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Immunogold–silver staining-on-a-chip biosensor based on cross-flow chromatography☆

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ABSTRACT

Immunogold–silver staining (IGSS) was adopted in cross-flow chromatographic analysis in which immunological reactions and silver intensification were sequentially conducted in the vertical and horizontal directions, respectively. Factors controlling the performance, except the silver substrate solution, were optimized to increase the signal-to-background ratio in measurements of cardiac troponin I as a model analyte. In generating the signal, the size of colloidal gold catalyst was critical; the smallest size (5-nm diameter) in the selected range yielded the highest colorimetric signal. To maintain the low background, two processes, blocking the remaining surfaces of membrane after antibody immobilization and washing the residual tracer after immunological reaction, were necessary. Self-nucleation of silver ions also caused a background signal and was controlled to some degree by decreasing the hydrodynamic force that arose when the substrate solution was supplied in the horizontal direction. Finally, a new chip (IGSS-on-a-chip; IOC) that allowed for convenient, efficient IGSS was produced by injection molding of plastic. This method enhanced the detection capability by 51-fold compared to the conventional rapid test kit using 30 nm-sized colloidal gold as the tracer. The IOC biosensor results also showed that silver intensification yield via cross flow after immunological reaction was 19% higher than that by traditional incubation.

1. Introduction

The chemical basis for silver intensification via colloidal gold, known as autometallography, dates back to 1930, and its application to gold tracer linked to an antigen–antibody reaction was demonstrated in the early 1980s [1]. In immunogold–silver staining (IGSS), gold provides a route for the transfer of electrons from the reducing agent in solution to silver ions bound to the gold surface. This results in specific deposition of metallic silver at the site of immunogold labeling, and the silver can then participate again in the catalytic reaction. In its earliest stages, the IGSS method was principally applied to immunohistochemical studies [1,2]. Such utilization in many studies served as a momentum for developing various related technologies including photography, histochemistry, and gold labeling, and in propagating them to other immuno-analytical fields [2,3]. The IGSS method has recently been utilized for signal intensification in different types of immunosensors based on electrochemistry [4,5], mass [3], topography [6], or colorimetry [7], generally leading to enhanced detection capability [2].

In recent immunoassay trends, analytes from various sources are increasingly being measured at the site where the specimen is furnished, allowing for rapid estimates of the analyte concentration [8]. The demands inherent to point-of-care testing (POCT) systems are well reflected by the spread of rapid test kits based on immuno-chromatography using membrane strips as a solid matrix [9,10]. Since the kits mostly utilize colloidal gold as a tracer, the detection capability can be enhanced by employing IGSS. This has been demonstrated in the analyses of bio-terrorism agents and infectious viruses for which sequential two-step procedures of antigen–antibody binding and silver intensification were employed [11]. Here the silver staining was carried out in an incubation mode by immersing the membranes in the bulk solution, which was a technical barrier that prevented us from using the method outside of the laboratory environment.

To resolve this technical limitation, cross-flow chromatography was developed in this laboratory [2]; in this method, immunological

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binding and silver intensification can be conducted under conditions suitable for POCT. To this end, a chip facilitating the two-step conduction in the vertical and horizontal directions, respectively, was produced by injection molding of plastic. Furthermore, the new chip was devised for assembly without adhesive, making the process simpler and, consequently, more reproducible. In this study, the color signal resulting from IGSS was detected and recorded using an inexpensive detector mounted with a digital camera. Such application of colloidal gold as signal generator combined the high detection capability with the structural stability of the non-proteinaceous label. As a model analyte for this research, cardiac troponin I (cTnI), a specific biomarker of acute myocardial infarction [13], was used.

2. Experimental

2.1. Chemicals and reagents

Cardiac troponin (cTn) I-T-C complex, cTnI, and a monoclonal antibody specific to cTnI (clone 19C7) were purchased from Hytest (Turku, Finland). Human anti-mouse antibody (HAMA) blocker (mouse IgG fraction) was obtained from Chemicon International (Temecula, CA). Casein (sodium salt type, extracted from milk), triton X-100, D(+)trehalose, human serum, sodium citrate, tannic acid, chloroauric acid ($HAuCl_4$), and silver enhancer solutions (A and B) were obtained from Sigma (St. Louis, MO). Nitrocellulose (NC) membrane (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) and glass fiber membrane (SA3J455I01) were purchased from Whatman (Maidstone, England) and Millipore (Bedford, MA), respectively. Other reagents used in this study were of analytical grade.

2.2. Equipment

To analyze the intensified silver signals on IOC after completing the whole procedure, a colorimetric detector with a digital camera (VIJE Talk CCD, SavitMicro, Korea), installed within a black chamber, was devised. The camera was mounted on the ceiling of the chamber, and light-emitting diodes were also installed to automatically illuminate when the IOC was inserted. The optical density of the captured image from the signal monitoring window was digitized in the vertical direction using software programmed in LabVIEW 7.1 (National Instrument, TX). The data was then transferred to a personal computer using a USB connector and stored in Microsoft Excel.

