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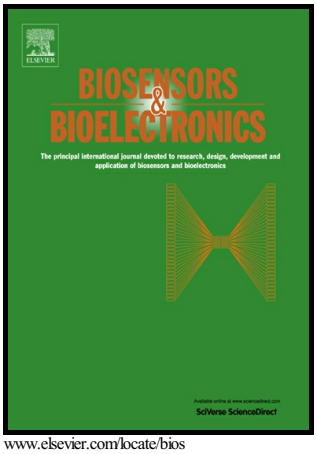

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Biochemical-Immunological Hybrid Biosensor based on Two-Dimensional Chromatography for On-site Sepsis Diagnosis

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Abstract

A hybrid-biosensor system that can simultaneously fulfill the immunoassay for protein markers (e.g., C-reactive protein (CRP) and procalcitonin (PCT)) and the enzyme assay for metabolic substances (e.g., lactate) in the same sepsis-based sample has been devised. Such a challenge was pursued through the installation of an enzyme-reaction zone on the signal pad of the typical immuno-strip for the rapid two-dimensional (2-D)-chromatography test. To minimize the mutual interference in the hybrid assays, a pre-determined membrane site was etched in a pattern and mounted with a biochemical-reaction pad, thereby allowing a loaded sample to enter and then stay in the pad for a colored-signal production over the course of an immunoassay. By employing such a constructed system, a serum sample was analyzed according to the vertical direction flowing along the strip, which supplied lactate to the biochemical-reaction zone and then protein markers to the immunological-binding area that

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was pre-coated with capture antibodies. Thereafter, the enzyme-signal tracers for the immunoassay and the substrate solution were sequentially furnished using a horizontal path for the tracing of the immune complexes that were formed with CRP or PCT. The color signal that was produced from each assay was detected at a pre-determined time and quantified on a smartphone-based detector. Under the optimal conditions, the dynamic ranges for the analytes covered the respective clinical ranges, and the total coefficient of variation was between 8.6% and 13.3%. The hybrid biosensor further showed a high correlation ($R^2 > 0.95$) with the reference systems for the target markers.

Keywords: Heterogeneous analytes, Multiplexed biosensing, Two-dimensional chromatography, Smartphone detector, Rapid sepsis diagnosis

1. Introduction

Point-of-care-testing (POCT) has been highlighted in the *in vitro* diagnostics (IVD) field due to the several advantages regarding its use; for instance, simplicity and a short turn-around time compared with the conventional laboratory approach (Von Lode, 2005). The immuno-chromatographic assay for which a membrane strip is used is one of the most practical formats and is widely used for POCT in the determination of different markers (e.g., hormones, proteins, and nucleic acids), which may indicate the onset or progress of a disease (Posthuma-Trumpie et al., 2009; Yang et al., 2017; Xu et al., 2017). The signal is usually produced as a color on an immuno-strip for which a colloidal gold tracer with a large extinction coefficient is employed, which can be recognized by the naked eye or quantified by the employment of the detection means (Kolosova et al., 2008). As the chromatographic method does not always offer a sufficient detection capability for low-abundant analytes in a sample, alternative signal generators such as enzymes and fluorescent dyes have been introduced to improve the limit of detection (LOD) (Cho et al., 2005; Lan et al., 2016). Nevertheless, the method was developed exclusively for immunological markers that are detectable based on antigen-antibody binding reactions, which may limit the diagnostic availability and not offer the clinical information on physiological aspects such as ions and metabolites (Levy, 2006). If necessary, the different marker species have to be analyzed on separate devices that are dedicated for the measurement of each species.

Diseases that require additional biochemical information for clinical decisions can be typically exemplified by sepsis. Sepsis is defined as a systemic inflammatory response syndrome (SIRS) of the body against infection, usually caused by pathogenic bacteria, and has caused millions of global deaths annually (Bone et al., 1997). Sepsis has been classically diagnosed via physical symptoms such as fever, leukocytosis, and an increased erythrocyte sedimentation rate (Lennard et al., 1982), which are non-specific to the disease or can only be assessed in a laboratory. Alternatively, the markers C-reactive protein (CRP) and lactate can be used as major indicators for the diagnosis of the disease onset and progress (Póvoa, 2002; Chen & Li, 2014). Hyperlactatemia typically occurs during severe sepsis or septic shock, and it may be an indication of a secondary anaerobic metabolism that is due to hypoperfusion or organ failure. However, the differentiation of the sepsis from non-infectious SIRS triggers is still difficult if only the existing methods are used. Recently, procalcitonin (PCT) has been suggested as a specific marker for sepsis, although it appears relatively late after the disease onset (Guven et al., 2002). According to the "Surviving sepsis campaign guidelines," at the least, triple blood-sample markers, which are directly related to sepsis, should be detected to make a clinical decision regarding the disease (Dellinger et al., 2013). Therefore, the following systematic diagnostic approaches are recommended for a more-effective clinical confirmation of sepsis (Henriquez-Camacho & Losa, 2014): 1) determination of CRP as a relatively specific inflammatory indicator that is present in the blood at increased levels, 2) detection of PCT that is augmented to a certain upper range, and 3) monitoring of a lactate-concentration elevation that is at least three times compared to the basal level.

Currently, the two protein markers, CRP and PCT, are analyzed using immuno-analytical systems, and the metabolite, lactate, is quantified by an enzyme-based biochemical sensor (Simon et al., 2004; Bardeletti et al., 1986). The performance of such separate biomarker analyses on different devices can potentially raise uncertainty in the making of a clinical decision, or it can impose an additional burden onto the patient. When the markers in the same sample are about to be measured, the devices might not be prepared in a timely manner. Any inconsistent delays, for instance, could cause changes in the sample conditions, analyte availability, or sample-matrix alteration. Although a fresh blood sample can be used for each analysis, the patient is increasingly subjected to pain, which is likely to be stressful, particularly for infants and the elderly. Furthermore, the separate measurements can mean that urgent sample testing is difficult to reliably accomplish in, for example, an emergency

room.

