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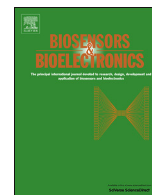

An automatic enzyme immunoassay based on a chemiluminescent lateral flow immunosensor

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ABSTRACT

Microfluidic integrated enzyme immunosorbent assay (EIA) sensors are efficient systems for point-of-care testing (POCT). However, such systems are not only relatively expensive but also require a complicated manufacturing process. Therefore, additional fluidic control systems are required for the implementation of EIAs in a lateral flow immunosensor (LFI) strip sensor. In this study, we describe a novel LFI for EIA, the use of which does not require additional steps such as mechanical fluidic control, washing, or injecting. The key concept relies on a delayed-release effect of chemiluminescence substrates (luminol enhancer and hydrogen peroxide generator) by an asymmetric polysulfone membrane (ASPM). When the ASPM was placed between the nitrocellulose (NC) membrane and the substrate pad, substrates encapsulated in the substrate pad were released after 5.3 ± 0.3 min. Using this delayed-release effect, we designed and implemented the chemiluminescent LFI-based automatic EIA system, which sequentially performed the immunoreaction, pH change, substrate release, hydrogen peroxide generation, and chemiluminescent reaction with only 1 sample injection. In a model study, implementation of the sensor was validated by measuring the high sensitivity C-reactive protein (hs-CRP) level in human serum.

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1. Introduction

The immunoassay is a biochemical test that is commonly used to measure the concentration of target molecules. The enzyme immunoassay (EIA) is a well-established *in-vitro* diagnostic technique using enzyme-labeled antibodies (Lequin, 2005; Wisdom, 1976). In clinical chemistry, EIA systems, such as the enzyme-linked immunosorbent assay (ELISA), are the most widely used analytic tools for determining various biomarkers, including the C-reactive protein (CRP), troponin, and cytokines in body fluids (Heeschen et al., 1999; Laurent et al., 1985; Leng et al., 2008), due to their ability to produce highly specific and sensitive results within 5 h. EIA-based microfluidic immunosensors have received increased attention from researchers and have recently been commercialized (e.g. i-STAT®, Abbott Laboratories., USA) (Hervás et al., 2012; Yakovleva et al., 2002; Zang et al., 2012). By using a microfluidic system, the EIA makes it possible to obtain faster and simpler measurements automatically, even with very small sample amounts. Therefore, microfluidic integrated EIA sensors are suitable for point-of-care testing (POCT). However, as a disposable sensor, microfluidic systems are not only relatively expensive, but also require a complicated manufacturing process (Yager et al., 2006). For these reasons, cheaper and simpler EIA-based POCT sensors are required for efficient on-site diagnostics. The lateral flow immunosensor (LFI) strip is one of the most widely used POCT systems for various types of detections (Ngom et al., 2010; Posthuma-Trumpie et al., 2009; Wongsrichanalai et al., 2007; Zuk et al., 1985) due to its rapid assay time, cost effectiveness, and ease of use. The conventional LFI strip uses antibody-labeled gold nanoparticles as a signal indicator, and fluorescent materials such as fluorescein, Q-dot, and europium are also used for obtaining better quantitative and sensitive results (Choi et al., 2004; Li et al., 2010; Xia et al., 2009). However, the EIA system is nonetheless difficult to implement in the LFI strip sensor because it requires additional steps during measurement, such as washing and injecting. Therefore, additional fluidic control systems are required for the implementation of EIA in an LFI strip sensor. For example, an ELISA-on-a-chip system has been reported for the detection of cardiac troponin I (cTnI) by using a cross flow of enzyme substrate solutions through a plastic fluidic channel (Cho et al., 2006). For this reason, no automatic EIA-based LFI strip immunosensor without additional fluidic control has yet been reported.

In this study, we developed a novel chemiluminescence LFI strip-based automatic EIA system using a delayed-release technique that could delay the release of enzyme substrates and change

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the reaction pH during the sample flow without the need for additional operations or complex manufacturing processes. To date, several types of horseradish peroxidase (HRP) catalyzed chemiluminescence LFI (CLFI) strip sensors have been reported (Cho et al., 2009; Kim et al., 2010; Mirasoli et al., 2012). However, these strip sensors require a substrate (luminol and hydrogen peroxide) addition step for generation of the chemiluminescent signal because of the following 3 problems. First, the optimal pH conditions differ for the immune response (neutral pH) and chemiluminescence (pH 8.5–9.5) (Dotsikas and Loukas, 2007). Therefore, the strip sensor pH needs to be changed from neutral to alkaline during the sample flow. Second, hydrogen peroxide has storage and stability problems. Third, if HRP-labeled detection antibodies are mixed with the substrates before the end of the immune reaction, an undesired signal will be produced. Therefore, the chemiluminescence reaction should occur after the sandwich immune reaction is complete. To overcome these problems, we designed the new LFI strip to sequentially control the immunoassay, delayed release of substrates with pH change, hydrogen peroxide generation, and the luminol reaction.