2.3. Characterization of colloidal gold as catalyst

Colloidal gold with a mean diameter of 5 nm or larger (10 and 30 nm) was synthesized using tannic acid or sodium citrate as the reducing agent, respectively [14]. Their catalytic reaction rates for silver intensification were then compared under conditions of equal mass or surface area of the gold particles in the liquid phase. First, the gold solutions were diluted with deionized water to a constant mass concentration (0.01 wt%) and added into separate microtiter wells (100 μL each). Silver enhancer solutions A and B were mixed in an equal volume (100 μL) and sequentially added to the colloidal gold solution. The gold-catalyzed silver signals were then spectrophotometrically monitored by measuring the optical densities at 450 nm for 20 min. The same procedure was then repeated with the gold solutions at equal total surface area as calculated by assuming that the colloidal gold particles were perfect spheres.

2.4. Labeling antibody with colloidal gold

A monoclonal antibody specific to cTnI (BD clone 12) was produced by the standard protocol [15] as described elsewhere [16]. The antibody was dialyzed in 10 mM phosphate buffer (PB), pH 7.4, and then added (15 μg) to 1 mL of gold solution, pH 8.0. After gentle shaking for 1 h, 5% (w/v) casein dissolved in PB (Casein-PB) was added (122 μL) to the mixed solution to block the residual surfaces of antibody-colloidal gold conjugate. The solution was further reacted for 1 h and then centrifuged at 14,000 rpm for 45 min. After discarding the supernatant, the gold precipitates were resuspended in Casein-PB (500 μL) and then separated under the same conditions again. After removing the liquid portion, Casein-PB was added to the precipitates to obtain 20-fold concentrate of the initial gold. The product was then stored at 4 °C until used.

2.5. Preparation of immunostrip

To perform an immuno-chromatographic assay, four different functional membrane pads were used (from the bottom): a glass fiber membrane (4 mm × 15 mm, GFB-R4) for sample application, another glass fiber membrane (4 mm × 6 mm, PT-R5) for the release of the detection antibody labeled with colloidal gold, NC membrane (4 mm × 25 mm) for signal generation, and cellulose membrane (4 mm × 15 mm, 17 CHR) for sample absorption. They were prepared as previously reported [17], except the conjugate release pad and signal generation pad. The conjugate pad was prepared by adding a solution containing the gold-labeled antibody conjugate (20-fold concentrate, 2 μL), HAMA blocker (5 μg), 10% Triton X-100 (0.5 μL), and 40% trehalose (5 μL), into the membrane. The signal pad was prepared as described earlier [17] but with a different concentration (1 mg/mL) of the capture antibody (clone 19C7) and different positions for the signal lines (6.5 mm for the analyte and 12.5 mm for the control). After drying the conjugate and signal pads at 55 and 37 °C, respectively, for 1 h, the four pads were then partially superimposed as reported [17] to maintain a continuous vertical flow.

2.6. Optimization of IGSS conditions

2.6.1. Analytical procedure via cross-flow chromatography

Standard samples for cTnI (I-T-C complex form) were prepared by serial dilution with human serum to mimic clinical conditions. The sample solutions (100 μL) were transferred onto the application pads of each immunostrip and then placed within glass tubes in an erect position. After 15 min, the immunostrips were removed from the tubes and, for horizontal flow initiation, assembled with glass fiber (15 mm × 15 mm, SA3J455I01) and cellulose (variable sizes for optimization, 17 CHR) membranes for solution supply and absorption, respectively. They were positioned on the lateral sides of the signal generation pads of each immunostrip. Deionized water (150 μL) was first supplied to remove any residual colloidal gold on the signal generation pad, and the mixture (150 μL) of silver enhancer solutions A and B was then added for signal intensification. The black-colored silver signals produced at predetermined times were captured as image files using a scanner (HP ScanJet 4670, Hewlett-Packard, Palo Alto, CA).

2.6.2. Blocking NC membrane

To minimize non-specific binding of reagents, the residual surfaces of the NC membrane after antibody immobilization were blocked by immersion in 0.5% casein-PB solution for 1 h. The membrane was dried at 37 °C for 1 h and then used to fabricate the immunostrip as described above.

2.6.3. IGSS by incubation

The silver intensification was carried out in incubation mode to measure the self-nucleation phenomenon. After immunological reactions in the vertical direction, the signal pad was washed by horizontal flow, taken apart from the immunostrip, and immersed in silver substrate solution (1 mL volume). The silver signals were captured at predetermined times using a digital camera and then quantified as optical densities using an image analysis program (Multianalyst version 1.1, Bio-Rad Laboratories, Hercules, CA). The density ratio of the analyte signal over the background signal between the analyte and control lines was calculated and plotted against the incubation time.

