to a particular concentration of analyte, e.g., 0.2 ng mL^{-1} cTnI, was monitored over time, the light intensity reached a maximum 30 s after the substrate was supplied and, thereafter, decreased slowly (Fig. 3, chemiluminometric). In contrast, during colorimetric determination the signal density increased continuously over time since the color continued to accumulate until the time of each measurement (3, colorimetric). For maintaining an approximately equal sensitivity between the two systems, the signals needed to be measured at 30 s for the light signal and 5 min for the color signal. Therefore, the employment of chemiluminometry may be advantageous for more rapid acquisition of the signal.

3.2. Immuno-chromatographic assay employing biotin–streptavidin capture

In order to construct the EOC with a high performance capability, capture technology based on streptavidin–biotin binding [27,28] was employed (refer to Fig. 1). The capture antibody used was an F(ab′)$_2$ fragment that was biotinylated via the sulfhydryl groups located at the hinge region according to the protocol we previously reported [20]. Such site-specific conjugates showed a higher antigen capture ability upon binding to streptavidin layers on solid surfaces than those made by the conventional, random procedure coupling biotin molecules to amino groups present all over the antibody.

3.2.1. Preparation of defined biotinylated antibody species

Compared to the random biotinylation protocol, site-directed biotinylation preferentially modified the functional groups within

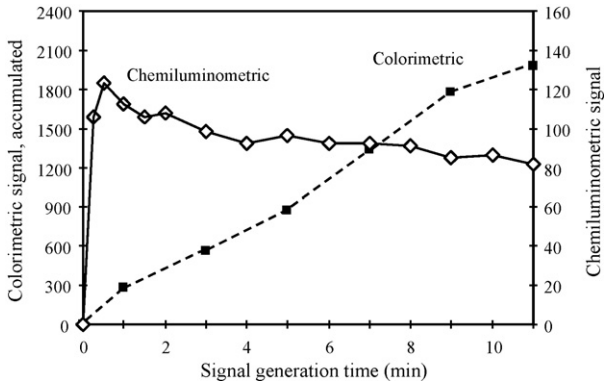

Fig. 3. Kinetic analyses of signal generation from the EOC using different enzyme substrates producing chemiluminometric and colorimetric signals, respectively. The chemiluminometric signal was measured at each time point without integration, whereas the color signal was determined by the accumulation of signal up until each time point. This analysis was carried out twice by employing samples containing a low concentration of cTnI for which the two kinetic patterns were contrasted. Variation of the two replicate measurements was negligible as low as less than 1.15%.

the hinge region of the $F(ab')_2$ fragment, resulting in a low conjugation yield. The presence of free antibody in the reaction mixture, nevertheless, does not interfere with the performance of most immunoassays such as ELISA in which the reactions are usually carried out stepwise. The deficiency caused by the presence of non-biotinylated species can be overcome simply by supplying an excess amount of the mixture. To the contrary, in the case of immuno-chromatographic assays where the antibody components simultaneously react with the sample, the free antibody also binds to analyte, but does not contribute to the signal generation. Therefore, the use of a mixed population of the capture antibody would cause a deterioration of the assay performance.

To separate the biotinylated species, an affinity chromatography step was carried out on a gel column with monomeric avidin immobilized on the gel surfaces. After loading the antibody mixture, the unbound portion was first washed with a sufficient amount of medium. The biotinylated antibody bound was then eluted from the column by competition with free biotin molecules for the binding sites of the immobilized avidin. For each elution fraction, the constituents were analyzed for the levels of protein, the presence of antibody, and the biotinylation state. From the chromatogram (data not shown), the first peak contained the free antibody and the second the biotinylated, indicating the two species were completely separated. Based on the integrated areas of each protein peak, the biotinylation yield for the antibody was about 83% under the reaction conditions used. It is noted that the yields of site-directed biotinylation for immunoglobulin G and Fab fragment were 68% or lower. Such levels were significantly inferior to those via the conventional, random protocol that were usually 90% or greater.

