Bead Packing and Release Using Flexible Polydimethylsiloxane Membrane for Semi-Continuous Biosensing

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Abstract: The continuous or semi-continuous biosensing of systemic inflammatory responses is important both during and after cardiopulmonary bypass (CPB) procedures. A bead packing and release method, which is able repetitively to capture and release receptor-coated beads within microfluidic channels, is herein advanced for use in semi-continuous biosensing. The receptor-coated beads are compacted and concentrated at specific locations in the device using an elastomeric valve. This concentration creates a localized bioreactor in which the binding of the antigen with the functionalized beads can be made more effective. After the reaction and detection have taken place, the beads can be released and a new assay carried out. We demonstrated the operation of our device using streptavidin-coated beads and biotin-4-fluorescein (B4F). The high sensitivity of the device allows it to detect a B4F concentration of 50 pg/mL after an incubation time of 5 min. We also tested our device in the semi-continuous immunoassay of interleukin (IL)-6, which is one of the proinflammatory cytokines. The assay demonstrated the linear dependence of the intensity of fluorescence at concentrations of IL-6 from 10 to 250 pg/mL, which is a physiologically important range for CPB procedures.

Key Words: Bead packing—Semi-continuous biosensing—Polydimethylsiloxane membrane—Inflammatory response—Interleukin-6.

The identification and quantification of the biomolecules that bind to a specific protein is an important part of a number of biological and clinical assays. An immunoassay relies on molecular recognition, and is one of the most widely used techniques in biological sensing as a result of its high sensitivity and selectivity. The recent introduction of micromachining technologies in life science has seen the integration of immunoassays within planar microfluidic chips (1–3). Integrated microfluidic chips, also known as υ-immunoassays, provide many advantages over conventional immunoassay systems. These include an improved sensitivity, a smaller sample consumption, a shorter analysis time, and easier automation (4–6). In particular, bead-based υ-immunoassay devices are currently being promoted due to their higher sensitivity compared with υ-immunoassays that use other methods, because the use of beads affords a higher surface-to-volume ratio for antigen-antibody binding, thereby providing a higher sensitivity (7).

A number of different bead-based biosensing or υ-immunoassay platforms have been reported. The simplest methods of bead-packing make use of rigid dam structures or solid filter pillars and result in highly sensitive immunoassays for the detection of carcinoembryonic antigens (8), human interferon-γ (IFN-γ) (9), C-reactive protein antigen (10), Tacrolimus (11), and marine iridoviruses (12) in microfluidic channels. Magnetic bead-based systems were introduced for semi-continuous immunoassays that utilize integrated micro-electromagnets (13) or external permanent magnets (14) to trap the beads. A continuous method using cytometric beads has also been
applied to continuous biosensing in a microfluidic device using particle cross over (15). Continuous and semi-continuous methods overcome the limitations of the single immunoassay of the foregoing methods, by utilizing rigid bead filters and thereby enhancing their applicability to those cases where consecutive assays are required, such as in cardiopulmonary bypass (CPB) procedures (15).

CPB procedures, which are commonly used in open heart surgery, may induce systemic inflammatory responses in a patient (16–19). These are the cause of a number of postoperative complications, including the dysfunction of vital organs that can lead to multiple-organ failure, and even death (20–22). Systemic inflammatory response is induced by activation of complement, neutrophils, and platelets, as well as the release of proinflammatory cytokines including tumor necrosis factor α and several different interleukins (IL-1, IL-6, and IL-8) (23). For example, the concentration of interleukin (IL)-6 normally increases from 10 pg/mL to 200 pg/mL during and after a CPB procedure (24). If the concentrations drastically increase more than that during the surgery, the probability of a number of postoperative complications also drastically increases. Moreover, if the concentration of IL-6 in the blood exceeds 1 ng/mL for several days, the risk of death is increased radically (25). However, so far, there is no effective method for preventing this systemic inflammatory response in cardiac surgery patients undergoing CPB procedures due to the lack of the technologies for timely measuring the concentration of blood plasma proteins. Currently, nurses collect patients’ blood samples during the surgery, and send them to the assay laboratory. The results are often come out after the surgery due to the long assay time. This may cause the increase in morbidity due to the lack of the timely medical interventions. Therefore, there is an unmet medical need to develop an online monitoring system for this indication and to get a better understanding of how CPB procedures might be modified to prevent its occurrence. Thus, it is desirable to develop a continuous or semi-continuous biosensing platform, which is able to detect inflammatory responses in either continuous or semi-continuous fashions, for the development of the online monitoring system. We herein introduce a semi-continuous bead-based biosensing platform that could be used for the online monitoring of systemic inflammatory responses. The platform can provide consecutive measurements of the clinically relevant proteins by assaying samples at given time intervals through the use of a flexible membrane that forms a dam-like structure, thereby enabling the packing and release of receptor-coated beads within microfluidic channels. The performance of the device was assessed using the simple and well-established binding event of streptavidin (SA) and biotin. We demonstrated the application of the device to the monitoring of systemic inflammatory responses through the semi-continuous measurement of the concentration of IL-6, which is one of the proinflammatory cytokines.