2.6.4. Optimization of horizontal flow conditions for IGSS

To maximize the signal-to-background ratio, the horizontal flow after the antigen–antibody binding was controlled by varying the length of the absorption pad. The signal images were captured and quantified as described above, enabling calculation of the signal-to-background ratios. These were then plotted against the silver enhancement time to determine the optimal size of the horizontal absorption pad.

2.7. Construction of IGSS-on-a-chip biosensor system

The IGSS-on-a-chip (IOC) consisted of two polycarbonate plastic plates, top and bottom, fabricated by injection molding. The bottom plate of the chip (32 mm × 76 mm × 8 mm) comprised fluidic channels arranged in the vertical and horizontal directions. The vertical channel was formed in the center of the width to hold the immunostrip. The horizontal consisted of the supply channel for silver substrate solution and a compartment for the horizontal flow absorption pad, placed on the respective side of the vertical channel. This design configuration provided a flow path for the substrate solution across the signal generation pad of the strip after integration. The supply channel was formed in a shape of a circular triangle expanding toward the vertical channel.

On the top plate of the chip, a window was installed for monitoring the produced signal, as were two pots for sample addition and silver substrate supply, respectively. The signal-monitoring window was furnished on the top plate surface (2.2 mm × 18 mm) corresponding to the ceiling of the signal generation pad of the immunostrip to be placed at the bottom plate. The sample application pot, aligned with the sample application pad of the immunostrip, was constructed as an open-bottom well (5 mm × 10.5 mm × 4.5 mm) to allow the sample addition (250 μL maximum). The substrate supply pot (5.3 mm in diameter) was installed at the far end of a supply channel as the inlet of the horizontal flow. The arrangement was shaped as a circular triangle devised to combine with the substrate channel on the bottom plate when integrated, providing a closed flow passage with a thin-layer outlet. Near the outlet, two air ventilation holes (1 mm in diameter) were located at both of the end projection areas.

Within the vertical channel and the horizontal absorption pad compartment of the bottom plate, the immunostrip and a flow absorption pad (cellulose membrane; 14 mm × 12 mm) were placed, respectively. The two plates were then firmly combined using groove joints without an adhesive. The assembled IOC was kept in a desiccator at room temperature until used.

2.8. Analytical performance

2.8.1. Analytical procedure of immunosensing

Using the IOC system, the cross-flow chromatographic analysis for cTnI was performed. A stock of cTnI (1 mg/mL; I-T-C complex form) was serially diluted to pre-determined concentrations with human serum that was previously determined to be negative for the analyte. The sample (100 μL) was transferred into the application pot of the IOC. After reaction for 15 min, the horizontal flow absorption pad was connected to the lateral side of the signal generation pad to induce the horizontal flow. Deionized water (100 μL) was first added into the substrate supply pot for washing and the mixture of silver substrate solution (200 μL) was sequentially supplied to intensify the signal for 8 min. For IGSS in incubation mode, the immunostrip was removed from the chip after washing and the signal intensification was carried out by immersing the strip in substrate solution for 7 min. The silver signals produced at the analyte and control sites were captured as images within the colorimetric detector, and the optical densities were digitized to obtain the signal value [17]. The same procedure was repeated with standards of different concentrations. The analyses at each analyte dose were repeated three times, and the mean of the signal values was used to prepare a dose response curve, enabling determination of the detection limit [17].

2.8.2. Conventional test kit

As reference, the conventional rapid test kit employing colloidal gold (30 nm diameter) as a tracer was constructed as previously described [12]. Identical samples to those used for the IOC were loaded on each kit and laterally migrated for 15 min. The colorimetric gold signals on the strips were quantified as described elsewhere [18]. The procedure was repeated three times for each sample, and a dose–response curve was prepared to determine the detection limit.

3. Results and discussion

3.1. Characterization of colloidal gold as catalyst

In IGSS, colloidal gold is used as a catalyst to chemically reduce silver ions to metallic silver on the gold surfaces [1,2]. As this reaction proceeds, the metallic particle dimension increases, which consequently enhances the color density that can be observed by the naked eye. Such staining yield mainly depends on the reactant composition and catalytic surface area. In this study, we used a ready-to-use silver substrate solution that included ionic silver and reducing agent (hydroquinone) so that the chemical concentrations were constant. The remaining factor that controls the performance is then the dimension of the catalyst, *i.e.*, the surface area. To determine an optimal size, three different colloidal gold particles with 5-, 10-, or 30-nm diameter were prepared [19] and then tested for their catalytic abilities at a constant total mass of gold added to the reaction solution (Fig. 1).

