Talanta 109 (2013) 20–25

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

On-chip immunoassay of a cardiac biomarker in serum using a polyester-toner microchip

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ARTICLE INFO

Article history:
Received 14 December 2012
Received in revised form
9 March 2013
Accepted 12 March 2013
Available online 20 March 2013

Keywords:
Polyester-toner microchip
Microfluidic immunoassay
C-reactive protein
Cleavable tag immunoassay

ABSTRACT

An on-chip immunoassay to detect C-reactive protein (CRP) was performed using a polyester-toner (PT) microchip. CRP is a highly conserved plasma protein responding to inflammation and is used for clinical purposes to diagnose an inflammatory state. For rapid analysis and specific interactions in immunoassays, extensive studies using microfluidic chips have been carried out. Recently, a simple technique to fabricate a disposable PT microchip by a direct printing process was developed and several applications were introduced. One major drawback of the PT microchip, however, is the poor separation performance due to the quality of the microfluidic structures. This problem for a PT microchip can be overcome using a cleavable tag immunoassay, which requires minimal separation performance. After analytes are conjugated onto antibodies which are immobilized on the surface of microbeads placed on the PT microchip, a second group of fluorescently tagged antibodies are added and complexed with the analytes. The tag is then cleaved and the solution containing the cleaved tag is analyzed by electrophoresis. The time needed for the complete analysis to be carried out on a PT microchip was less than 35 min. The dynamic range of the CRP in 10-fold diluted serum was 0.3–100 mg/L and the limit of detection was 0.3 mg/L, which demonstrated the possibility of a quantitative analysis of CRP in serum in clinical trials.

1. Introduction

Over half of all cardiovascular-related diseases do not show early symptoms. Therefore, biomarkers are used as pre-diagnostic indicators [1]. C-reactive protein (CRP) is a highly conserved plasma protein responding to inflammation, and an increase in its plasma concentration is clinically considered to indicate an acute inflammatory state [2]. The Centers for Disease Control and the American Heart Association acknowledged the correlation between a CRP increase in serum and future cardiovascular events and thus endorsed CRP as an inflammatory marker for clinical diagnoses of cardiovascular diseases such as myocardial infarction, ischemic stroke, and sudden cardiac death [3]. About a quarter of the US population has plasma CRP levels higher than 3 mg/L, and a CRP level higher than 10 mg/L may reflect an acute phase of a cardiovascular disease that would call for a closer investigation [2,4].

In clinical laboratories, the CRP level has usually been measured by an immunoassay due to the outstanding specificity of antigen/antibody interactions [5]. Conventional immunoassays are carried out in microwell plates requiring a long reaction time with labor-intensive manual procedures or an expensive robotic system [6]. When a microfluidic device with integrated sample treatment steps is used, the immunoreaction time, labor, and consumption of the sample and reagents can be greatly reduced [7–15]. Furthermore, microfluidic devices are promising tools for high throughput assays and good portability [16].

Microfluidic devices for immunoassays are fabricated using various materials, including silicon, silicon nitride, glass, quartz, metal, polystyrene, cyclic polyolefin, PDMS, and PMMA [17]. The fabrication processes, however, are usually complicated and require special facilities. Recently, a very simple process of fabricating a polyester-toner (PT) microchip by direct printing on a transparent polyester film using an office-type laser printer was introduced, and various applications, such as microchip electrophoresis [18–24], a micromixer [25], a microreactor [26], microzone plates for immunoassay [27], DNA analysis [28], and clinical diagnostics [29] have been demonstrated. In this work, we fabricated a PT microchip capable of performing a cleavable tag immunoassay (CTI) [30] and used it to analyze CRP in a serum sample. In the CTI, only the fluorescent tag cleaved from the detection antibody was injected into the electrophoresis channel. Therefore, we were able to overcome the low separation quality associated with a typical PT microchip. The overall analysis was completed within 35 min. The dynamic range, limit of detection (LOD), and limit of quantification (LOQ) of CRP in a 10-fold diluted sample of serum were 0.3–100 mg/L, 0.3 mg/L, and 0.6 mg/L,

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http://dx.doi.org/10.1016/j.talanta.2013.03.028

respectively, showing the capability of clinical analyses of CRP in a real sample.

