Microchip Electrophoresis Utilizing In Situ Photopolymerized Thrombin-Immobilized Preconcentrator Gels for Specific Entrapment and Analysis of Thrombin Aptamers

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Abstract
A method was developed for the specific entrapment and separation of thrombin aptamers using a thrombin-immobilized polyacrylamide gel fabricated at the channel crossing point of a microfluidic electrophoresis chip. The channel intersection of the poly(methyl methacrylate) (PMMA) microchip was filled with a solution comprising thrombin, acrylamide, N,N-methylene-bis-acrylamide, and 2,2’-azobis[2-methyl-N-(2-hydroxyethyl)propionamide], which functioned as a photocatalytic initiator. In situ polymerization at the channel crossing point was performed by irradiation with an LED laser beam. The fabricated thrombin-immobilized gel (100 ×100 × 30 µm) contained approximately 40 fmol of thrombin and therefore could entrap thrombin aptamers at the femtomolar level. The electrophoretically trapped thrombin aptamers were released from the gel by switching the voltage, which delivered high concentrations of phosphate ions in a background electrolyte. The broad sample band eluted from the gel was effectively reconcentrated at the boundary of a pH junction generated by sodium ions delivered from the outlet reservoir. The reconcentrated sample components were then separated and fluorometrically detected at the end of the separation channel. Under the optimized conditions, the thrombin aptamers were concentrated by a factor of 1,000-fold, and the peak resolution was comparable to that obtained by pinched injection. This method was successfully utilized to preconcentrate and analyze thrombin aptamers.

Keywords: Aptamer; Thrombin; Microchip electrophoresis; On-line preconcentration

1. Introduction
Aptamers are single-stranded (ss) DNA or RNA oligonucleotides that bind with high affinity and specificity to a wide range of targets, such as proteins [1,2], oligosaccharides [3], small molecules [4], cells [5], and therapeutic monoclonal antibodies [6]. The aptamer-target molecule interaction occurs via a variety of interaction types including electrostatic, hydrophobic, and hydrogen bonding. Specific aptamers are generated by in vitro solid-phase synthesis; an inexpensive, scalable, and easily automated technique with excellent batch-to-batch reproducibility. Additionally, aptamers are highly stable over wide ranges of pH, temperature, and ionic strength. They also spontaneously refold after denaturation [7]. The systematic evolution of ligands by exponential enrichment (SELEX) method is commonly used to generate aptamers [8]. Usually, SELEX involves repeated rounds of binding, separation, partitioning, and PCR amplification. Typically, SELEX requires up to 20 selection rounds to generate aptamers and is very laborious and time-consuming.

Microchip electrophoresis (ME) has emerged as a promising tool for rapid analysis of various substances. Electrokinetic effects provide both efficient transport and separation of sample components in microfluidic channels. Translation of traditional capillary zone electrophoresis to a microchip platform enables analysis of various types of sample components. In addition, affinity ME may reduce non-specific and off-target binding, which may allow for the omission of steps to remove non-specific binding factors [9].

We have previously reported the fabrication of a polyacrylamide gel at the channel crossing point of two microfluidic channels. The gel was formed by irradiation with an argon ion (Ar+) laser. This gel allowed for the entrapment of specific ions in a sample solution [10-13]. In addition, we have reported the fabrication of a lectin-impregnated gel at the channel crossing point of two microfluidic channels [14]. Moreover, we have constructed
trypsin- or peptide-N-glycosidase F-impregnated polyacrylamide gels in a pipette tip [15]. These affinity polyacrylamide gels functioned as highly efficient concentration devices and allowed for the entrapment of specific compounds in a mixture solution. These results indicated that protein activity was maintained in a polyacrylamide gel.