In general, the major factors that control the catalytic reaction rate are the activation energy, the binding constant of catalyst to reactant, and the area of the gold surface where the active sites are present [19]. Since the first two factors are thought to be the same for colloids of different sizes, the rate would then be determined by the total surface area of the gold transferred in the reaction mixture. This may qualitatively explain the results obtained under the conditions of constant total mass (ratio of total surface area for 5-:10-:30-nm gold = 6:3:1) as shown in Fig. 1. The postulation, however, was not further evidenced in the subsequent tests in which the total surface area was kept constant (see Fig. 1, inset). Furthermore, the particle numbers of each gold colloid under the respective conditions were significantly different, *i.e.*, the particle ratios for 5-:10-:30-nm gold at constant mass were 216:27:1 and 36:9:1 at the same total surface area. These ratios were consistent with the relative patterns of the kinetic curves in Fig. 1. It is conceivable that with larger numbers of colloidal particles, the chance of collision with the silver ionic molecules was higher [20]. Furthermore, the smaller gold colloid seems to move more actively in

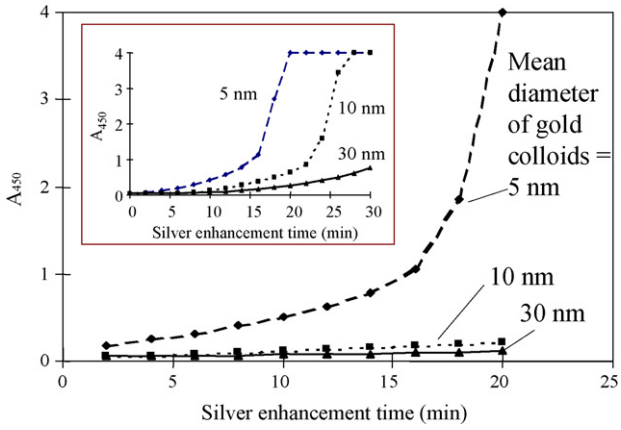

Fig. 1. Comparison of the gold-catalytic abilities of colloidal gold of different sizes. The catalytic reactions were performed using a pre-prepared silver substrate solution under conditions of equal gold mass. The enhancement of color signals by each gold particle (5 nm, 10 nm, and 30 nm in the mean diameter) was measured spectrophotometrically at 450 nm against time. Further characterization for each gold particle was performed at equal surface area (the inset). 5-nm gold colloid showed the highest catalytic capability among those tested.

aqueous solution, which might contribute to the supply of energy to overcome the activation barrier against the reaction [21]. Further investigations are needed to explore this hypothesis; however, such experiments exceed the scope of this study.

Based on our results, colloidal gold with a 5-nm diameter was best for the silver intensification and was therefore used as the catalyst in subsequent experiments. It is noted that the 30-nm-sized gold has usually been used as a tracer in conventional, qualitative rapid tests based on immuno-chromatography [22].

3.2. IGSS employing cross-flow chromatography

The same analytical concept of cross-flow immuno-chromatography [12] can be applied to IGSS so that this staining procedure can be conducted anywhere the specimen is furnished, particularly outside of the laboratory (Fig. 2). To this end, an immunostrip was prepared as in the conventional test kit [22] except that the detection antibody was labeled with 5-nm gold.

To use the gold catalysis process in the assay, the sample containing analyte was first absorbed by capillary action from the bottom of the strip (Fig. 2a). The vertical flow induced a sandwich immune complex formation with the capture antibody immobilized on the signal generation pad. After the binding was complete, the two horizontally arranged pads were subsequently placed on the lateral sides of the signal generation pad (2b). Upon supply of the silver substrate, the horizontal flow was initiated to trigger the catalytic reaction of the gold contained in the sandwich complex. The silver staining process via the catalytic reaction can be divided into three steps as shown in Fig. 2b: initiation, propagation, and termination [23]. This produced an intensified color signal in proportion to the analyte concentration, and a control signal was also generated to monitor the consistency of tests.

3.3. Optimization of analytical conditions

Among a number of parameters that characterize the performance of an analytical system, the specificity and sensitivity are major properties to consider, particularly when the system is under construction [24]. We selected monoclonal antibodies to cTnI that maintain high specificities, even in human serum, to approximate a real sample [16,17]. Signal enhancement and background control were the goals of this study since the signal-to-background ratio would determine the sensitivity, defined as the detection limit of the analyte concentration [25].

3.3.1. Blocking the NC membrane

To increase the signal-to-background ratio, it is essential to regulate the background on the signal generation pad. Thus, the pad preparation conditions for IGSS may differ from those used for the conventional rapid test kit. In the conventional process, the antibody solutions are first dispensed at predetermined sites on the NC membrane, which is dried and then assembled straight with other membrane pad components. This process was adopted to fabricate an immunostrip for cTnI that was then used for the cross-flow chromatography with IGSS for signal intensification as shown in Fig. 2 (Fig. 3a, left). The result revealed high background staining with metallic silver appearing all over the signal generation pad. This resulted from the non-specific adsorption of the gold on the pad surfaces during the vertical flow and the subsequent catalytic reaction on the gold during the horizontal flow.