2. Experimental

2.1. Materials and reagents

Polyethylene terephthalate (PET) transparency films (CG3300) were from 3M (Austin, TX, USA). Sulfo-NHS-SS-biotin, sulfo-NHS-biotin, and tris(2-carboxyethyl)-phosphine (TCEP, 0.5 mM) were purchased from Thermo Pierce (Rockford, IL, USA). Non-porous silica microbeads (100 mg/mL) with a diameter of 2.56 μm were from Bangs Laboratories (Fishers, IN, USA). 3-Aminopropyltriethoxysilane (APTES, 99%), boric acid, fluorescein-5-isothiocyanate (FITC), 25 wt% aqueous solution of glutaraldehyde, hexamethylenediamine, sodium chloride, sodium tetraborate, triethylamine (TEA), ethanol, avidin from egg white, CRP from human plasma, 29 wt% aqueous ammonia solution, phosphate buffered saline (PBS) dry powder, Tween 20, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal antibodies to CRP produced in mouse (clones 5 and 7) were from Exbio (Praha, Czech Republic). CRP-free human serum was from Sunny Lab (Sittingbourne, UK). Methanol was from Merck (Darmstadt, Germany) and tris(hydroxymethyl) aminomethane (Tris) was from ICN Biomedicals (Aurora, OH, USA).

The borate buffer used for the antibody immobilization reaction and electrophoresis was prepared by titrating 20 mM sodium tetraborate with 1 M NaOH to pH 9.2 at 25 °C. 1 × PBS was prepared by dissolving PBS dry powder in water and titrating it to pH 7.4 with 1 M HCl. A stock solution of CRP as a model analyte was prepared in a PBS–Tween 20 mixture (PBS–T, 1 × PBS with 0.05 vol% Tween 20). PBS–T and borate buffer were used for washing during the on-chip immunoreaction. To prevent the non-specific binding of serum proteins, a blocking buffer (PBS–T with 0.01 wt% BSA) was used before the CRP-antibody binding reactions and for the dilution of serum.

2.2. Synthesis of the labeled detection antibody

10 mg of FITC and 20 mg of hexamethylenediamine were reacted overnight at room temperature in 1 mL of methanol containing 10 μL TEA to prepare fluorescein thiocarbamyl hexamethylenediamine (FTHD) [31]. After the reaction, the precipitated product was washed with 1 mL of methanol/acetonitrile (1:10 vol/vol) and dried under ambient conditions. The precipitate was then reacted with 16 mg sulfo-NHS-SS-biotin (~1:1 M ratio with FTHD) in 1 mL 1 × PBS for 4 h at 4 °C to synthesize the tag, fluorescein hexamethylenediamine biotin (FHB). 100 μL of 1 mg/mL CRP detection antibody (clone 5) in 1 × PBS was biotinylated with 50 μL of 100 μg/mL sulfo-NHS-biotin in 1 × PBS for 2 h at 4 °C. The biotinylated detection antibody in 1 × PBS was then reacted with 150 μL of 5 mg/mL avidin in 1 × PBS for 2 h at room temperature. The molar ratio between the biotin and the avidin was 1:1. Subsequently, 450 μL of 25-fold diluted FHB solution in 1 × PBS was added to the avidin-biotinylated detection antibody and bound to the remaining sites of the avidin at 4 °C overnight. The reaction products were used as detection antibodies.

2.3. Immobilization of the capture antibody onto silica microbeads

50 μL of a 100 mg/mL silica microbead solution was suspended in 1 mL of ethanol. 60 μL of 29 wt% ammonia solution and 50 μL APTES were added to the suspension and the mixture was agitated overnight at room temperature. The microbeads were separated by centrifugation at 1100 *g* for 60 s, washed three times with 1 mL of borate buffer, and suspended in 1 mL of 0.25 mM Tris solution containing 10 μL of a 25 wt% glutaraldehyde solution. The suspension was stirred for 2 h at room temperature and the reacted microbeads were collected by centrifugation and washing with a borate buffer as above. The glutaraldehyde-modified microbeads were suspended in 1 mL of 1 × PBS. After adding 10 μL of 1 mg/mL CRP capture antibody (clone 7), the mixture was stirred for 1 h at room temperature. The capture antibody-immobilized microbeads were washed three times with 1 × PBS and then centrifuged at 1100 *g* for 60 s, dispersed in 1 mL of 1 × PBS, and stored at 4 °C until their use [31].