Therefore, a hybrid-biosensor system has been developed for this study to simultaneously carry out the immunoassay and the enzyme decomposition-based metabolite assay (named as biochemical assay throughout this report) and to characterize them to determine the feasibility of a rapid sepsis diagnosis. To this end, the POCT version of the enzyme-linked immunosorbent assay (ELISA) was adopted as the analytical platform on which the two protein markers can be typically analyzed based on 2-D chromatography (Cho et al., 2006). For this platform, cross-flows are employed, and the vertical flow along the immuno-strip is used for the antigen-antibody bindings, while the horizontal channel is allotted for the washing and the signal generation. The lactate-assay zone was additionally constructed on the signal pad through the patterned etching of the membrane and the attachment of the enzyme-reaction pad onto the engraved plastic back sheet (refer to Figure 1, A). This design can fulfill the entrance of the loaded sample into the defined zone, where it can stay for an independent reaction regardless of the cross-flows. Such a hybridized system was constructed for different analyte species, proteins and metabolites in this case, and it was used to simultaneously measure the three sepsis markers in the same sample (1, B). For the quantification, the color signals that were produced for the markers were measured using a smartphone-based detector and they were digitized using an application (app; 1, C).

2. Materials and Methods

All reagents used in this study were of analytical grade and are listed in the Supplementary Information. Although experimental details are also described in Supplementary Information, procedures are outlined in the Results and Discussion.

3. Results and Discussion

3.1. Analytical concept exemplified by the detection of the sepsis markers

A certain type of hybrid biosensor was designed to simultaneously conduct both a biochemical analysis and an immunoassay on the same membrane strip via simple analytical steps (e.g., sample loading and signal generation). For the biochemical analysis, i.e., lactate

assay in this study, the patterned reaction zone where the enzyme-substrate reaction occurred is positioned near the bottom of the signal-generation pad of the conventional immuno-strip (refer to Figure S1). The pad was modified through the configured etching of the nitro cellulose (NC) signal pad and the attachment of the patterned cellulose membrane to the engraved site. The pattern was designed with a drawing program (AutoCAD 2012) and the physical etching was performed with a carbon-dioxide-driven focused laser (see Figure S2 for details). The reaction zone was formed to leave the edge as an empty space containing only plastic backing to provide a site surrounded by a barrier that prevents the vertical flow that is driven by the capillary action through the membrane pores. This zone contains a chromogenic substrate (potassium iodide) and an enzyme mixture (lactate oxidase (LOX) and horseradish peroxidase (HRP)). Upon the application of the lactate-containing sample, a colorimetric signal that is proportional to the analyte concentration was produced via serial enzyme-substrate reactions (Tatsuma & Watanabe, 1991). Hydrogen peroxide was first produced from the lactate via a catalytic reaction of the LOX, and in the presence of the intermediate product, a colorless potassium iodide was oxidized by the HRP into a brown-color iodine.

Using the hybrid-signal pad, a sample that might contain the different biomarker types (e.g., proteins such as PCT and CRP, and metabolites such as lactate) that indicate a disease (e.g., sepsis) was analyzed. The hybrid-signal pad was first installed in the place of the conventional immuno-strip pad (Figure 1A), which was loaded in the plastic cartridge and particularly prepared for the 2-D chromatography-based analysis (1B). For the diagnosis of sepsis, the immuno-strip was prepared through the immobilization of the captured antibodies for the respective protein markers, PCT and CRT, on each analyte site and a secondary antibody at the control site (1B, inset; the left). In addition, the biochemical-reaction site for lactate was fabricated through the impregnations of the LOX, HRP, and the chromogenic substrate in a dry state. A clinical sample (e.g., serum) that was combined with the biotinylated detection antibody to PCT was then applied to the hybrid immuno-strip, and immunological and biochemical reactions were simultaneously induced at pre-determined locations using the lateral flow in the vertical direction. Thereafter, the enzyme tracers for the immunoassays were supplied using a sequential, horizontal flow, which did not disturb the lactate assay in the biochemical-reaction zone that was surrounded by the plastic barrier that works against the cross-flow. Two horizontal membrane pads for the tracer supply and the absorption were placed on each lateral side of the signal pad (1B, inset; the middle). The

enzyme conjugates (streptavidin (SA)-linked HRP polymer and HRP-labeled detection antibody to CRP) were added to the supply pad and transferred using the horizontal flow to form further binding complexes with the protein markers. After the replacement of the used horizontal pads with the new ones (e.g., using tweezers), the enzyme substrate for the HRP was supplied in an identical manner for the colorimetric-signal generation (1B, inset; right). The signal intensities at the respective sites were subsequently determined by placing the cartridge in the smartphone-based detector and capturing the image for the quantification (1C; see Figure S3 for details).

It should be noted that the biochemical reaction was not affected by the immunoassay procedure and vice versa. The signal-generation processes of the immunoassay for which the cross-flow was used did not disturb the determination of the biochemical analysis; this is based in the design of the laser-patterned plastic gap, as its surrounding of the biochemical-reaction zone prevented the reagent solution that was supplied in the horizontal direction from overflowing the patterned barrier. In the vertical flow, even though the sample consumption can initially occur in the biochemical zone, the antigen-antibody reaction was minimally affected because only a small portion of the loaded sample entered the patterned area. Shortly after the filling of the biochemical zone, most of the sample was migrated along the outside of the area, and it participated in the immuno-reactions with the capture antibodies on the signal pad.