3.2.2. Determination of optimal antibody concentrations

The analytical conditions maximizing the performance of the chemiluminometric EOC may be different from those for the colorimetric EOC. This may result from the adoption of disparate reaction mechanisms of each enzyme substrate for signal generation and differences in the detection protocols of the signals produced. Since the antibodies used for capture and detection were the same as those in the previous report that utilized colorimetry [1,14,15], their concentrations were merely optimized by fixing one to determine the other (Fig. 4). For an enhanced signal, an optimal amount of the capture antibody prepared by biotinylation was 5.45 pmol per assay under the conditions used although this factor did not dramatically alter the signal intensity (Fig. 4a). The detection antibody labeled with HRP, in contrast, increased the signal in proportion to the quantity employed (Fig. 4b). However, since the noise level, i.e., the signal at the zero dose, was also elevated due to non-specific binding, the optimal concentration furnishing the highest signal-to-noise ratio was determined to be 0.87 pmol per assay. It is noteworthy that the optimal amounts of each antibody used in the chemiluminometric EOC were 2-fold higher than those used for the colorimetry-based assay.

As shown in Fig. 4a, an increase in concentration of the capture antibody did not lead to significant enhancement of the chemiluminometric signal in the low concentration range and even reduced the signal in the high concentration range, which may be particularly related to the chromatographic assay utilizing the biotin–streptavidin reaction. In the low concentration range (Fig. 5a), the vertical flow induced by the addition of sample carried the capture antibody to the streptavidin layer to immobilize it onto the surfaces (a-1). The analyte contained in the sample bound to the immobilized antibody (a-2) and the detection antibody labeled with HRP sequentially reacted with the bound analyte to form sandwich complexes (a-3). The complexes eventually contributed to the production of the light signal in a peak pattern of regular shape as shown in the results (Fig. 5a, Analyte). When a high concentration of capture antibody was used (e.g., higher than 8.18 pmol per assay; Fig. 5b), this condition did not allow the antibody to be immobilized to the streptavidin area prior to reacting with analyte, but instead some of the antibody reacted with the analyte in the liquid phase prior to immobilization (b-1). These complexes may be transferred along with the remaining free analyte and will be required to find binding sites on the streptavidin region not already occupied by unbound capture antibody that arrived ahead of these complexes. Such an arrangement of the capture antibody may cause diffusion of the sandwich complexes across the length of the streptavidin region (b-3) and consequently result in an asymmetric signal pattern with a hump or shoulder at the time of signal generation (Fig. 5b, Analyte). This dispersion effect could cause a decrease in the signal yield and, thus, limit the effective amount of the biotinylated antibody needed for high performance of the assay.

3.3. Analytical assessment

Under the optimal conditions determined above, the chemiluminometric EOC biosensor system shown in Fig. 2 was assessed for its analytical performance ability in detecting a particular biomarker. To this end, we first obtained a dose-response curve of the sensor to human sera spiked with variable concentrations

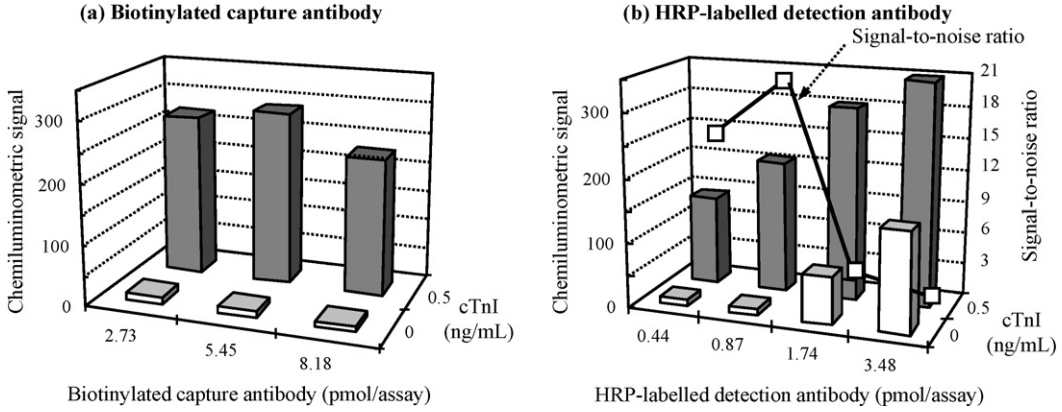

Fig. 4. Determination of antibody concentrations offering maximal analytical performance of the EOC. Using a fixed amount of the detection antibody labeled with HRP (0.87 pmol per assay), the optimal concentration of biotinylated capture antibody was determined to be 5.45 pmol per assay at which the highest light signal was emitted in response to a fixed analyte dose (a). Using this quantity of the capture antibody, the detection antibody concentration was varied, and an optimal signal-to-noise ratio was observed at a concentration of 0.87 pmol per assay (b). Duplicate experimental tests in the optimization showed a maximum 17.9% variation for the capture antibody and 20.9% for the detection antibody.