Here, we describe a thrombin-immobilized polyacrylamide gel formed at the channel crossing point of two microfluidic channels. The gel was formed by in situ photopolymerization via irradiation with an LED laser. The thrombin aptamers in a sample solution were specifically and continuously trapped in the thrombin-immobilized polyacrylamide gel by applying electrical voltage across the gel plug. The entrapped thrombin aptamers was released from the gel by delivering acidic phosphate ions to the gel. The obtained broad sample band was effectively reconstituted with a pH gradient generated by a highly concentrated sodium phase that was delivered from the anodic end of the separation channel. This method provided a sensitive analysis of thrombin aptamers in a sample solution.

2. Experimental

2.1. Reagents and materials

Acrylamide, \(N,N'\text{-methylene-bis-acrylamide,}\)
\(N,N,N',N'\text{-tetramethylethlenediamine (TEMED,}\)
hydroxypropylcellulose (HPC), and 2,2'\text{-azobis[2-methyl-N-(2-hydroxyethyl) propionamide]}
were purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). A standard, cross-pattern microchip synthesized from PMMA with channels 100 μm wide and 30 μm deep as well as a disposable injection syringe (5 mL) with a cone-shaped rubber cap that was used for exchanging the solution in the channel, were purchased from Hitachi Kasei Polymer Co., Ltd. (Tokyo, Japan). Human thrombin derived from plasma was purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Fluorescein isothiocyanate (FITC)-labeled thrombin aptamer was obtained from Aptamer Sciences Inc. (Gyeonggi, Korea). This thrombin DNA aptamer (Apt-29; 5'-AGT CCG TGG TAG GCC AGG TTG GGG TGA CT-3') is 29 nucleotides (nt) in length and has high affinity (\(K_a \sim 0.5 \text{nM}\)) [16] for the heparin-binding site of human thrombin [17]. A 5-carboxytemethylrhodamine (5-TAMRA) labeled random 29 nt DNA aptamer was purchased from Integrated DNA Technologies Inc. (Coralville, IA, USA).

2.2. ME apparatus

All ME experiments were performed utilizing a system built in our laboratory. The microchip was placed on the stage of a fluorescence microscope (BX50WI; Olympus, Tokyo, Japan), and a 365 nm LED light source (approximately 2.5 mW) was focused on the stage for photopolymerization of the acrylamide. An Ar' laser (488 nm) was used for fluorometric detection of the FITC-labeled aptamers. The Ar' laser beam was focused into the iris diaphragm and recollimated using a set of convex lenses and a set of block filters (U-MWB; Olympus, Tokyo, Japan). The block filters (Ex/Em 450-480/515 nm) only passed the central maximum of the laser beam's diffraction pattern. The laser beam had a 25 μm diameter for 20× magnification and a 100 μm diameter for 4× magnification. The fluorescence emission from the sample was collected directly with a PMT tube. A power supply (HVS448 1500 V; LabSmith, Livermore, CA, USA) was used to apply the separation voltage. The fluorescent microchip images were obtained using a digital microscope (All-in-One fluorescence microscope BZ-X700; Keyence Corp., Osaka, Japan). The images were photographed using a cooled color CCD (VB-7010; Keyence Corp., Osaka, Japan) installed at the front port of the microscope. All experiments were performed at 25 ± 2°C.

2.3. Fabrication of a thrombin-immobilized preconcentrator gel on a cross-channel type microchip

All experiments were performed on commercial PMMA microchips. The distances from the intersection of the channels to the inlet of reservoirs R1, R2, R3, and R4 [see Fig. 1(a)] were 5.2, 5.2, 5.7, and 37.5 mm, respectively. The channel width and depth were 100 μm and 30 μm, respectively. A freshly prepared acrylamide solution (20% T/20% C) consisting of 5.8% Tris, 16% acrylamide, 4%
N,N'-methylene-bis-acrylamide, 0.75% TEMED, and 0.6% 2,2'-azobis[2-methyl-N-(2-hydroxyethyl) propionamide] was chilled in an ice bath and deaerated by connection to a vacuum line to prevent bubble formation in the fabricated gels. The acrylamide solution (10 μL) was poured into the sample outlet reservoir (R2) of the chip and introduced to all the channels by applying pressure. After placing the chip on the microscope stage, the channel crossing position was irradiated with LED light (365 nm) using a 4x magnification objective lens. The gel was polymerized within 3 min. The fabricated polyacrylamide gel is shown in Fig. 1(b). Next, all the reservoirs were filled with electrophoresis buffer, and the acrylamide solution remaining in the channels was replaced with 25 mM Tris-acetate buffer (pH 7.0)/0.5 w/v% HPC that was evacuated from reservoir R4, and 500 V was applied to reservoir R2 for 3 min. Unfavorable adsorption of fluorescent reagents and weak EOF were prevented by addition of HPC in the running buffer.