2. Materials and methods

2.1. Materials

C-reactive protein (CRP)-free serum (90R-100), surfactant 10G (95R-103), CK-MB (30-AC66), troponin I (30-AT43), proBNP (30C-CP1149) and myoglobin (30-AM20) were purchased from Fitzgerald Industries International (Acton, MA). CRP was purchased from Wako Chemicals (309–51191; Osaka, Japan), and anti-CRP polyclonal antibody and monoclonal antibody-HRP conjugate were purchased from Abcam Inc. (Cambridge, MA, USA). The nitrocellulose (NC) membrane was purchased from Millipore (HFB02404; Billerica, MA). The sample pad (P/N BSP-133-20) and asymmetric polysulfone membrane (ASPM, Vivid plasma separation-GX, USA) were purchased from Pall Co. (Port Washington, NY). Choline oxidase (ChOx) was purchased from TOYOBO Co., Ltd. Luminol, choline chloride, p-coumaric acid, p-iodophenol, polyvinylpyrrolidone (PVP55K), sucrose, human serum albumin, thrombin and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All buffers and reagent solutions were prepared with water purified using the Brema water purification system.

2.2. Preparation of the substrate pad

A solution containing 100 μL luminol (0.5 M in 50 mM NaOH), 30 μL choline chloride (1 M in DW), and 1 μL p-coumaric acid (0.5 M in dimethylformamide (DMF)) was mixed with 870 μL 0.1 M carbonate buffer (pH 9.2). After mixing, the 75 μL mixed solution was loaded on the pad (50×3.8 mm^2) and was subsequently dried in a dry oven at 65 °C for 30 min. A solution-treated pad was attached to the middle of a piece of sealing tape (50×15 mm^2) and was cut to obtain a 3.8×3.8-mm^2 section by using a cutter. All solution components were optimized for the chemiluminescence lateral flow immunosensor (LFI).

2.3. Preparation of detection, capture, and control antibody solutions

2.3.1. Detection antibody solution

The solution containing 5 μL anti-CRP monoclonal antibody-HRP (2 mg mL^{-1}) was mixed with 95 μL of 2% (v/v) surfactant 10G, 3.75% (w/v) PVP 55 K, and 0.5% (w/v) sucrose-added 1X pH 7.2 phosphate-buffered saline (PBS) which is composed of 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4.

2.3.2. Capture and control antibody solutions

The solution containing 5 μL anti-CPR polyclonal antibody (10 mg mL^{-1}) for capture or 5 μL anti-mouse IgG (1 mg mL^{-1}) was mixed with 45 μL choline oxidase (100 unit mL^{-1})-added PBS.

2.4. Dispensing of the detection, capture, and control antibody solutions

An NC membrane (0.25×30 cm^2) was used to immobilize the capture and control antibodies and to load the detection antibody-HRP conjugate to different zones by using a dispenser (DCI100; Zeta Corporation, Kyunggi-do, South Korea); the detection antibody (2.5 μL cm^{-1}) and capture and control antibodies (1 μL cm^{-1} for both) were added consecutively. The distance between the test and control zones was approximately 3 mm, and the distance between the detection antibody and the test zone was approximately 5 mm. The dispensed NC membrane was dried for 1 h in a desiccator at room temperature.

2.5. Preparation of the chemiluminescence LFI (CLFI) strip

The strip was composed of 6 parts, including the sample pad, inter pad, substrate pad, asymmetric membrane, antibody-dispensed NC membrane, and absorbent pad. An absorbent pad (1.5×30 cm^2) was attached to the top of the NC membrane, and the inter pad (5 mm × 30 cm) and sample pad (1.5×30 cm^2) were consecutively assembled on a plastic adhesive backing (60×300 mm^2). The assembled strips (3.8 mm in width) were cut with a cutter device. After cutting, the asymmetric membrane (5×3.8 mm^2) and substrate pad were stacked on the NC membrane between the inter pad and the antibody-HRP loaded line. The larger pore side of the asymmetric membrane made contact with the substrate pad.

2.6. Optimization of luminol, p-coumaric acid, choline chloride, and choline oxidase concentrations

2.6.1. Luminol

One microliter of 20 μg mL^{-1} Ab-HRP was spotted on the NC membrane and dried at 37 °C in a dry oven for 15 min. Various concentrations of luminol in pH 9.2 0.1 M carbonate buffer were loaded on the substrate pad and dried at 65 °C in a dry oven for 30 min. The luminol-stored substrate pads were attached to the Ab-HRP-spotted NC strip, and 100 μL of 1 mM hydrogen peroxide-added CRP free serum was loaded. The concentration of 50 mM showed the highest signal intensity (Figure S2A). The strip preparation process was performed as described above.

2.6.2. p-coumaric acid

p-coumaric acid was dissolved in DMF (0.5 M). Various concentrations of p-coumaric acid were prepared on substrate pads containing 50 mM luminol. Strip preparation and measurement were performed in the same manner as described for the luminol optimization steps, but with a different Ab-HRP concentration (0.2 μg mL^{-1}). The signal (I) was compared to that of the no enhancer condition (I_0). The concentration of 0.5 mM showed the highest signal intensity (Figure S2B).

2.6.3. Choline chloride

One microliter of 2 μg mL^{-1} Ab-HRP in 100 units mL^{-1} choline oxidase-added PBS was spotted on the NC membrane. Various concentrations of choline chloride-added substrate pads with 50 mM luminol and 0.5 mM p-coumaric acid were prepared. The test strip was prepared in the same way as described above in section (a). The signal intensity was saturated at concentrations of more than 10 mM choline chloride (Figure S2C).

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