3.2. Optimization of the immuno-analytical systems

3.2.1. Characterization of the immuno-reagents

Since the triple markers for sepsis were employed in the hybrid-biosensor system, it was necessary to test and optimize the associated components including the antibodies and the bio-conjugates prior to the lateral-flow chromatographic assay. First, the antibody specificity that is one of the most crucial immunoassay factors was examined using an ELISA with an analyte immobilized on a microtiter plate (Figure S5). From the results, two antibodies that are highly specific to the corresponding analyte were selected as the capture and detection binders for each marker, as follows: C2 and C6 for CRP and 16B5 and 42 for PCT (see below for details). The two antibodies used for each marker forms the sandwich complex with the same analyte molecule, which has been preferred due to the provision of high sensitivity.

Next, as shown in Figure 1A, the hybrid strip was constructed and used to optimize the analytical conditions for the immunoassay of each of the markers, CRP and PCT. The major variables that were tested are the amount of capture antibodies that were immobilized on the signal pad and the concentration of the bio-conjugates that were used for the signal generation (e.g., biotinylated anti-PCT antibody, HRP-labeled anti-CRP antibody, and streptavidin-poly-HRP20). Furthermore, the additives that enhanced the assay performance (e.g., trehalose, bovine serum albumin, and detergent) and reaction times of each step (refer to Figure 1B) were also determined.

3.2.2. Single-dose response of the biosensor to CRP

Under the optimal conditions, the performance of the hybrid biosensor was initially assessed for CRP, which is the generic anti-inflammation protein marker (Schols et al., 1996). As mentioned, the two monoclonal antibodies, C2 and C6, were used as the capture and detection binders, respectively, for the analysis. Standard samples were prepared in the range of 0 μg/mL to 100 μg/mL, and each sample was applied to the sample port of the sensor cartridge. As described in Figure 1B, the lateral flow was maintained in the vertical direction for 15 min, and the HRP-labeled detection antibody was then supplied in a cross-flow for 5 min. The subsequent addition of the enzyme substrate to the HRP produced a color signal and it accumulated for another 5 min. The color image was captured using the smartphone detector (Figure 2A, inset) and it was then digitized along the longitudinal line of the signal pad using the app (Figure 2A). The optical density within the analyte line was integrated and allotted as the signal value that was then plotted against the analyte concentration (Figure 2B). The dose-response curve showed a sigmoidal pattern and was further linearized via a log-logit conversion (Park et al., 2016) that revealed the correlation coefficient (R^2) > 0.95 (2B, inset). From the linear curve, the dynamic range of the hybrid biosensor for CRP was determined as a value between 0.01 μg/mL and 100 μg/mL, which covered the clinical concentration range of CRP (0.1 μg/mL to 50 μg/mL) in terms of sepsis (Póvoa, 2002). The limit of detection (LOD) was also determined as approximately 0.07 μg/mL corresponding to three times the standard deviation for triplicate measurements of the blank value. It is noted that the analyte line thickness was widened upward proportionally to the concentration of analyte (see the inset of Figure 2, A). As the sample was transferred in the vertical direction across the analyte line, the antigen-antibody binding occurred on the solid surface from the

bottom of the line. At a high dose of analyte, the initial reaction zone is saturated and the zone can be widened upward.

3.2.3. Single-dose response to PCT in the signal-amplification manner

As PCT is normally present in human blood in trace concentrations that are unlike those of the other inflammatory markers (Gaïni et al., 2006), the conventional sandwich immunoassay that is exemplified by the CRP detection may not be sufficient for the PCT assay. Therefore, for the signal amplification, a polymeric enzyme tracer (i.e., poly SA-HRP conjugate) was employed in the immunoassay through a coupling with the detection antibody via a biotin-SA linkage (Lim et al., 2015). Since this approach could enable the detection of the marker at the early stage of sepsis, it has been initially tested for the performance for which a microtiter plate is used as a solid matrix (Figure S6). In the PCT assay, the antibody clones 42 and 16B5 were used as the capture and detection binders, respectively. The signal resulting from the assay (S6, SA-HRP polymer) was amplified compared with that of the conventional system (SA-HRP monomer), showing an approximately 10-fold LOD (0.05 ng/mL) enhancement. The hybrid biosensor for which the signal-amplification technology was adopted was characterized under the optimal conditions toward the analytical performance for PCT. Since PCT is normally present at < 0.5 ng/mL at the basal blood level (Guven et al., 2002), the biosensing for the marker was particularly carried out using the poly-HRP tracer, as has already been mentioned. The standard samples in the range of 0.01 ng/mL to 50 ng/mL were combined with the biotinylated detection antibody and immediately loaded into the cartridge. After the sandwich-complex formation was conducted with the capture antibody on the signal pad, the enzyme tracer and substrate were serially supplied in a cross-flow. The produced color signal was detected, and the optical density was then profiled along the longitudinal axis of the signal pad (Figure 3A). The signal value was determined, allotted for each standard sample, and then plotted against the PCT dose at the semi-log scale (3B). The dose-response curve was then linearized in the concentration range of the standard samples (R^2 > 0.95; 3B, inset). As the signal was saturated at 50 ng/mL of PCT (see Figure 3A), the dynamic range for the marker lies between 0.01 ng/mL and 10 ng/mL, covering the clinical concentrations in terms of sepsis (0.05 ng/mL to 2 ng/mL; (Harbarth et al., 2001)). The PCT LOD that was determined in the single assay is 0.009 ng/mL. This is lower than that of the microtiter plate-based immunoassay, consistent with a previous result (Lim et al., 2015), which may result from the use of high-capacity solid matrix for the capture antibody

immobilization. It is noted that as the basal level of PCT in the dialyzed serum was not detectable using an ELISA, the serum was treated as PCT-free medium.