2.4. PT microchip fabrication

The PT microchip fabrication procedure was previously reported in detail [18] and will be summarized here briefly (Fig. 1). A microchip layout was prepared using Adobe Illustrator CS5 (Adobe Systems, San Jose, CA, USA). The channel width was 200 μm, the electrophoresis channel was 16.0 mm long, and other connecting channels were 8.0 mm long. A double-T injector with a 450 μm offset for the pinched injection was located between the sample reservoir (SR) channel and the sample waste reservoir (SW) channel. Three weirs each with a width of 120 μm were prepared at the SR outlet in order to prevent leakage of microbeads from the SR to the connecting channels during the assay. An opening of 80 μm was formed on the channel wall so that fluid could flow through whereas the microbeads could not. The layout was printed on a polyester transparency film using an HP LaserJet 2100 (Hewlett-Packard, Boise, ID, USA) with a C4096A toner cartridge at a maximum resolution of 1200 dots per inch. The printed transparency film and a blank polyester film were then subjected to a corona discharge treatment [32] using a Tesla coil (BD-10A; Electro-Technique Products, Chicago, IL, USA) for ~20 s at ~5 mm height from the film under the condition of 35–40% humidity so that the channel surfaces were made hydrophilic for easy injection of the run buffer and sample. The printed transparency film and the blank film were then joined using a heating laminator (SKY-325R6, Peach Laminating, Seoul, Korea) at 130 °C and at a feeding rate of 1000 mm/min to form channels in the blank regions. Following the lamination step, 200 μL pipette tips were attached using hot melt stick glue (Keumsung, Daejeon, Korea) for use as liquid reservoirs. The chip fabrication process was completed within 15 min, from printing to the reservoir preparation step.

2.5. Sandwich immunoassay of CRP

A PT microchip was placed on a microscope (IX71, Olympus, Tokyo, Japan). After gravity filling a run buffer in the buffer reservoir (BR) into the channels of the PT microchip, the flow of the liquids was electrokinetically controlled using a high-voltage power supply (DBHV-100, Digital Bio Technology, Seoul, Korea) connected to an IBM-compatible PC through a COMI-CP301 D/A board (COMIZOA, Daejeon, Korea). The sandwich immunoassay of the CRP consisted of two parts: CTI and electrophoresis. The voltage programming of each step is given in Table 1.

The procedures for the on-chip CTI were adapted from earlier work [31], as illustrated in Fig. 2. 30 μL of the suspension of capture antibody-immobilized microbeads in 1 × PBS was injected into the SR. After rinsing the microbeads in the SR three times each with 100 μL of PBS–T using a micropipette, 45 μL of CRP sample solution was added to the SR and the reaction was carried out for 10 min at room temperature. The electroosmotic flow (EOF) was induced from the SR toward the rinse reservoir (RR) by applying a lower voltage to the RR compared to that applied to other reservoirs. After the reaction, the solution in the SR was

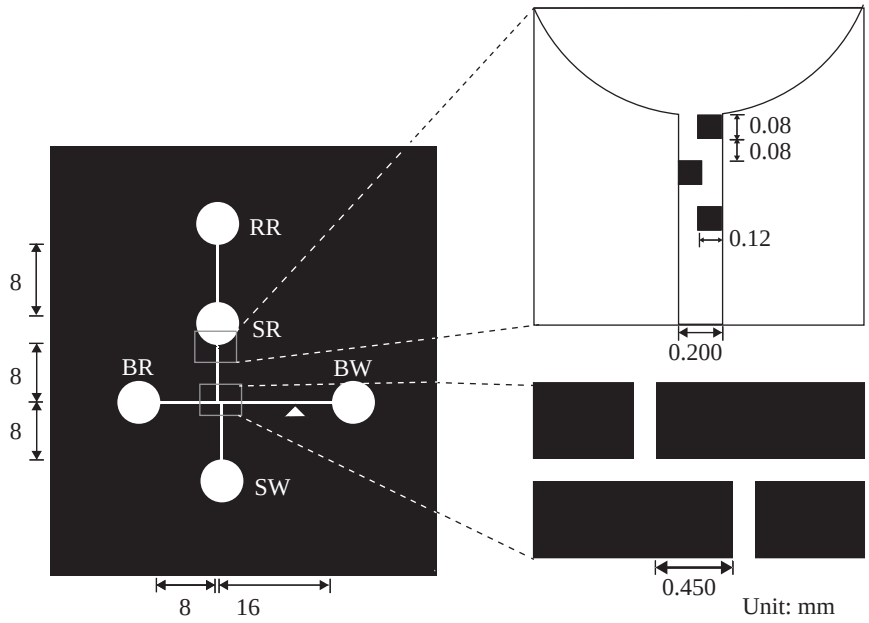

Fig. 1. Schematic of the PT microchip layout. SR; sample reservoir, SW; sample waste reservoir, BR; buffer reservoir, BW; buffer waste reservoir, RR; rinse reservoir. The triangle located 10 mm from the double-T injector toward BW represents the detection point.