The online monitoring of systemic inflammatory responses using this semi-continuous biosensing platform could be easily realized by simply connecting the platform (antigen inlet) to the blood circulation path, for example, to the CPB machine directly (see electronic supplementary information [ESI], Fig. S1 for a more detailed description of the online monitoring system). The computer-aided control can realize the automatic sampling and assaying. Therefore, the blood sample can be collected and analyzed at a given time interval without changing the device. And, the system will not need nurses’ support. Additionally, since this microanalytical system requires a small volume of blood sample, it might be beneficial for neonate patients (total blood volume of a 3 kg neonate is only 240 mL).

**MATERIALS AND METHODS**

**Assay principle**

The method of bead packing as a means of semi-continuous biosensing is implemented on a single chip by the partial closure of a flexible membrane valve within a microfluidic channel. The principle of operation of the proposed method of immunoassay is presented schematically in Fig. 1. The device was composed of two elastic polydimethylsiloxane (PDMS) layers (Fig. 1a). The fluidic layer contained microfluidic channels, in which the analytes and reagents could be introduced and interrogated. The pneumatic layer contained microchannels to which pressurized air could be applied in order to cause the deflection of a thin membrane situated between the pneumatic and fluidic channels. As the pressure in the pneumatic channel increases, the thin membrane deflects downwards and partially blocks the microfluidic channel, thereby forming a bead-blocking zone, as shown in Fig. 1b. Following the formation of the bead-blocking zone, the antibody-coated beads are injected. Then, the beads accumulate in the bead-packing zone. The bead-carrying solution passes through the bead-blocking zone because it is not completely sealed by the thin membrane. After the resulting packing of the beads is complete, the target
analytes are introduced and incubated to allow the specific binding reaction to occur between the antibodies and the target antigens (Fig. 1c). After rinsing any unreacted antigens with buffer solution, the fluorophore-labeled detection antibodies are infused to detect the target analytes using a fluorescent light detector (Fig. 1d). After any unreacted fluorophore-labeled detection antibodies have been rinsed through with buffer solution, the intensity of the fluorescence emitted from the beads is measured (Fig. 1e). In order to carry out the subsequent assay, the applied pressure in the bead-blocking zone is then relieved and the beads are flushed out (Fig. 1f).

**Device layout and fabrication**

Figure 2a shows the layout of the device. The blue and black lines represent the fluidic and pneumatic channels, respectively. The fluidic layer had four inlets and one waste outlet in order to permit a sandwich immunoassay to be carried out. Four inlets were used in order to allow the introduction of the antibody-coated beads, target antigen, detection antibodies and buffer solution.