To circumvent this problem, after immobilizing the antibodies on the NC membrane, the residual bare surfaces were treated with casein, an inert protein that is widely used as a blocking agent in immunoassays (3a, right). The background was almost eliminated by the additional blocking step, intercepting the intrinsic hydrophobicity of the NC membrane. Thus, the coating of the membrane surfaces with such an agent is crucial to control the non-specific binding prior to the silver intensification. This requirement for casein suggests a higher non-specific adsorption of the gold tracer than with an enzyme signal generator, which does not require a blocking agent [12,17,18].

3.3.2. Substrate supply modes

In a conventional IGSS procedure, the gold-catalytic reaction is carried out in the bulk substrate solution and can be continued until a background color begins to be obvious. The background is developed via the non-catalyzed reaction between silver ions and the reducing agent, *i.e.*, self-nucleation, in the bulk solution [14]. Leaving this non-specific effect increases the background color and, thus, deteriorates the analytical sensitivity. To identify the effect, after induction of antigen–antibody binding on the signal generation pad by lateral flow as shown in Fig. 2a, the pad was removed and immediately incubated in the substrate solution. The signal and background color generated between the signal and control were measured, and the ratio, a parameter indicating the analytical sensitivity, was plotted against the incubation time (Fig. 3b, Incubation). Although both densities were augmented throughout the measurement time period, the ratio reached a maximum after 7 min, the optimal termination time, and decreased thereafter.

To enhance the analytical sensitivity, it is necessary to suppress self-nucleation by managing the chemical and physical factors (*e.g.*, temperature) that control the IGSS. The non-specific event may be regulated by adding a stabilizing agent, called a protective colloid, such as gum arabic, bovine serum albumin, gelatin, dextran, or polyethylene glycol [14]. However, such agents not only retarded the appearance of background color, but also the generation of signal, which consequently resulted in an insignificant improvement in the ratio of the two optical densities (data not shown). The concentrations of silver ions and reductant were also varied by dilution, but did not yield any enhancement. Among the physical factors, temperature, which governs the rate constant of the catalytic reaction, selectively restrained the self-nucleation to some degree at low temperature, *e.g.*, 4 °C (data not shown). However, this condition is better suited for analysis within a laboratory rather than in the field for POCT.

In the conventional IGSS procedure, a hydrodynamic force resulting from mixing of the substrate solution may arouse the self-nucleation, and the volume may determine the amount of nucleated

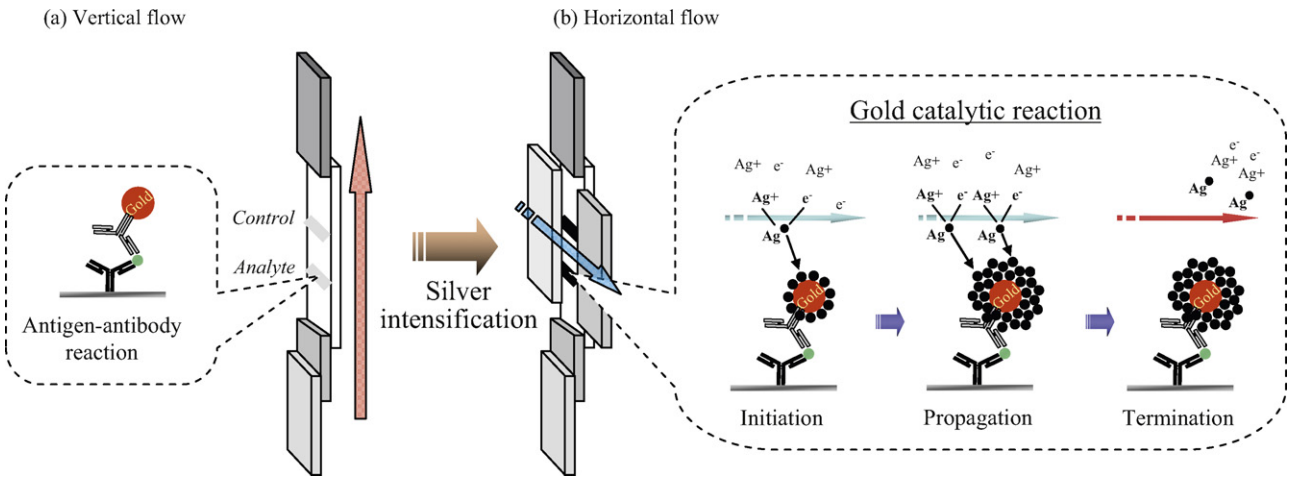

Fig. 2. The analytical concept of silver-intensified immuno-chromatographic assay based on sequential cross flow. The constitution of the assay system is similar to that of the conventional rapid test kit except that 5-nm gold and additional cross-pads for the supply of silver substrate solution were used. The analytical procedure consists of vertical flow for antigen–antibody reactions (2a), as in the conventional rapid tests, and horizontal flow for signal generation via the catalytic reaction on the gold (2b). The catalytic reaction proceeds in sequential steps of initiation, propagation, and termination.