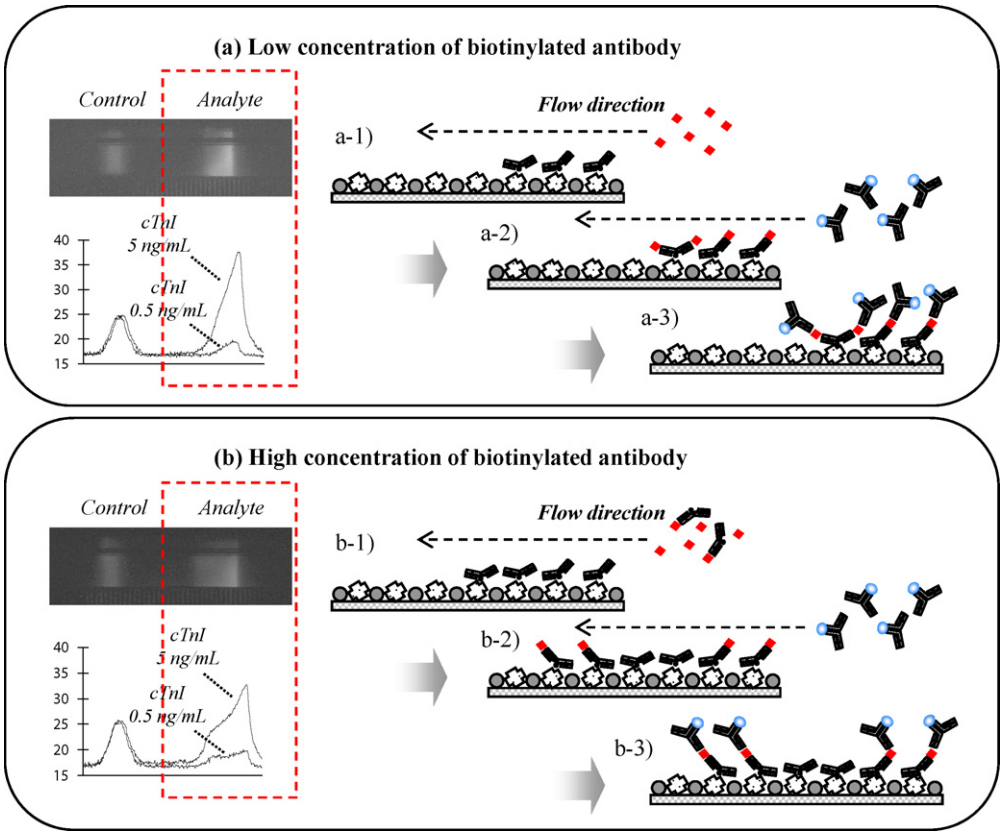

Fig. 5. Dispersion effect of the signal according to an increase in the concentration of the biotinylated antibody employed for capture. At a low concentration of the antibody (a), the vertical flow of the sample carried the analytical components to the streptavidin layer on the signal detection pad where they subsequently formed immunocomplexes. The complexes generated an evenly shaped band of light signal. When the capture antibody was used at a high concentration (b), this condition did not allow all of the antibody molecules to reach the streptavidin area prior to binding to analyte, and instead immunocomplexes were formed in the liquid phase. This resulted in an asymmetric pattern of light signal with a hump or shoulder. See the text for details.

of cTnI, a specific biomarker for AMI, in order to closely mimic clinical conditions [14,16]. In fact, we have previously verified that the EOC system itself highly correlated in measuring cTnI with a clinical standard system, Beckman Coulter Access [21]. The analyte in the form of the cTnI-T-C complex was commercially purchased from Hytest. According to the manufacturer's data, this molecular form corresponded to the actual nature of the molecule present in specimens of AMI patients [29,30]. Following the predetermined protocol, the samples were analyzed on the EOC and images of the luminescent signals were subsequently captured using the cooled