Table 1
High-voltage program used in on-chip immunoassay.

	Rinse reservoir (V)	Sample reservoir(V)	Buffer reservoir(V)	Sample waste reservoir (V)	Buffer waste reservoir (V)	Duration (s)
Reaction	0	10	10	10	10	2000
Injection	0	50	100	200	1000V	60
Electrophoresis	500	600	100	1500	600V	40

removed and rinsed five times each with 100 μL of PBS–T, as described above. During the rinsing procedure, a constant potential from the SR to the RR was applied to remove unbound reactants. 90 μL of the detection antibody solution (~1 mM) with a cleavable tag, which was double of the analyte amount, was added to the SR. This step was followed by a reaction for 10 min at room temperature. Unreacted detection antibodies also migrated toward the RR due to the EOF during the reaction. After the reaction, the rinsing procedure was repeated five times each with 100 μL of a run buffer. The SR was then filled with 100 μL of run buffer containing 5 vol% TCEP and the cleavage reaction was carried out for 10 min. The fluorescent tag from the detection antibody was cleaved with TCEP by reducing the disulfide bond in the tag moiety. As a result, the cleaved tag was dispersed in the sample solution while the antigen and the detection antibody were immobilized on the microbeads.

The cleaved tag was loaded into a double-T injector by flowing the solution in the SR toward the SW, after which electrophoresis with laser induced fluorescence (LIF) detection was carried out. Through a 10× objective of a microscope, the excitation beam from an argon-ion laser (488 nm, 10 mW, Melles Griot, Carlsbad, CA, USA) was loosely focused onto the detection point located 10 mm from the double-T injector toward the buffer waste reservoir (BW) covering the full width of the channel. The fluorescence emission was filtered through a 520 nm band pass filter (CVI, Albuquerque, NM, USA), focused by the same objective and then detected using a photomultiplier tube module (PMT; HC 120-01, Hamamatsu, Bridgewater, NJ, USA). The PMT signal was collected and processed using a PCI-MIO-16XE-50 board (National Instruments, Austin, TX, USA) on an IBM-compatible PC controlled with in-house software written using LabVIEW 7.0.1 (National Instruments).

As a reference, off-chip CTI was also carried out in the same manner used with the on-chip CTI process except for immunoreactions of 2 h each and the collection of the cleaved tag with a 3 kD Nanosep centrifugal concentrator (Pall Corporation, Port Washington, NY, USA). The cleaved tag was then analyzed either with a PT microchip as described above or with a commercial CE instrument (P/ACE MDQ, Beckman Colter, Fullerton, CA, USA). For conventional CE, a bare fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm ID and with a total/effective length of 40/30 cm was rinsed with 0.1 M NaOH, water, and borate run buffer for 3 min, each at 80 psi. A reverse potential of −20 kV was applied across the capillary, which was maintained at 25 °C, and the tag was monitored with LIF.

3. Results and discussion

3.1. Characteristics of a PT microchip

For the microfluidic immunoassay on a PT microchip, several features were implemented. First, the channel surfaces were made hydrophilic by a corona discharge treatment. The contact angles of water on a blank and a printed transparency polyester films increased from $29 \pm 3^\circ$ to $53 \pm 2^\circ$ and from $46 \pm 3^\circ$ to $107 \pm 3^\circ$ ($n=6$), respectively. As a result, the gravity injection of liquids into the PT microchip was done without difficulty. Second, a RR was added to the SR. During the reaction steps, a lower potential was applied to the RR than the other reservoirs to prevent the leakage

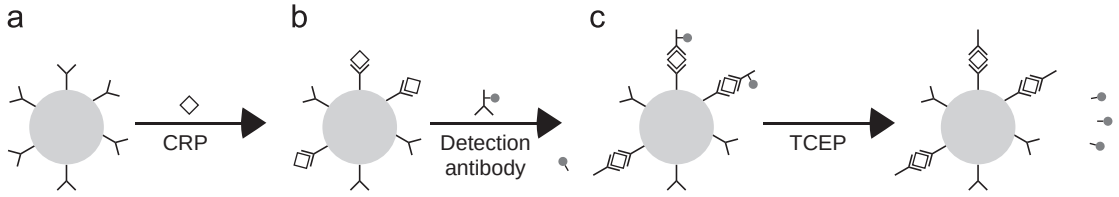

Fig. 2. Immunoassay procedure. (a) Addition of CRP to the antibody-immobilized bead solution, (b) addition of the detection antibody with a fluorescent tag and (c) cleavage of the tags.