silver metal that deposits on the membrane surfaces. We tested the effect associated with hydrodynamic force and volume by controlling the horizontal flow while conducting IGSS using cross-flow chromatography (refer to Fig. 2). To this end, the length of the horizontal absorption pad was varied from 1 to 6 cm, managing the two variables at the same time, and the cross-flow chromatographic analyses were performed (Fig. 3b). The signal-to-background ratios measured against time under each condition revealed that the ratio steadily increased as the pad length was reduced. The analytical performance in incubation mode was approximately equal to that of the flow method using a 6-cm-long absorption pad, which produced the lowest signal-to-background ratio. This result suggests that the performance of the flow system can be further enhanced compared to the incubation mode, provided the pad length is shortened.

In the silver staining mechanism, the colloidal gold particles captured within the membrane pores furnish the catalytic sites and transfer electrons from hydroquinone, a reducing agent, to silver ions in the substrate solution [14]. Since the membrane pores have a small space in which to hold the substrate solution, the catalytic reaction rate might be limited by mass transfer. This postulation, however, was not supported by the results in Fig. 3b, which show that increased substrate supply induced self-nucleation of silver ions rather than the catalytic reaction, which could be caused by high turbulence of the flow. Therefore, the IGSS procedure was limited by the reaction rate, and lower substrate supply would be advantageous under the settings used. Furthermore, dilution of the substrate did not affect the reaction.

3.4. Construction of IGSS-on-a-chip biosensor system

To simplify the immuno-chromatographic assay using cross flow (refer to Fig. 2), we constructed a new plastic IGSS-on-a-chip (IOC) by injection molding of polycarbonate to improve the device [17] (Fig. 4). The vertical channel (Fig. 4a) held a 4-mm-wide immunostrip, as shown in Fig. 2a, that fit tightly within the channel; this channel also had a sample application pot and a signal monitoring window on the chip surface. The horizontal channel (4b) consisted of a silver substrate supply passage with an inlet pot and an absorption pad compartment that were placed on the lateral sides of the signal generation pad of the strip. Using the IOC, we performed the cross-flow chromatographic analysis of cTnI as an analyte by transferring the sample in the vertical direction and subsequently supplying the substrate solution in the horizontal direction.

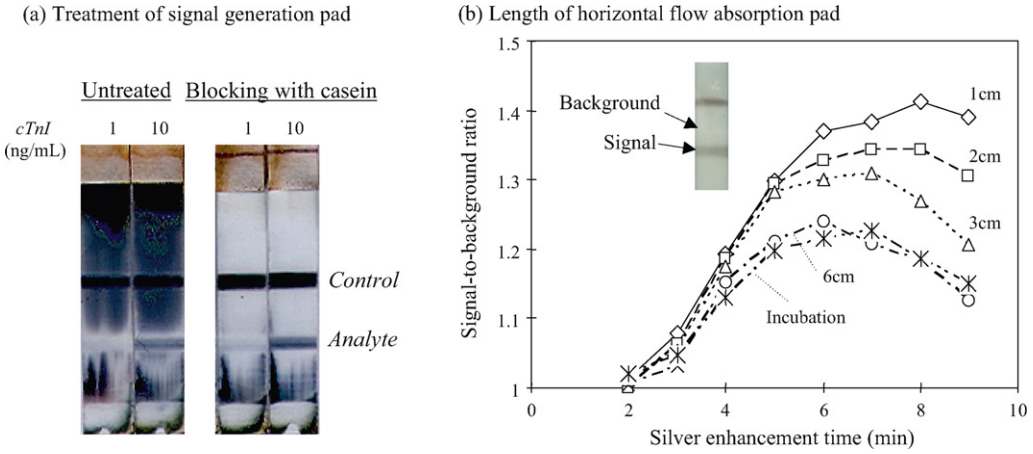

Fig. 3. Optimization of variables for the flow-based IGSS performances. First, blocking the residual surfaces of the signal generation pad with casein remarkably reduced the background in the silver-intensified immuno-chromatographic assay (3a). Secondly, the horizontal flow absorption pad length also controlled the background that the maximum signal-to-background ratio attainable increased as the pad length was shortened in the range of 1–6 cm (3b). The performance of the system with the longest pad was similar to that obtained in incubation mode where a conventional IGSS procedure was employed as control.