2.4. On-line affinity ME

Specific preconcentration of the FITC-labeled thrombin aptamers on the thrombin-immobilized gel (fabricated on the microchip) was performed as follows: (1) all channels were filled with 25 mM Tris-acetate buffer (pH 7.0) evacuated from reservoir R4; (2) reservoirs R3 and R4 were replaced with 25 mM Tris-phosphate buffer (pH 2.0), and 200 mM sodium borate buffer (pH 11.0), respectively; (3) a dilute solution of FITC-labeled thrombin aptamers was poured into R1; and (4) 0 and 200 V were applied to reservoirs R1 and R2, respectively. The FITC-labeled thrombin aptamers were electro-transferred to the channel crossing point, and the samples with an affinity for the thrombin impregnated in the gel were specifically entrapped and concentrated in the thrombin-immobilized gel. Components with no affinity for thrombin passed through the gel. After the concentration of the thrombin aptamers in the gel reached saturation (approximately 5 min), the trapped FITC-labeled thrombin aptamers were released from the gel. Next, the FITC-labeled thrombin aptamers were concentrated and separated in the separation channel by applying voltage of 200, 200, 0, and 1000 V for 5 min on R1, R2, R3, and R4, respectively. The separated components were detected 5 mm from the end of the separation channel. Samples subjected to ME were compared to samples subjected to pinched injection analysis. Pinched injection analysis was conducted on samples in 25 mM Tris-acetate buffer (pH 7.0) lacking a polyacrylamide gel. The samples were introduced into channel cross for 2 min before separation.

3. Results and discussion

3.1. In situ photopolymerization of thrombin gels and preconcentration of thrombin aptamers

We developed a method to capture and concentrate specific aptamers in a sample mixture utilizing a thrombin-impregnated polyacrylamide gel. This system also separated and detected the aptamers on a microfluidic chip. This method included the following four steps: (1) affinity matrix fabrication; (2) specific aptamer concentration; (3) aptamer elution; and (4) aptamer separation and detection. Figure 2 shows the images at the channel crossing point obtained with 10^9 M FITC-labeled thrombin aptamers electrophoresed through a thrombin-impregnated gel. A thrombin-containing acrylamide solution was delivered to the channels from R2 under pressure. A round thrombin-impregnated gel was fabricated at the intersection of the channels. After washing the channels with 25 mM Tris-acetate buffer (pH 7.0, neutral buffer), R1 was filled with a solution containing FITC-labeled thrombin aptamer. Next, R2, R3, and R4 were filled with the neutral buffer. After washing and preconditioning the thrombin polyacrylamide gel, no fluorescence was detected from the gel [inside of square in Fig. 2(a)]. After 200 V was applied across R1 and R2, with R2 as the anode, the fluorescence intensity in the thrombin polyacrylamide gel gradually increased [Fig. 2(b)], expanded, and finally reached a maximum [Fig. 2(c)-(d)]. This indicated the specific trapping of the FITC-labeled thrombin aptamers. This step is referred to as the aptamer concentration step. The
maximum FITC-labeled thrombin aptamer fluorescence intensity at this stage is likely limited by the amount of thrombin immobilized in the gel. Following the aptamer concentration step, an electric field (1000 V, 5 min) was applied across R3 (acidic buffer) and R4 (basic buffer). The concentrated FITC-labeled thrombin aptamers were released from the gel to the separation channel by delivering phosphate ions to the gel, which induced thrombin denaturation. This step is referred to as the elution step. The sample components were finally separated and detected at the end of the channel. This step is referred to as the separation and detection step.