In the next subsection, the lactate-assay system for which the enzymes are optimized and characterized for the construction of the hybrid biosensor is described.

3.3. Optimization of the biochemical-assay conditions

3.3.1. Optimization of the enzymatic reaction for the lactate analysis

Lactate, known as one of the major inflammation markers, is a metabolic product, and the prognostic value (over 36 mg/dL) in septic-shock patients has been well established (Harbarth et al., 2001). To analyze the lactate on a membrane pad, the two enzymes, LOX and HRP, and a chromogenic substrate, potassium iodide, were employed on the pad in a dry state (Figure S7, A). The assay conditions were then mainly optimized through a varying of the enzyme and substrate concentrations, and also the reaction time.

The effect of the enzyme concentrations on the analytical performance was first tested through the maintenance of the other conditions as constant. The same amounts of the two enzymes were mixed to obtain the quantities of 200 U/mL, 600 U/mL, and 1500 U/mL, and they were then loaded on the cellulose-membrane pad together with the chromogen. The results indicated that the intermediate amount of the enzyme mixture is optimal for the timely control of the biochemical-reaction rate (data not shown). The signal level is low with the smaller amount, yielding a shallow dose-response curve, and alternatively, the level was nearly saturated with the higher quantity when the high lactate doses were used. The excess enzymes may also induce a natural oxidation in the absence of lactate, causing a non-specific signal generation (Auriol et al., 2007).

The potassium-iodide concentration was further fine-tuned through the fixing of the optimal enzyme amounts. At the various chromogen levels (0.6 M to 1.8 M), the repetition of the same experiment with the various standard samples showed the dose responses to lactate, which allowed for a comparison of the dose-response patterns that were obtained under each condition (S7, B). Although all of the patterns appeared to be proportional to the analyte dose, the response curve that was obtained at the middle concentration (i.e., 1.2 M) provided the

best resolution between the measurements. It is noted that the standard samples were prepared through the spiking of the analyte in human serum, which had been previously dialyzed against PBS using a cellulose dialysis bag (MWCO 4,000); this could minimize the influence of the background-level lactate in the preparation of the standard curve (see below for further discussion).

Under the optimal reagent conditions, the reaction time for the lactate assay that maintains the enhanced performance was finally determined. After the standard lactate-containing samples were loaded, the assays were individually proceeded for different time periods (15 min to 35 min). The signal produced from each unit was detected, and the dose-response patterns that vary according to the assay time were then compared (Figure S8, A). After the digitization to the optical density, the response curves were obtained, revealing that the curve at the middle period of the reaction time (i.e., 25 min) offered the steepest slope, compared to the other tested durations (S8, B). The shorter or longer assay time diminished the steepness, thereby determining the analytical resolution. For the longer assay time used, triiodide, the brown-colored product of the iodide oxidation by HRP, was produced in excess, which caused bleaching of the extinction of triiodide via reduction by HRP (Banerjee, 1989).

3.3.2. Single-dose response to the lactate

Lactate is a marker for cellular hypoxia, and its level is elevated in serum due to either an impaired lactate clearance or its excessive production (Mikkelsen et al., 2009). According to the "Surviving sepsis campaign guidelines," the basal level of lactate in the serum of a healthy donor is maintained within the range between 4.5 mg/dL and 18 mg/dL (0.5 mmol/L to 2 mmol/L), and the lactate level increases during septic shock to 36 mg/dL or more (Levy, 2006). Under the above-determined optimal conditions, the quantitative analysis for lactate was conducted using the hybrid biosensor to prepare the dose-response curve. The produced color image was captured using the smartphone-based detector (Figure 4A) and was converted to the optical density via digitization; the image was then plotted against the lactate concentration in the range of 9 mg/dL to 72 mg/dL (4B). From the dose-response curve, the dynamic range of the hybrid biosensor covered the clinically acceptable range of lactate (> 18 mg/dL; > 2 mmol/L), indicating that the hybrid-biosensor system can be utilized for clinical samples (Ferrer et al., 2009).

Although the dialyzed serum was used as the medium, it is unlikely that the change of the sample matrix would be significant enough to alter the immunoassay conditions compared with the non-treated sample. Since the dialysis bag that was used for the lactate removal has an MWCO of 4,000, only small molecular compounds such as ions and metabolites were excluded and the large chemical matrix remained intact. The ionic concentration was supplemented with the ions in the buffer to maintain the physiological level (e.g., 150 mM); therefore, the dialysis effect on the antigen-antibody bindings could not be substantial. In the next section, the hybrid-biosensor performances are characterized from the testing of the mutual interferences among the assays and the further correlation of these performances with those of the reference systems for the respective analytes.

3.4. Characterization of the hybrid-biosensor performances

3.4.1. Test for the interferences among multiple assays

To determine whether the multiple assays for the PCT, CRP, and lactate in the hybrid system influenced each other, the assays were first carried out simultaneously using the same samples that contain those analytes in varying concentrations (Figure 5, A). The standard samples for the triple markers were prepared by diluting each stock with the dialyzed human serum to the following concentration ranges: 0.01 ng/mL to 10 ng/mL for PCT, 0.01 μg/mL to 100 μg/mL for CRP, and 4.5 mg/dL to 144 mg/dL for lactate (see the figure for their combinations). The standard samples that were prepared in seven different concentrations, including the background, were separately loaded into the hybrid-biosensor system, and the results were then analyzed using the procedure that is described above. Here, the control signal was designed to be consistent regardless of the variable analyte concentrations in each sample.