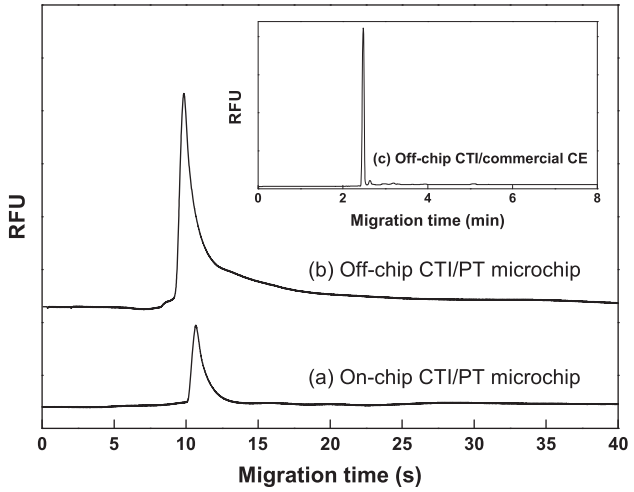

Fig. 3. Electropherograms of cleaved tags. (a) Off-chip CTI of CRP (10 mg/L) using a commercial CE instrument. 50 μm ID × 40/30 cm fused silica capillary; 20 mM sodium tetraborate run buffer of pH 9.2; LIF λ_{ex}=488 nm, λ_{em}=520 nm; 25 °C; −20 kV, (b) off-chip CTI and (c) on-chip CTI of CRP (10 mg/L) using a PT microchip. 20 mM sodium tetraborate run buffer of pH 9.2; LIF, λ_{ex}=488 nm, λ_{em}=520 nm; room temperature; voltage programming as in Table 1. RFU; relative fluorescence unit.

of reactants and products into the separation channel. Third, a single-toner layer chip [18] was used to reduce the risk of bubble formation caused by Joule heating accumulated from the long application of electric potentials during several reaction steps. Due to the smaller cross-sectional areas of microchannels, a single-toner layer chip produces less Joule heating and thus fewer bubbles. Moreover, the chip fabrication process was simplified since the exact alignment of the channel structures printed on the two polyester films was unnecessary. Finally, to prevent the leakage of microbeads from the SR, weirs were added at the outlet of the SR, simply by drawing additional lines in the chip layout. Note that the separation performance of the cleaved tag carried out after immunoreactions were not interfered by the weirs.

3.2. Analytical performance of the on-chip sandwich immunoassay

The EOF profiles in the microchannels of the PT microchip are not flat, as the top and bottom surfaces of the microchannel consist of polyester films and the sidewalls of the microchannel consist of toner materials. In addition, the channel surfaces are rough and a number of random toner spots exist in the channels due to the limitation of the printing process by a laser printer. Therefore, the separation quality obtainable with a PT microchip is much worse than that from a quartz, glass, or polymer microchip [23]. As a result, it is quite difficult to carry out an immunoassay of a biological sample in a complex matrix on a PT microchip. One remedy to this problem is CTI [30]. When using this method, a fluorescent tag detached from the detection antibody was injected into the electrophoresis channel, while other components remained immobilized on the microbeads or were removed by rinsing. The difficulty of protein separation could

Table 2
Comparison of CTI/CE of CRP (n=3).