In order to control the fluid flow and to capture the beads, the pneumatic layer was composed of five ports, each of which triggered one of five valves installed over each fluidic channel. Each valve was formed from the overlap between the fluidic and the pneumatic channels. The first valve provided the bead-packing and -blocking zones. It was designed to be sufficiently stiff to control the aperture of the fluidic channel, and was installed at the front of the waste outlet. The width of the fluidic channel was 100 μm and the length of the valve was 100 μm, yielding an area of overlap of $100 \times 100 \mu m^2$. The remaining four valves (numbers 2 to 5 in Fig. 2a) were designed to prevent the cross-contamination of the analytes, as well as to seal off the fluidic channel completely. The width of the fluidic channel was 150 μm and the length of the valve was 300 μm, yielding an area of overlap of $150 \times 300 \mu m^2$. A magnified view of the bead-packing and -blocking zones is shown in the inset of Fig. 2a. The cross-sectional view of the bead-blocking zone shows the rounded shape of the fluidic channel, which allows the complete sealing of the fluidic channel by the other four valves.
The device was fabricated using the multilayer soft lithography process (26), which allows the integration of a number of micromechanical valves within a microfluidic chip. The molds for the top and bottom PDMS layers were fabricated by the photolithographical patterning of the negative photoresist (DNR-L300). The patterned photoresist was reflowed by baking on a hotplate at 160°C for 5 min to achieve the rounded channel shape. Following this stage, the width and height of the channel were typically 100 µm and 15 µm, respectively. In order to minimize the degree of adhesion between the replica and the mold, the mold was treated with 0.2% Teflon by spin-coating it at 2000 rpm for 30 s, and then baking on a hotplate at 160°C for 5 min. Each PDMS layer was replicated separately from the relevant mold, and the layers were then bonded together. The fluidic layer was produced by spin-coating the PDMS mixture on a micromachined mold at 2000 rpm for 30 s in order to obtain a layer thickness of about 50 µm. This produced a membrane that was approximately 35 µm thick. The PDMS mixture was prepared using a base:curing agent ratio of 10:1. Each layer was baked individually for 1 hour at 90°C. The pressure channel layer was bonded onto the thin fluidic channel layer after O₂ plasma treatment, and the two bonded layers were then fixed on a slide glass using another O₂ plasma treatment. Once the device was complete, a 3% solution of bovine serum albumin was introduced into the microfluidic channels at a rate of 1 µL/min for 10 min before the experiments began in order to minimize the occurrence of nonspecific binding.

Reagent and materials
SA and biotin were used to demonstrate the potential of the proposed method for semi-continuous biosensing. The capture probe consisted of 8 µm-diameter beads coated with SA (Bangs Laboratories, Inc., Fisher, IN, USA) at a concentration of 1.38 × 10⁶ beads/mL in deionized (DI) water. The target molecule was represented by six sets (50, 100, 250, 500, and 2500 pg/mL) of biotin-4-fluorescein (B4F, Sigma-Aldrich), also prepared using DI water. IL-6 antibody-coated beads (7.5 µm-dia), IL-6 antigen, phycoerythrin-labeled detection antibody, their diluents, and buffer solution (BD Biosciences, Inc., IN, USA) were selected and purchased for the μ-immunoassay. The IL-6 antibody-coated beads were diluted to a concentration of 5 × 10⁵ beads/mL in capture bead diluent. Five sets (10, 50, 100, 150, 250 pg/mL) of IL-6 antigen solutions were prepared in assay diluent. The phycoerythrin-labeled detection antibodies were reconstituted using PE diluent.

Apparatus
The switching and control of pressurization was achieved using three-way solenoid valves (GAG311-1-10-H2G-DC24V; CKD, Japan) and regulators (MVRB500B-LLC4-10; CKD, Aichi, Japan). An external pressure source was connected to the normally closed port of the three-way valves. The pressure was controlled by the regulators that were installed between the outlet of the three-way valves and the pressure inlets of the device. The flow of the analytical samples was controlled by microsyringe
Assay and measurement

In the SA and B4F binding experiment, the SA-coated beads, B4F, and buffer solution were injected into the antibody-coated bead, antigen, and buffer inlets, respectively (Fig. 2a). The detection antibody inlet was not used in this part of the experiment. Because B4F has a fluorophore, the assay does not require a secondary binding process. Ultra-high-purity DI water was the buffer solution used throughout the experiment. Each inlet was filled with its reagents before the assay was conducted. A flow rate of 5 mL/min was maintained throughout the procedure, except during flushing (20 µL/min). The operating pressure was 200 kPa at valve 1 and 400 kPa at valves 2–5.