The signals for each marker were distinguishable according to the stepwise elevation of the concentration, and the graph pattern was increased proportionally to the marker dose (Figure 5, A). For the quantitative analysis, the sigmoid-type curves were converted into linearized graphs via a log-logit transformation (Meddinqs et al., 1989), which showed high correlations with the respective regression lines ($R^2 > 0.96$; Figure S9). The dynamic range for each marker corresponded to the range that was determined regarding the single assay earlier. Within the dynamic range, the total CV was calculated as follows: 8.6% for PCT, 10.1% for

CRP, and 13.3% for lactate. The LOD for each marker (0.008 ng/mL for PCT, 0.065 μg/mL for CRP, and 3.2 mg/dL for lactate) was also determined by multiplying the standard deviation of the background by three (Schols et al., 1996). These results indicated that the standard curves are sufficient for the purpose of the quantification of the markers that are present in the dynamic range.

The evaluations of the interference presence among the triple assays were carried out at the same time through a comparison of the dose responses (Figure 5, A) with those obtained from each single assay (Figures 2 to 4). To this end, the signal levels that were measured in the two different modes were plotted together at identical marker concentrations (Figure 5, B). Both of the dose-response patterns are consistent regardless of the measurement mode that was used, although the intensities that were determined at the same dose are somewhat variable in different degrees (1.5% to 15.7%). Such differences may be due to the fact that the detection components that are additional to that for the single assay were employed in the simultaneous determinations; these might raise not only non-specific interactions among the matrix constituents of the medium, but also the non-specific binding to the solid surfaces. Other contributions may also include the experimental errors in the preparation and handling of the increased number of components.

3.4.2. Correlation with the reference-system performances

As part of the assessment of the hybrid biosensor, the analytical performances were compared with those of the reference systems that are dedicated for each marker through a simultaneous analysis of the standard samples containing the triple analytes in human serum. To this end, the conventional reference systems of the ELISA for CRP and PCT were constructed using a microtiter plate as the solid substrate, and a commercial analyzer (AccutrendPlus^{TM}) was employed for the lactate. The used concentration ranges are 0.01 ng/mL to 10 ng/mL for PCT, 0.01 μg/mL to 100 μg/mL for CRP, and 4.5 mg/dL to 144 mg/dL for lactate. To test the performance correlations for each marker, the analytical results that were obtained from the two different systems for the same sample were plotted at the respective axes of the graph and a regression analysis was then performed (Figure 6). From the graphs for the target markers, highly linear relationships were obtained with the correlation coefficient (R^2) > 0.95 between the hybrid biosensor and the reference system, indicating that the two systems for the given analyte are quantitatively matched all over the used dose range; therefore, the

results supported the further utilization of the hybrid biosensor as an analytical means for clinical samples that have been drawn for the on-site diagnosis of sepsis.

Although such hybrid biosensor for sepsis was developed to suitably use it at the points of care, further study is needed for a pilot-scale production and subsequent clinical tests. Prior to the tests, as the sensor utilizes the protein tracer, an enzyme, for signal generation, it has to be stabilized in the dry immuno-strip to retain a long shelf-life (e.g., > 1 year at room temperature). This may shift an additional burden relatively to other sensors using non-protein tracer (e.g., fluorophore).

4. Conclusion

A hybrid-biosensor system that enabled the simultaneous conduction of both immunoassay and biochemical analyses was devised and then applied to the detection of the triple biomarkers, i.e., PCT, CRP, and lactate, for sepsis. The POCT version of the enzyme immunoassay system that is based on 2-D chromatography was adopted as the platform of the novel biosensor, and a biochemical-reaction zone was then allotted on the signal-generation pad. The color signals produced at the pre-designated sites were detected and quantified using a smartphone via a plastic-holder installation. As the analytical performances of such a hybrid system are highly correlated with those of the reference systems, it could be applied to the on-site diagnosis of the disease without the need for duplicate blood drawings, the complexity from the use of different devices, and even inadvertent delays. It is further expected that the hybrid platform could also be used for the management of various diseases for which it is necessary to concurrently measure the biochemical and immunological markers. The examples here include diabetes, which can be diagnosed by glucose and glycosylated proteins, and cardiovascular diseases, which can be managed with measurements of cholesterol and the brain-natriuretic-peptide (BNP) species. It should also be notable that, in case of using smartphone as signal detector, biosensing results for the target analytes can be collected in real-time through the internet, an urgent patient located in a remote place can be instantly recognized and treated within the gold time period (Seo et al., 2016).

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Figure 1. Analytical concept of the simultaneous biosensing for the biochemical as well as the protein markers in an independent manner. The biosensor cartridge for the diagnosis of sepsis (see Figure S1) was designed to measure the different types of biomarker, proteins (e.g., PCT and CRP), and a biochemical (e.g., lactate; A). The immuno-strip was prepared by immobilizing the capture antibodies for PCT and CRT on the signal pad and also a secondary antibody at the control site (B, left). In addition, the biochemical-reaction site for lactate was fabricated on the bottom of the signal pad in a manner that created a surrounding barrier (refer to the text for details). For the analysis, a clinical sample (e.g., serum) was applied to the cartridge in the vertical direction and the enzyme tracers for the immunoassays were subsequently supplied using the horizontal flow (B, middle). The enzyme substrate for HRP was finally supplied along the identical path (B, right), and the color signal intensities were determined using the smartphone-based detector (C).

Figure 2. Dose-response curve of the hybrid biosensor for CRP. The hybrid biosensor was constructed under the optimal conditions with the use of two monoclonal antibodies, C2 and C6, as the capture and detection binders, respectively. According to the protocol shown in Figure 1B, for the separate analyses of the standard CRP samples, the concept of 2-D chromatography was employed. Each color signal was captured as an image and digitized to the optical density, and the density was profiled along the relative distance of the signal pad (A). The integrated value under the analyte line was plotted against the CRP concentration in a semi-log graph, and the curve was then linearized via a log-logit transformation ($R^2 > 0.95$; B). This showed the dynamic range between 0.01 μg/mL and 100 μg/mL, covering the clinical concentrations in sepsis.