	Peak height (S/N)	Number of plates	RSD	
			Migration time (%)	Peak height (%)
Off-chip CTI/PACE MDQ	14000 ± 560	13000 ± 390	3	4
Off-chip CTI/PT microchip	270 ± 11	680 ± 10	17	16
On-chip CTI/PT microchip	100 ± 15	600 ± 33	23	26

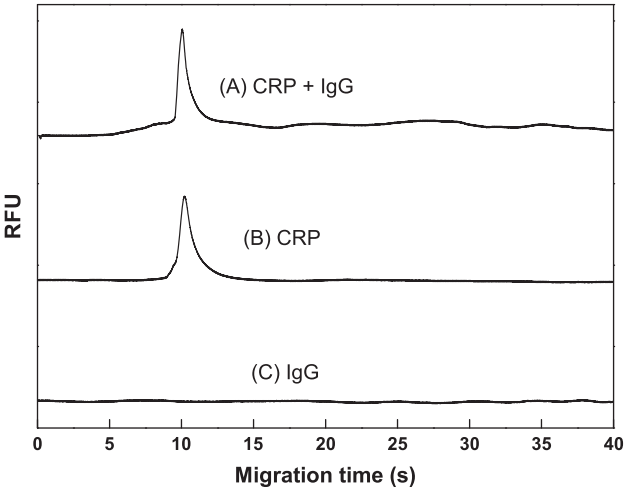

Fig. 4. Electropherograms from on-chip immunoassay. Sample; 85 nM CRP and/or IgG in a blocking buffer. Electrophoresis conditions as in Fig. 3c.

therefore be avoided and CRP in serum could be assayed using a PT microchip. Advantages of adding the electrophoresis step are the possibilities of on-line sample preconcentration and multi-analyte testing using a single PT microchip, which we are preparing to demonstrate in separate reports.

In order to confirm whether the immunoreactions proceeded well on a PT microchip, the electrophoresis results of cleaved tags obtained through off-chip immunoreaction were compared with the on-chip immunoassay result. Fig. 3a shows an electropherogram of a tag from off-chip CTI obtained with a commercial CE instrument. Figs. 3b and c show electropherograms with PT microchips of tags from off-chip CTI and on-chip CTI, respectively. The peak height in terms of S/N ratio and the number of plates (n=3) for the peak of tag obtained with the commercial instrument, off-chip CTI, and on-chip CTI were (14000 ± 560, 13000 ± 390), (270 ± 11, 680 ± 10), and (100 ± 15, 600 ± 33), respectively. The relative standard deviations of the migration time and peak height were (3%, 4%), (17%, 16%), and (23%, 26%), respectively for the commercial instrument, off-chip CTI, and on-chip CTI (see Table 2). As expected, the quality of the CE results

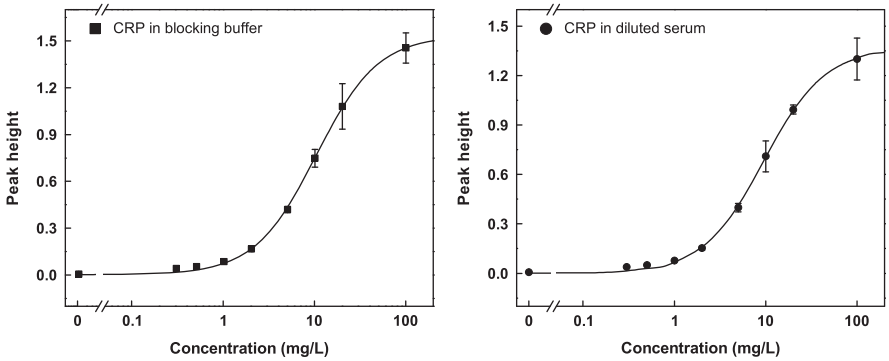

Fig. 5. Hill equation calibration curves for CRP (a) in a blocking buffer and (b) in serum 10-fold diluted with the blocking buffer. Electrophoresis conditions as in Fig. 3c. Error bars represent the standard deviations ($n=4$).

with the PT microchips was greatly inferior to that from the commercial CE instrument. However, the on-chip CTI results obtained within 35 min were quite comparable to those from off-chip CTI obtained within 4.5 h, demonstrating the feasibility of the sandwich immunoassay on a PT microchip.

The specificity of our immunoassay was checked using IgG (pI 7.3 [33]) as a control. Since either IgG or the detection antibody could not be immobilized on the microbeads and migrated to the RR by EOF, cleaved tags were not detected in the electrophoresis channel, as expected. The interference of IgG was also tested using a mixture sample of CRP and IgG. Fig. 4 shows that our sandwich immunoassay responded only to CRP.