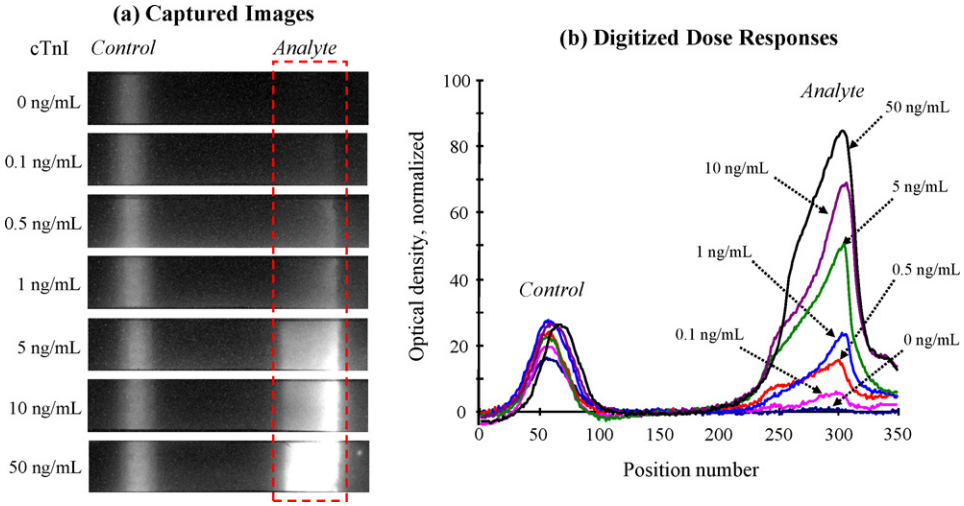

Fig. 6. Typical dose responses of the chemiluminometric EOC biosensor to the analyte, cTnI. Under the optimized conditions, human serum samples spiked with various concentrations of analyte were analyzed on the EOC biosensor system as shown in Fig. 2. The image captured by the sensor (a) was digitized across the length of the signal generation pad to generate optical densities that were normalized and plotted against their positions on the biosensor using a computer program (b). The area under the peak was measured as the signal proportional to the analyte concentration.

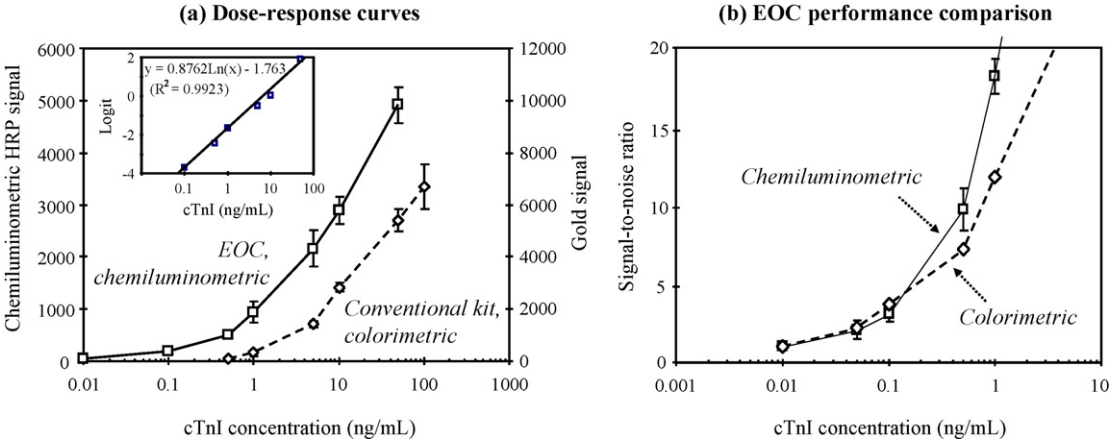

Fig. 7. Comparison of the detection capability of the EOC biosensor with those of the conventional analytical systems. Standard curves for each system were obtained under the respective optimized conditions using different signal production and detection elements. For the luminescent EOC, the HRP signal was determined using chemiluminometry and, for the conventional test kit, the colloidal gold signal was measured using colorimetry (a). The curve for the EOC was also linearized by logit–log transformation to determine the dynamic range (the inset, 7a). Furthermore, from the same batch of EOC, two different signals, the chemiluminescence and color using a chromogenic substrate, were produced and the resulting signal-to-noise ratio was then plotted against the analyte concentration (b). By comparing the results, the both EOC systems showed about the same performance regarding sensitivity and were about 30 times more sensitive than the conventional kit. Standard deviations of triplicate measurements at each point are indicated.