In the IL-6 immunoassay, IL-6 antibody-coated beads, IL-6 antigen, and PE-labeled detection antibody, buffer solution was injected into the antibody-coated bead, antigen, detection antibody, and buffer inlets, respectively (Fig. 2a). For rinsing, the buffer solution was used throughout the IL-6 immunoassay (see ESI, Table S1 and S2 for a more detailed description of the assay sequence).

In order to maintain the quantities of trapped beads in each experiment, the height of the bead-packing zone was fixed at 350 µm in the B4F assay and at 200 µm in the IL-6 assay. Semi-continuous biosensing was achieved by increasing the concentration of the target analytes in each device. This increase in concentration of the target analytes is similar to that which occurs during CPB procedures, in which it is known that the concentration of IL-6 increases during and after the procedure. An average intensity of fluorescence was obtained over the fixed region of interest (ROI). In order to quantify the intensity in each case, the intensity of the packed beads was measured both before injection and after the injection and incubation of the target analytes. The second value was then subtracted from the first, and the results of the assay were then plotted using the means and standard deviations of the intensities obtained from at least three different experiments.

RESULTS AND DISCUSSION

Figure 2b shows an image of the fabricated device, and Fig. 2c,d show the operation of the fluidic and bead-blocking valves, respectively, for a range of different pressures. When pressure is applied to the pneumatic channel, the membranes begin to deflect (Fig. 2b,c). When the pressure is increased further, the membranes make contact with the bottom surface of the fluidic channel. Due to the rounded shape of the channel, the deflected membrane begins to make contact with the side wall first and the bottom surface of the fluidic channel second, thereby forming an “hourglass” shape (Fig. 2e at 200 kPa). The recovery of the membrane from its deflected state is achieved simply by removing the applied pressure. As long as the flow rate is maintained at 5 µL/min, the beads begin to be captured when a pressure of 180 kPa is applied to the bead-blocking valve, and a stable bead-packing zone was achieved at a pressure of 200 kPa. In fact, we chose to operate the fluidic valve at 400 kPa in order to ensure the complete sealing of each fluidic channel even at the high pressures of the injected fluid.

Figure 3a shows the sequential bright-field images when the SA-coated beads (8 µm-diameter) were in the bead-packing zone. When a pressure of 200 kPa was applied to the bead-blocking zone, the capture and accumulation of the SA-coated beads began. The bead-packing zone was completely filled with SA-coated beads within 3 min. The beads were packed in multilayers in the rounded channel due to the fact that the depth of the channel (15 µm) was about twice the diameter of the beads. After the SA-coated beads had filled the bead-packing zone completely, the analyte solution (B4F) was introduced, and the SA-coated beads began to fluoresce as a result of the binding event between the SA and the B4F. A gradual increase in the intensity of the fluorescence took place as the incubation progressed (Fig. 3b). Varying levels of brightness were observed in the packed beads for a given concentration of B4F. This nonuniformity in intensity could be attributed to variations in the density of the SA immobilized on each bead. After each assay was complete, the beads were flushed out by removing the pressure applied to the bead-blocking zone and by the simultaneous injection of DI water at a flow rate of 20 µL/min (Fig. 3c).
Semi-continuous biosensing was achieved by increasing the concentration of B4F from zero to 2500 pg/mL. To quantify the intensities of the fluorescence, the intensity of the packed beads before injecting B4F was subtracted from that observed after incubation of the B4F. This method minimized the influence of the background signal caused by nonspecifically bound proteins and any bead debris generated during the previous assay. In addition, because the use of beads with high surface-to-volume ratios improves the binding of B4F, the fluorescent light that is emitted from the beads dominates that emitted from the nonspecifically bound proteins on the PDMS wall (see ESI, Fig. S2 for a more detailed description). Figure 4a shows the relationship between the intensity of fluorescence and the concentration of B4F for various incubation times. The intensity that corresponds to 50 pg/mL is from the first assay using a fresh device and that corresponding to 2500 pg/mL is from the sixth semi-continuous assay. As expected, the higher the concentration of B4F, the brighter the intensity of the fluorescence. This may be seen in the inset of Fig. 4a, which shows the fluorescence images obtained from the packed beads as obtained at various concentrations of B4F for an incubation time of 10 min. The fluorescent light emitted from beads exposed to a B4F concentration of 50 pg/mL for 10 min can just be distinguished in the predominantly dark figure. This response is more sensitive than that afforded by the continuous biosensing platform (16). The dashed box shown in the image of 2500 pg/mL (inset of Fig. 4a) indicates the fixed ROI, and the average intensity of the pixels was extracted in order to calculate the intensity of fluorescence. The intensity increased with the concentration of B4F from 0 to 500 pg/mL, it then began to saturate around 1000 pg/mL, and became almost fully saturated at 2500 pg/mL. The intensity of the fluorescence also increased as the incubation time was increased from 5 to 20 min. This behavior is a typical characteristic of the second-order affinity binding between receptor (R) and ligand (L) (4,27), and may be expressed as:

\[
[RL](t) = [R]_0 \left(1 - e^{-k_a t[L]_0}\right)
\]

By fitting our data to Eq. 1, the association rate constant, \(k_a\), can be calculated. The solid lines (Fig. 4a) represent the curves obtained using Eq. 1. The average association rate constant \(k_a\) calculated from our three sets of experimental data (for incubation times of 5, 10, and 20 min) was \(2.42 \times 10^6 \text{ (M·s)}^{-1}\), with a standard deviation of \(0.95 \times 10^6 \text{ (M·s)}^{-1}\), values that are comparable with those reported for free SA and biotin (28) \((k_a = 5 \times 10^6 \text{ [M·s]}^{-1})\). Our value is within the acceptable range for the association constant for the interaction between immobilized-SA and B4F because it is known that adsorbed protein has a lower \(k_a\) (29).

There is quite a large variation in the intensity of fluorescence for the given conditions. The percentage coefficient of variance (\% CV), defined here as the standard deviation expressed as a percentage of the mean value, is about 20\%. The variation that this figure represents could be due to variations in bead-packing density, immobilized SA surface density, and the amount of bead/PDMS debris. The variation in packing density is mainly due to the change in
hydraulic pressure generated in the bead-packing zone. At the same time, the change in hydraulic pressure is also caused by the change in packing density. The change in hydraulic pressure can be reduced by capturing small quantities of beads. The variation in packing density can be further reduced by using a shallower channel, thereby ensuring that the beads are captured within a monolayer. This may generate a more consistent intensity of fluorescence and thereby improve the accuracy of the assay. An uneven level of brightness caused by variation in both the immobilized SA surface density, and by the presence of varying quantities of debris, may be resolved by image processing.

The results of the foregoing SA-B4F binding experiment show that μ-immunoassays may be performed semi-continuously. In order to demonstrate this capability, we tested for the detection of IL-6, which is one of cytokines that plays an important role in inflammation response. The quantification of IL-6 was performed using IL-6 antibody-coated beads, an IL-6 antigen, and a phycoerythrin-labeled detection antibody. Because the concentration of IL-6 normally increases from 10 to 200 pg/mL during and after a CPB procedure, five different concentrations (10, 50, 100, 150, 250 pg/mL) of IL-6 antigen solution were used. The assay was performed semi-continuously by gradually increasing the concentration of IL-6. The fluorescent intensity measured prior to the injection of phycoerythrin-labeled detection antibody was subtracted from that measured after incubation, in a similar manner to the SA-B4F binding experiment (see ESI, Fig. S3 for a more detailed description). Figure 4b shows the relationship between the intensity of fluorescence and the IL-6 concentration for an incubation time of 20 min. The fluorescence intensity increases linearly with the concentration of IL-6. The lower detection limit was determined to be 10 pg/mL after an incubation time of 20 min. The linear relationship clarifies the linearity in the IL-6 concentration in the range 50–250 pg/mL. The capability of detecting IL-6 semi-continuously in the range 50–250 pg/mL strongly suggests that it is possible to monitor inflammation response at given time intervals both during and after a CPB procedure. It is noteworthy that our bead-based method, which uses a short (20 min) incubation time, exhibits a sensitivity that is comparable with that of typical enzyme-linked immunosorbent assays, which involve incubation times of several hours (30). In addition, it is possible to increase the sensitivity of the detections or reduce the incubation times by adopting a method that uses an electrochemical, chemoluminescent, or photomultiplier tube according to the number of packed beads and their ability to amplify the electrochemical or chemoluminescent signal. Furthermore, since this system utilized the multilayer lithography, it has high expandability. It can be expanded to a parallel assay system by simply installing an additional bead-blocking zone in parallel. It would help to acquire multiple data points during the surgery thereby providing accurate quantification. It is also possible to capture various probe beads in several bead-blocking zones placed in