Figure 3. Dose-response curve of the hybrid biosensor for PCT. The biosensing for the marker was carried out with the particular use of poly-HRP as the tracer in the sandwich

immunoassay with the use of the antibody clones 42 and 16B5 as the capture and detection binders, respectively. The standard samples that were mixed with the biotinylated detection antibody were analyzed through the sandwich-complex formation on the signal pad, and then poly-HRP binding was conducted through the use of a biotin-SA linkage (refer to Figure 1B). The color signal was produced from the enzyme and the optical density was profiled along the longitudinal axis of the signal pad (A). The signal value was allotted for each standard sample and plotted against the PCT dose, which was further linearized ($R^2 > 0.95$; B). The dynamic range was determined between 0.01 ng/mL and 10 ng/mL.

Figure 4. Analytical performance of the hybrid biosensor for lactate. Standard samples (0 to 72 mg/dL) were prepared using the normal human serum that had been dialyzed against a buffer and was used as an assay under the optimal conditions. The color signal was produced for each sample, and it was captured (A) and analyzed on the smartphone-based detector to draw the dose-response curve (B). From the linearized curve (B, inset), the dynamic range of the hybrid biosensor at least includes the range of 9 mg/dL to 72 mg/dL. The LOD of the lactate in the single assay was determined as approximately 3 mg/dL.

Figure 5. Simultaneous analyses of the triple sepsis markers for which the hybrid-biosensor system was used (A) and comparison of the dose responses with those from each single assay (B). The dose responses that were measured in the multiple mode (see Figure S9) or in each single determination (presented in Figures 2 to 4) were plotted in the same graph for the respective markers. The mean signals at the identical analyte level were varied up to 15.7% according to the measurement mode, and otherwise, the patterns are very close each other.

Figure 6. Performance correlations of the hybrid biosensor for the triple sepsis markers with the reference systems. Microtiter-plate-based ELISA systems were built in-house as the references for CRP and PCT, and a commercial device for lactate (AccutrendPlus™; Roche) was purchased. Standard samples, prepared in a dose range for each marker (see the graph), were analyzed using the hybrid biosensor and the reference system. The analytical results from the two systems were then plotted along the respective axes for the same sample. The graphs for the respective markers show linear relationships with the correlation coefficient (R^2) > 0.95. Variations of the duplicate measurements for each determination are indicated.

Highlights:

- A biosensor that can simultaneously fulfill immunoassay and enzyme assay was devised.
- Triple markers (CRP, PCT, and lactate) in the same sepsis-based sample were targeted.
- The color signal produced from each assay was quantified on a smartphone detector.
- The dynamic ranges for the analytes covered the respective clinical ranges.

Figure 1

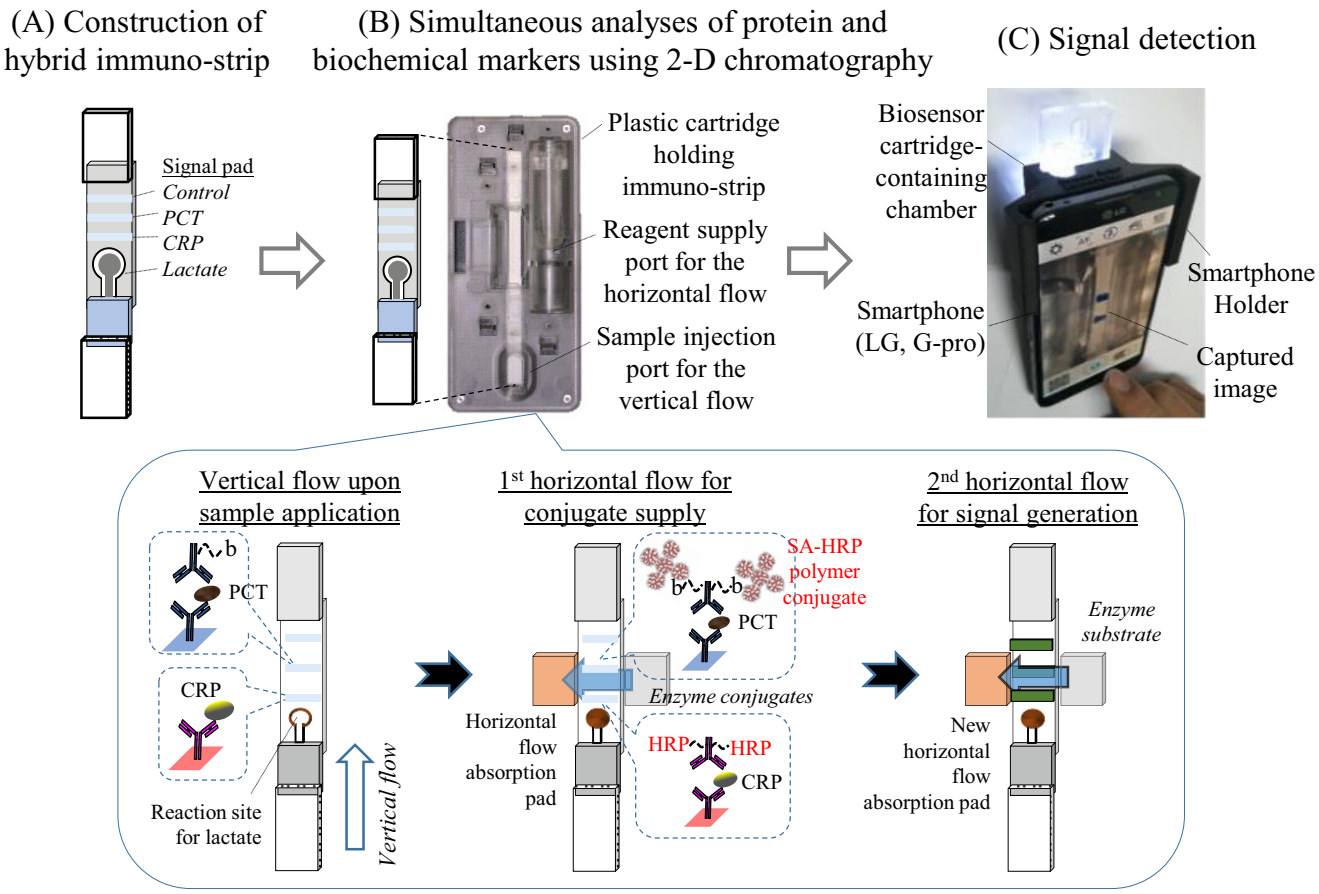
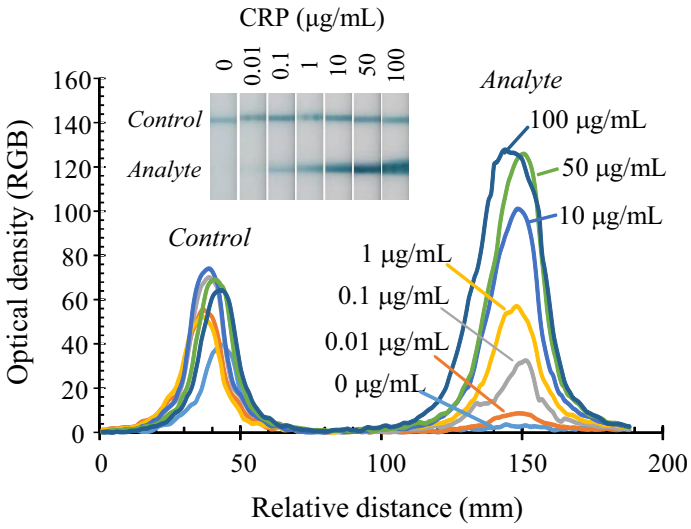