We also investigated the specificity of aptamer entrapment in the thrombin-immobilized polyacrylamide gels. Thus, we fabricated an albumin-immobilized polyacrylamide gel and introduced the FITC-labeled thrombin aptamers in the same manner as described for Fig. 2. Figure 3(a) shows the image of the channel crossing point with FITC-labeled thrombin aptamers electrophoresed through an albumin-immobilized gel via applying 200 V for 10 min. No fluorescence was observed in the albumin-immobilized polyacrylamide gel. Thus, the thrombin-immobilized gel enabled specific entrapment and concentration of the thrombin aptamers. This indicated that the signals derived from DNA components with no affinity for thrombin could be removed from an electropherogram because these components will not be trapped but instead will pass freely through the thrombin gel.

As a further test of specificity, a mixture of a specific aptamer and non-specific ssDNA were electrophoresed through a thrombin-immobilized gel. The thrombin-immobilized gel was prepared as described above. Next, a mixture of $10^8$ M FITC-labeled thrombin aptamers and $10^7$ M 5-TAMRA-labeled random ssDNA were delivered to the gel from the anode and cathode.

respectively. Figure 3(b) shows the image of the channel crossing point obtained with a mixture of FITC-labeled thrombin aptamers and 5-TAMRA-labeled random ssDNA electrophoresed through a thrombin-impregnated gel via applying 200 V for 10 min. The green fluorescent image was obtained with the FITC filter set. FITC was highly concentrated even though the levels of FITC-labeled thrombin aptamer were 100-fold less than the levels of 5-TAMRA-labeled random ssDNA. On the other hand, no red fluorescence was observed in the gel even when the detection filter was changed to TAMRA (data not shown). These results showed that this method was effective for trapping specific DNA aptamers in complex DNA mixtures.

3.2. Entrapment and concentration of thrombin aptamers

Figure 4 shows a time-course of fluorescence intensity changes at the channel crossing point due to the entrapment of $10^{-10}$ or $10^{-11}$ M FITC-labeled thrombin aptamers subjected to thrombin-immobilized polyacrylamide gel electrophoresis. The fluorescence intensity of $10^{-10}$ M FITC-labeled thrombin aptamer appeared at 35 s, reached a maximum at 2 min, slightly decreased at 4 min, and reached a plateau after 5 min. The fluorescence intensity of $10^{-11}$ M thrombin aptamer appeared at 10 s, gradually increased at 2 min, reached a maximum at 4 min, and plateaued after 5 min. We also monitored the fluorescence intensity of $10^{-10}$ M FITC-labeled thrombin aptamers electrophoresed through an albumin-immobilized polyacrylamide gel under the same conditions as the reference. No increase in the fluorescence intensity of FITC-labeled thrombin aptamers was observed with the albumin-immobilized polyacrylamide gel. In addition, albumin-immobilized
polyacrylamide gel and pinched injection without polyacrylamide gel had a nearly identical fluorescence profile.

In this experiment, the gel contained 5 mg/mL of thrombin and the volume of the gel was approximately 3.0 \(10^{-13}\) m\(^3\). Thus, approximately 40 fmol of thrombin was immobilized in the gel. The number of moles present in 10 \(\mu\)L of the \(10^{-10}\) M FITC-labeled thrombin aptamer solution (approximately 1 fmol) was low relative to the number of moles in the thrombin polyacrylamide gel. Therefore, the gel should contain enough thrombin to trap all the thrombin aptamers. However, the excess FITC (from the labeling reaction) and a small fraction of the thrombin aptamers electrokinetically passed through the thrombin-immobilized polyacrylamide gel. Moreover, the fluorescent intensity of the FITC-labeled thrombin aptamers in the gel slightly changed after 1.7 min. Similarly, the number of moles present in 10 \(\mu\)L of the \(10^{-11}\) M FITC-labeled thrombin aptamer solution (approximately 100 amol) was very low relative to the number of moles in the thrombin polyacrylamide gel. Thus, almost all the \(10^{-11}\) M FITC-labeled thrombin aptamers were entrapped by the thrombin-immobilized polyacrylamide gel. The concentration of FITC-labeled thrombin aptamer in the thrombin-immobilized polyacrylamide gel approached a linear increase kinetically from 10 s to 2 min and the maximum fluorescent intensity was maintained after 2 min.