FIG. 4. (a) Semi-continuous B4F detection characteristics showing the change in intensity of fluorescence with concentration of B4F and incubation time. The inset shows the fluorescence images of the packed beads for concentrations of B4F of 50, 100, 250, 500, 1000, 2500 pg/mL for an incubation time of 10 min. The region of interest (ROI) for the extraction of the fluorescence intensity for 2500 pg/mL of B4F is indicated by the dashed box. (b) Characteristics of semi-continuous IL-6 detection showing the change in intensity of fluorescence with IL-6 concentration for an incubation time of 20 min. All the plots for B4F and IL-6 result from the analysis of 8-bit and 16-bit images, respectively, and the error bars show the standard deviations of the intensities measured in at least three experiments.

parallel or sequentially. Therefore, the multiplex assay would allow us to monitor pro-inflammatory cytokines simultaneously and to diagnose the systemic inflammatory response accurately during the CPB procedures.

CONCLUSION

We have herein described the simple operation of an elastic PDMS membrane that creates a flexible dam in a microfluidic channel for the capture and release of receptor-coated beads. The repeated capture and release of the beads provides a semi-continuous biosensing function that is applicable to the online monitoring of clinically relevant proteins during CPB procedures. Our semi-continuous microfluidic platform exhibits a highly sensitive biosensing capability by detecting B4F down to concentrations of 50 pg/mL using SA-coated beads. The simple replacement of SA and biotin with antibody and antigen renders this platform applicable to clinical applications. We have demonstrated the ability of the platform to detect IL-6, which is one of the proinflammatory cytokines, down to a concentration of 10 pg/mL using an IL-6 antibody-coated bead and a phycoerythrin-labeled detection antibody. The device showed a linear dependence of fluorescence intensity on IL-6 concentration in the range 10–250 pg/mL. Semi-continuous bead-based biosensing platforms are thus highly applicable to the online monitoring of systemic inflammatory response during and after CPB procedures.

Acknowledgments: The research reported herein was partially supported by the Korean Research Foundation (KRF-D00583), the Ministry of Education (World Class University Program, Project No. R31-2008-000-10026-0), and the institute of Medical System Engineering (imSE), GIST, Republic of Korea.

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**ELECTRONIC SUPPLEMENTARY INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Conceptual drawing of an online monitoring system using a semi-continuous biosensing platform

**Fig. S2.** (a) Characteristics of semi-continuous B4F detection showing the change in intensities of fluorescence with concentration of B4F and incubation time. The intensity measured at 0 min (before injection of B4F) is the background intensity. The error bars are the standard deviations obtained from at least three experiments. (b) Variation in background intensity for six semi-continuous assays involving an increase in concentration of B4F from zero to 2500 pg/mL in a single chip.

**Fig. S3.** (a) Characteristics of semi-continuous IL-6 detection showing the change in fluorescence intensity with IL-6 concentration for an incubation time of 20 min. The intensity measured at 0 min incubation (before injection of IL-6) is the background intensity. The error bars are the standard deviations from at least three experiments. (b) Variation in background intensity during nine semi-continuous assays in which the concentration of IL-6 was increased from 100 to 250 pg/mL in a single chip.

**Table S1.** Assay procedure for the detection of B4F.

**Table S2.** Assay procedure for the detection of IL-6.

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