Figure 2

(A) Dose responses of the biosensor to CRP



(B) Dose-response curve for CRP

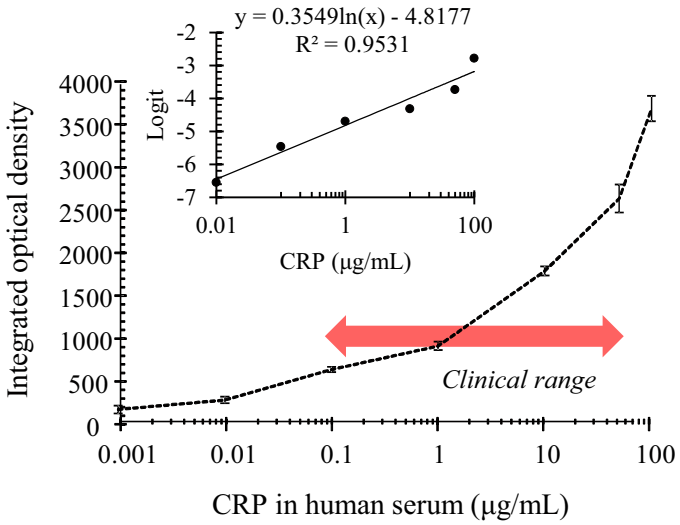
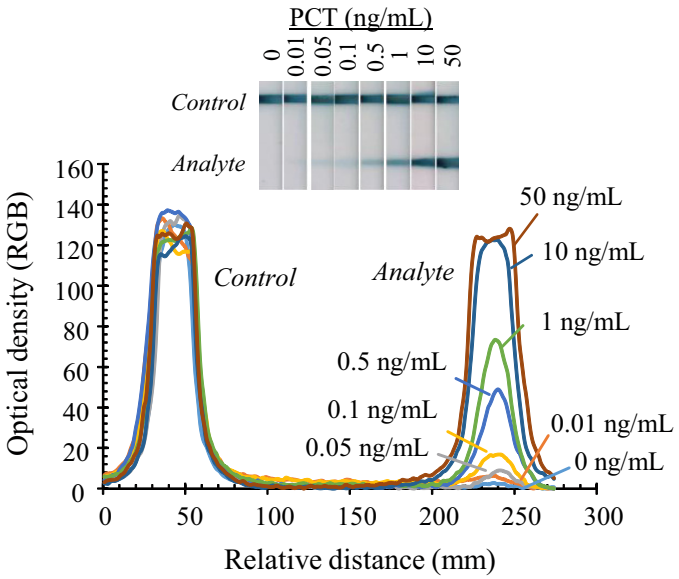


Figure 3

(A) Dose responses of the biosensor to PCT



(B) Dose-response curve for PCT

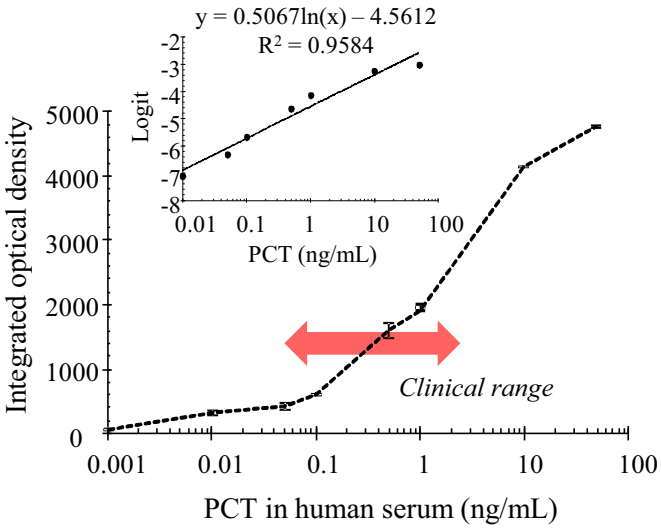
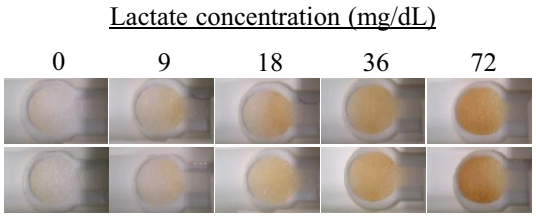