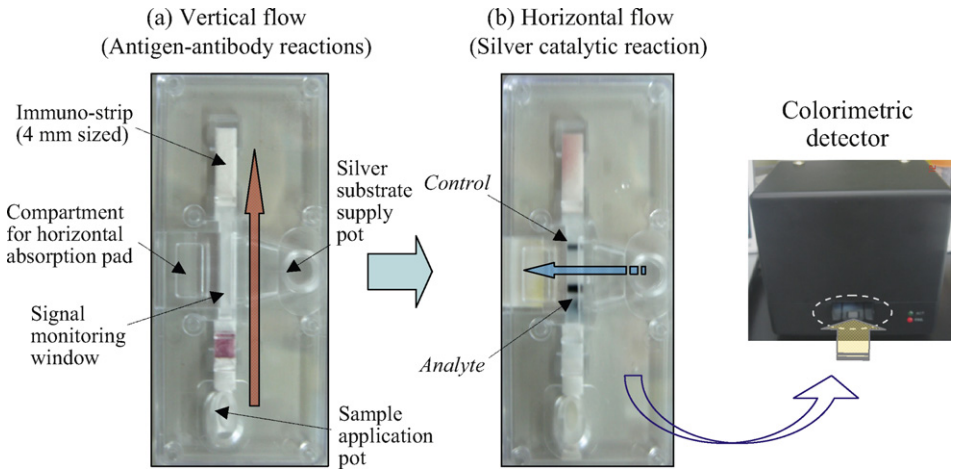

Fig. 4. Construction of IGSS-on-a-chip (IOC) biosensor system with cross-flow chromatography. Upon sample addition into the application pot, the flow was transferred by capillary action along the strip (4a). The silver substrate solution was then added into the supply pot and the horizontal flow absorption pad was immediately connected to the signal generation pad. This initiated the flow in the horizontal direction and consequently produced a signal in proportion to the analyte concentration (4b). The signal was quantified using a digital camera-based colorimetric detector.

3.5. Analytical assessment

Under the optimal conditions determined above, the IOC performance was characterized using cTnI spiked in human serum to closely imitate a real specimen. The two monoclonal antibodies selected for this study were specific to cTnI [17]. They were used as capture and detection antibodies, respectively, in fabricating the immunostrip that was installed within the plastic chip to produce the functional IOC. After the analytical procedure, the black signals that appeared on the signal generation pad of the IOC were captured as images using a digital camera mounted within the colorimetric detector (Fig. 5a). The signals at each analyte concentration were subsequently digitized to optical densities in the vertical direction using software developed in this laboratory, and the values were plotted against the position after normalization (5b). The peak size, which reflected the dose response of the IOC sensor system, was directly proportional to the analyte concentration.

The signal peaks corresponding to each cTnI concentration were used to make a dose–response curve that was used to determine the sensitivity or detection limit [26]. The peak areas were calculated by integration and plotted against the cTnI concentration (Fig. 6a; IGSS, cross flow). For comparison, the same analytical procedure for silver intensification was repeated using the incubation mode instead of cross flow (6a; IGSS, incubation). The performance was also compared with that of the conventional rapid test kit utilizing 30-nm-diameter colloidal gold as tracer using the same capture and detection antibodies under optimal conditions [12]. The rapid test was performed using only the lateral flow in the vertical direction, and the resulting gold signal was measured by colorimetry that was essentially the same as for the silver signal (6a; Gold, rapid test).

From the dose–response curves, the detection limits for each analytical system were determined as previously reported [17]. The limits were 0.016 ng/mL for IGSS with cross flow, 0.019 ng/mL for IGSS by incubation, and 0.82 ng/mL for the conventional kit. These results indicate that both IGSS systems improved sensitivity more than 43-fold compared to the conventional test kit. To compare the two IGSS systems in depth, the curves were recomposed by plotting the signal relative to noise, *i.e.*, signal measured at the zero