CCD detector (Fig. 6a). The image was scanned across its length to convert the optical densities to digital values by employing a computer software program and the values were then plotted against the position on the signal generation pad after normalization (Fig. 6b). The peak size reflecting the response of the sensor was directly proportional to the analyte concentration, whereas the peaks obtained from the control remained approximately constant, merely indicating the consistency of the assay regardless of the analyte fluctuation.

The peaks corresponding to each cTnI concentration were used to generate a standard curve for the determination of sensitivity defined as the detection limit according to established methods [31,32]. The peak areas were calculated by integration and plotted against the corresponding analyte concentrations (Fig. 7a; EOC, chemiluminometric). As a reference system, the conventional rapid test kit was constructed employing the same antibodies but a different tracer, i.e., colloidal gold, under optimal conditions [1,6,15]. In the conventional kit, the assay was conducted using lateral flow only in the vertical direction, and the resulting color signal was measured by means of colorimetry [6]. A standard curve was prepared utilizing the same standard samples as those used for the chemiluminescent EOC (Fig. 7a; Conventional kit, colorimetric). From the two curves, the detection limits determined using the method previously reported [1,20] were 0.027 ng mL^{-1} for the chemiluminescent EOC and 0.82 ng mL^{-1} for the conventional kit, which revealed a 30-fold improvement in sensitivity with the novel biosensor. The standard curve for the EOC was linearized by logit–log transformation (the inset of Fig. 7a; [33]), which showed a dynamic range of 0.1–100 ng mL^{-1} and a variation less than 16% in triplicate measurements.

In comparison with the previous studies, the sensitivity of the chemiluminometric EOC was about three times higher than that of the colorimetric EOC using a chromogenic substrate for HRP, also developed in this laboratory [14], where the same antibody pair to cTnI was employed. This may probably result from the introduction of biotin–streptavidin capture here, yielding in a high antigen–antibody binding complex formation. To test such a postulation, the both EOC signals were produced under the same conditions employing the capture technology and converted to signal-to-noise ratio for a direct comparison (Fig. 7b; [34]). The results revealed the two EOC systems to have an identical performance regarding sensitivity. It is then notable that the EOC could be approximately 10-fold more sensitive than the conventional test kit unless the capture technology was used. Other POCT systems for cTnI have also been developed by utilizing variable tracers such as fluorescent beads [35] and magnetic particles [36] that showed sensitivities about 0.04 ng mL^{-1} and 0.5 ng mL^{-1}, respectively. Compared to these products, the EOC systems still demonstrated a superior performance.

4. Conclusions

In conclusion, the chemiluminometric EOC biosensor in combination with an image detector equipped with a cooled CCD camera demonstrated significantly enhanced performance compared to the conventional rapid test kit. The EOC design has indeed been innovated to offer fluidic channels for reproducible cross-flow without using a chemical bonding process in the fabrication. The biotin–streptavidin capture technology employing a defined biotinylated antibody also contributed to the improvement of the assay, but resulted in a unique dispersion pattern of the light signal in the chromatographic step, which consequently limited the amount of the biotinylated antibody that could be used in order to achieve high performance levels. The production of the luminescent signal, in contrast to the color signal produced by the same enzyme, would be favorable to analytical settings requiring a rapid acquisition of the response. In addition, the CCD camera could be replaced by a miniature sensor such as complementary metal-oxide-semiconductor image sensor without sacrificing the detection capability provided it is physically integrated with the signal generation pad. Since the sensor can be manufactured at a low cost by mass production, this may allow us to make the resulting analytical system disposable together with the EOC. Such system construction based on the chemiluminometric signal detection would be suitable for POCT.

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