Figure 4

(A) Dose responses of the biosensor to lactate



(B) Dose-response curve for lactate

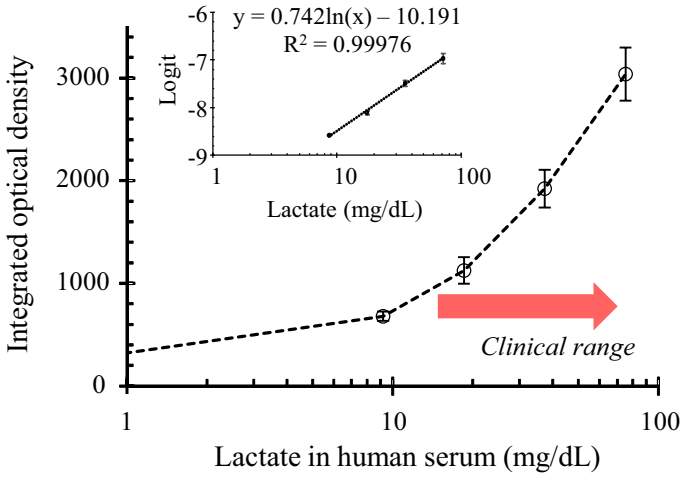
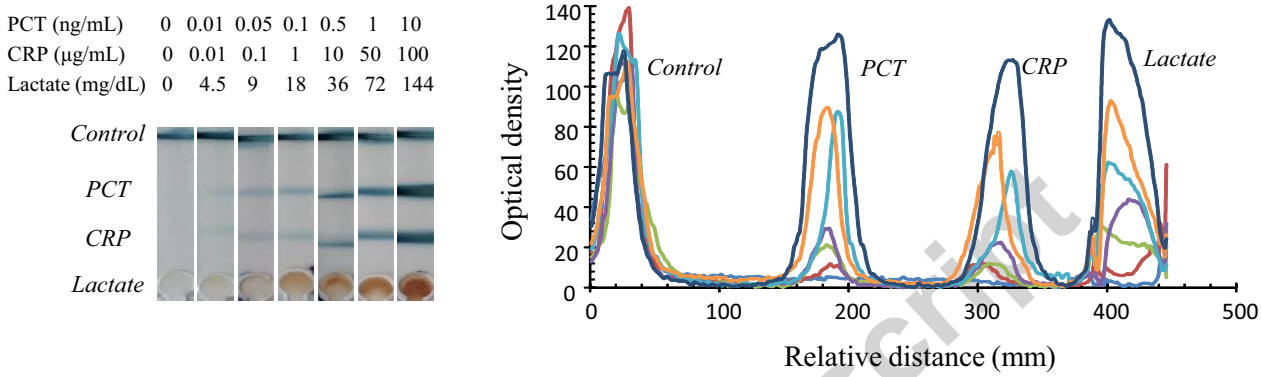


Figure 5

(A) Dose responses of the biosensor to triple markers at the same time



(B) Comparison of the dose responses of the hybrid biosensor with those from each single assay

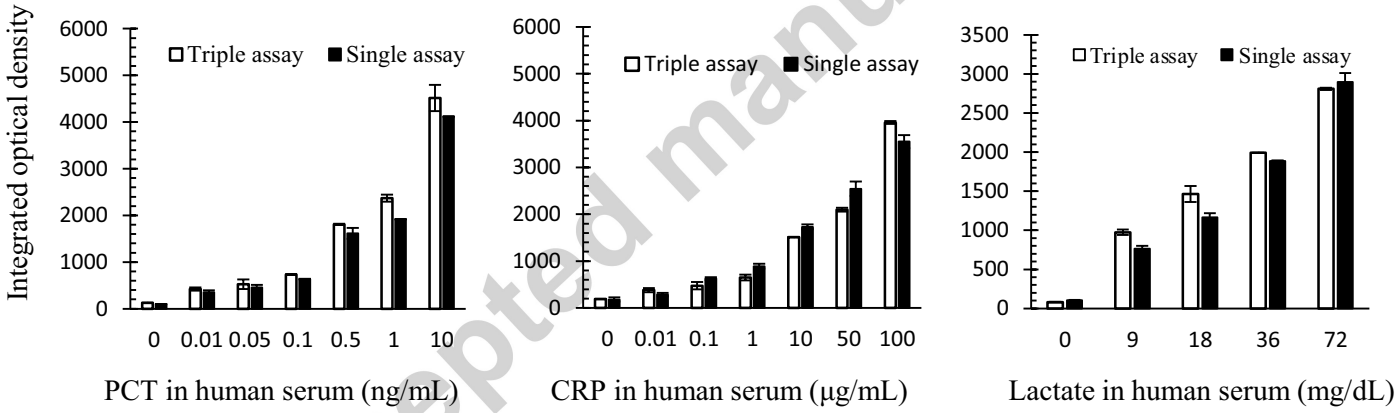


Figure 6