These results indicated that there was enough thrombin present in the gel to bind all the FITC-labeled thrombin aptamers.

3.3. Elution step

One of the most effective methods for rapid release of thrombin aptamers from thrombin-impregnated gels is the induction of thrombin denaturation by altering the pH. Most proteins are denatured and lose their activity in acidic or basic conditions (pH < 4 or pH > 8). Therefore, thrombin-ssDNA complexes should dissociate when the gel is immersed in an acidic or basic buffer. In order to denature the thrombin in the present study, 25 mM phosphate buffer (pH 2.0) was delivered to the thrombin-impregnated gel from reservoir R3. This treatment eluted all the thrombin aptamers from the gel. However, the resulting thrombin aptamer band length was approximately 300-400 \(\mu\)m in the separation channel, which may interfere with separation. In order to solve this problem, we applied a transient stacking mode to obtain a narrow elution band as described previously [18]. When the eluted sample band reached the boundary of the sodium ions delivered from the anode, the sample band was recompressed from the front end of the band and finally converted into a concentrated sharp band. The concentrated sample components were then separated and fluorometrically detected at the end of the separation channel.

3.4 On-line concentration of thrombin aptamers

Next, we utilized the established conditions described in the previous section to analyze a sample containing thrombin aptamers. The separation of the \(10^{-7}\) M FITC-labeled thrombin aptamer by pinched injection analysis is shown in Fig. 5(b). The FITC-labeled thrombin aptamers appeared at 1.0 min. In contrast, pinched injection analysis of \(10^{-8}\) M FITC-labeled thrombin aptamers did not produce any peaks on the electropherogram because the aptamer concentration is lower than the detection threshold (data not shown). On the other hand, the thrombin-immobilized polyacrylamide gel ME preconcentration method enhanced the thrombin aptamer detection sensitivity. Figure 5(a) shows the electropherogram derived from \(10^{-10}\) M FITC-labeled thrombin aptamers subjected to thrombin-immobilized polyacrylamide gel ME. Using this method, the FITC-labeled thrombin aptamers appeared at 4 min. The peak intensity obtained utilizing thrombin-immobilized gel ME of the thrombin aptamers was greater than that obtained by pinched injection. Therefore, the ME method enhanced the sensitivity for thrombin aptamer detection by approximately 1,000-fold. The thrombin aptamer peak (\(10^{-7}\) M) obtained by pinched injection analysis appeared at 1.0 min, while the thrombin aptamer peak (\(10^{-10}\) M) subjected
to thrombin-immobilized polyacrylamide gel ME appeared at 4 min. These results indicated that the detection time was delayed as the concentration of the thrombin aptamer decreased. One possible explanation for this finding may be the slow elution of the thrombin aptamers from the thrombin-immobilized gel.

4. Conclusions

A thrombin-immobilized polyacrylamide gel was constructed at the channel crossing point of two microfluidic channels. The gel was photopolymerized by irradiation with an LED laser, and polymerization was complete after 3 min. This thrombin-immobilized polyacrylamide gel specifically and efficiently entrapped thrombin aptamers after the application of voltage for a few minutes at both sample channels. This method also enabled the analysis of femtomolar levels of thrombin aptamer with high sensitivity. The total analysis time was approximately 10 min. This method may be utilized to screen and profile aptamer content in complex DNA samples.

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