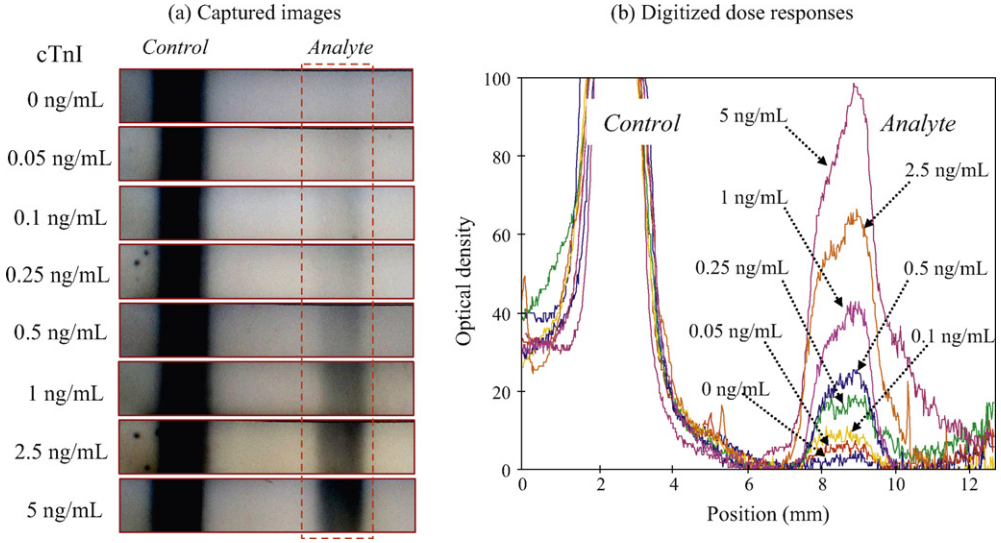

Fig. 5. Dose responses of the IOC sensor system for cTnI under optimal conditions. After sample analyses, the silver-intensified signals produced from the chip were captured on the colorimetric detector (5a) and the signals were then digitized along the length of the strip to convert them to optical densities. The densities were plotted against the position, which revealed dose responses proportional to the analyte concentration (5b). The signal measured at 0.05 ng/mL cTnI was distinguishable from the noise at the zero dose.

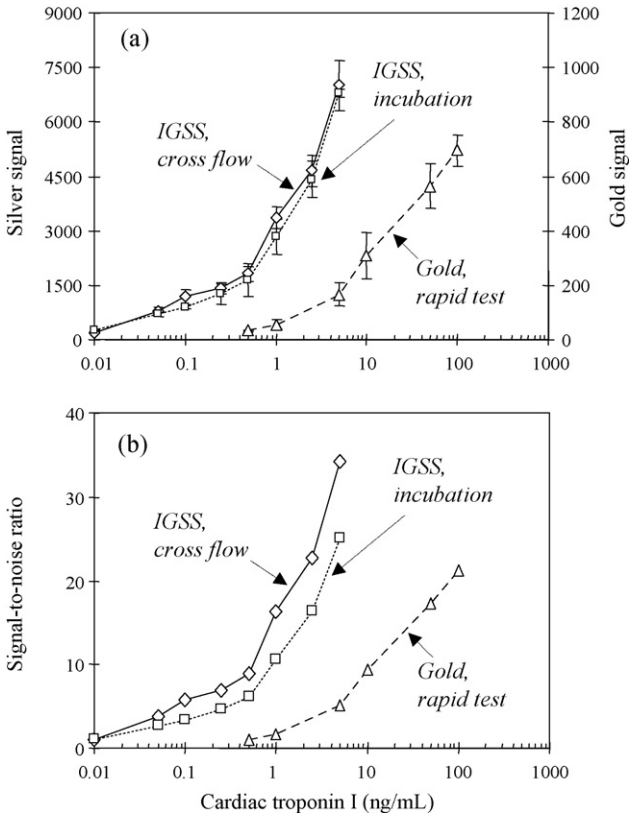

Fig. 6. Comparison of the detection capability of the IOC sensor employing IGSS in cross flow with those of two other reference analytical systems. The signal peaks as dose responses of IOC shown in Fig. 5b were integrated and plotted against the cTnI concentration (6a; IGSS, cross flow). These were compared to IGSS conducted in the incubation mode (IGSS, incubation) and the conventional rapid test kit with 30-nm colloidal gold as tracer and the same antibodies (Gold, rapid test). The analytical systems utilizing IGSS enhanced the detection capabilities by more than 43-fold compared to the conventional kit. To contrast the IGSS systems, the signal-to-noise ratio was plotted against the cTnI concentration (6b). This plot revealed that the IGSS version employing cross flow performed better. Standard deviations of triplicate measurements at each point are indicated.

dose, against the analyte concentration (Fig. 6b). The results clearly showed that the detection capability of the POCT version using cross flow for IGSS was 19% higher than that of the laboratory version by incubation, *i.e.*, 51-fold higher than the rapid test.

4. Conclusion

The IOC sensor system using cross-flow chromatography enhanced the detection capability of cTnI spiked in human serum 51-fold compared to the conventional rapid test kit depending on colloidal gold-based signal generation. The signal intensification by IGSS conduction was even more efficient than that in traditional laboratory tests. Furthermore, the IOC performance was about 5 times better than that of the system employing the same analytical concept but an enzyme tracer (*e.g.*, horseradish peroxidase) [17]. Such an improvement with the IGSS procedure could result from two additional steps, blocking of the immunosorbent and washing of the residual tracer after reactions, which minimized the non-specific signal generation. The background staining was also caused by self-nucleation of silver ions, which was regulated to some degree by quantitatively controlling the substrate solution supplied in the horizontal flow. In future studies, an improved version of IOC can be devised to automate the cross-flow steps and to measure the silver signal by means of electrochemistry (*e.g.*, electric conduction) using electrodes [27].

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