In addition, in order to examine the quantitativity of CRP analysis, the on-chip immunoassay process was carried out with CRP samples of different concentrations from 0.3 to 100 mg/L (Fig. 5a). The calibration curve for CRP using the Hill equation takes the following form [34]:

$$y = Max\frac{[x]^h}{[x]^h + [EC_{50}]^h} \quad (1)$$

The curve was plotted with a logarithmic-scale x-axis with the Max being the maximum intensity, with the half-maximal effective concentration (EC_{50}) serving as the inflection point on the calibration curve and h representing the Hill coefficient. The parameter values obtained from fitting to the data were as follows: $Max=1.54\pm0.02$, $EC_{50}=10.4\pm0.3$, $h=-1.28\pm0.04$ and $R^2=0.999$ ($n=9$). The LOD and LOQ for CRP in a blocking buffer estimated as three and ten times of the standard deviation of blank signal were 0.2 and 0.5 mg/L, respectively. The migration time was 10 ± 3 s.

3.3. On-chip sandwich immunoassays in human serum samples

To verify the applicability of the proposed on-chip sandwich immunoassay to a real sample, CRP samples in human serum were analyzed. CRP was spiked into CRP-free human serum 10-fold diluted with a blocking buffer. The dilution was to prevent long immunoreaction times and the clogging of the microchannels due to the high-viscosity serum matrix. As shown in Fig. 5b, the results obtained with the samples of 0.3–100 mg/L CRP in 10-fold diluted serum and the non-CRP spiked serum as a control ($n=4$) were in good agreement with those from CRP samples in the blocking buffer. The parameter values estimated from fitting to the data were: $Max=1.37\pm0.03$, $EC_{50}=9.53\pm0.48$, $h=-1.30\pm0.06$ and $R^2=0.998$ ($n=9$). The LOD and LOQ for CRP in serum estimated from the standard deviation of control signal were 0.3 and 0.6 mg/L, respectively. As shown in Fig. 6, the migration time of CRP in the diluted serum also agreed with that of CRP in the blocking buffer. Inspite of the poor reproducibility and resolution obtained with a PT microchip, the effect of the diluted serum matrix did not significantly interfere with the sandwich immunoassay. Moreover, our PT microchip immunoassay provided a sensitivity level that was sufficient for the clinical cut-off level (10 mg/L) and is thus feasible for use as a point-of-care device for risk screening for CRP-related diseases.

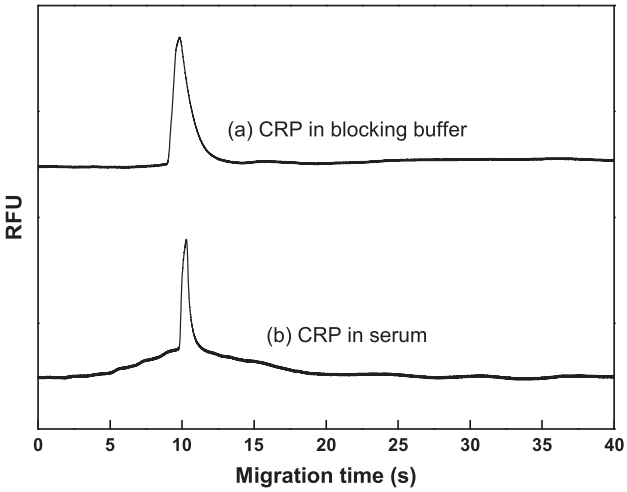

Fig. 6. Electropherograms from on-chip immunoassay of 10 mg/mL CRP (a) in a blocking buffer and (b) in serum 10-fold diluted with the blocking buffer. Electrophoresis conditions as in Fig. 3c.

4. Conclusions

A PT microchip capable of being used with microfluidic sandwich immunoassays was developed. In order to overcome the problem of the low separation performance of a PT microchip as compared to a glass or polymer microchip, CTI was employed. After immobilizing CRP with the capture antibody on the surface of a microbead, the detection antibody was conjugated with the CRP. The fluorescent tag cleaved from the detection antibody was injected into the separation channel for electrophoresis with LIF detection. The microfluidic immunoassay using a direct printing process has the advantages of completing microchip fabrication and immunoassay within 15 and 35 min, respectively. With this on-chip immunoassay, the cardiac marker CRP in (undiluted) serum could be detected in the concentration range of 3–1000 mg/L, demonstrating its potential as a point-of-care device in clinical diagnosis. Taking advantage of the electrophoresis step of the PT microchip, we plan to apply on-line sample preconcentration and add different fluorescent tags for multi-analyte testing and internal standardization.

Acknowledgment

The authors would like to thank the National Research Foundation of Korea (2012–0005479).

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