A microfluidic protease activity assay based on the detection of fluorescence polarization

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Abstract
This article describes a fluorescence polarization (FP)-based protease assay on a microfluidic device that is compatible with fast and reproducible analyses of protease activities. The optical systems were arranged for simultaneously measuring fluorescence intensities of vertical and horizontal polarization planes, and the binding of tetramethylrhodamine (TMR) labeled-biotin with streptavidin was utilized for optimizing FP detection in continuously flowing solutions within 74-μm wide, 12-μm deep microchannels of a glass chip. In developing off-chip FP-based assays for proteinase K, trypsin, papain and elastase, TMR conjugated-casein protein (TMR-casein) was employed as a universal substrate. After optimization of the hydrodynamic flow control to allow complete mixing of TMR-casein and short proteolysis time as possible, and of buffer composition to minimize protein sticking problems, the developed assay was transferred to the microfluidic chip by monitoring FP changes of TMR-casein in the main microchannel. The results indicate that the proposed device would serve as an integrated microfluidic platform with automated injection of reacting species, diffusion-controlled mixing, reaction and detection for protease activities without the need to separate the products.

Keywords: Protease assay; Interaction analyses; Fluorescence polarization; Microfluidic device; Activity screening system

1. Introduction
The need to reduce the reagent consumption and to increase the analysis speed in biochemical assay has motivated the miniaturization of analysis, which has been enabled by microscale device fabrication and microfluidics [1–3]. In addition, the microfluidic technology offers great promise of integrating a complete assay on a single microfluidic chip. Despite these attractive features, substantial research needs to be done for more sensitive and versatile high-performance detection, as more biochemical protocols are being implemented on microfluidic chips with more fluidic functions.

Fluorescence, especially fluorescence intensity, has been widely employed as an optical detection method for microfluidic chips [4,5]. The use of fluorescence polarization (FP), on the other hand, has not been extensively explored for applications in microfluidic devices although it serves as a valuable technique for analyses of biomolecular interactions in conventional formats [6,7] and by capillary electrophoresis [8–10], and its utility has been demonstrated for some portable devices sensing chemicals [11] and oxygen [12] as well as for immobilized aptamer-based biosensors [13,14]. Only very recently, biosensing of homogeneous molecular binding by FP detection using a commercial fluorescence spectrometer has been attempted in microfluidic devices containing relatively large 500-μm microchannels [15]. FP is sensitive to changes in the rotational motion arising from molecular interactions/reactions. A small fluorescently labeled molecule that rotates rapidly in solution emits depolarized fluorescence upon excitation by a polarized light, resulting in low FP. On the other hand, a large molecule exhibits a higher polarization because of its slower motion under the same condition. Therefore, changes in FP can reflect association or dissociation between molecules of interest as well as breakdown of large molecules containing fluorescence labels.

The present work demonstrates the feasibility of an on-chip assay based on FP detection, integrated in a microfluidic sys-
tem containing narrow microchannels. In order to optimize the FP measurement system for microfluidic chips, binding of biotin with streptavidin is employed as a simple model assay. The measurement system is then applied to a protease assay using a fluorescently labeled universal protein substrate, TMR-α-casein. Various techniques on identification and quantification of the activity of proteolytic enzymes have been developed, including measurements of absorbance or fluorescence liberated in the supernatant of precipitation assays [16], homogeneous fluorimetric assays using fluorescence-quenched, hyperconjugated fluorescein derivatives of protease substrates [17,18], and FP-based assays of a fluorescently labeled-protein substrate [19,20]. The former suffers from the need for careful sampling intervals, control of sample volumes, and separation of labeled hydrolysis products from unhydrolyzed protein, while the latter two appear to provide a rapid and convenient measurement system. Since FP is independent of fluorescence intensity thus more tolerant of fluorescence intensity fluctuations, the FP-based protease activity assay is chosen to test the suitability of our constructed microfluidic detection system for a reliable activity evaluation tool. After optimization of off-chip assay, and on-chip mixing and flow control, on-chip reactions of four different proteases are performed. The results demonstrate that our FP-based microfluidic chip would provide a quick and reliable miniaturized system for a wide variety of interaction and enzymatic assays.

2. Experimental

2.1. Reagents

Proteinase K, papain, elastase and α-casein were purchased from Sigma–Aldrich (St. Louis, MO, USA). Trypsin was obtained from Boehringer Mannheim (Mannheim, Germany), and streptavidin from Calbiochem (Darmstadt, Germany). TMR succinimidyl ester, TMR–biotin and D-biotin were from Molecular Probes (Eugene, OR, USA). Other chemicals were of the highest grade commercially available. All the buffers were filtered through 0.22-μm disposable Millipore syringe filters. TMR-α-casein was prepared by reacting α-casein with TMR succinimidyl ester (7:1 mol/mol) in 0.1 M sodium bicarbonate buffer, pH 9.0 at room temperature for 2 h, similarly as previously reported [19]. The resulting product was purified by passing the reaction mixture over a Sephadex G-25 desalting column (Amersham Bioscience, USA), followed by eluting with 0.1 M sodium phosphate buffer, pH 7.0. The collected TMR-α-casein was spectroscopically quantified and stored at −70°C in aliquots until use. The molar ratio of TMR to protein was determined to be 0.18.

2.2. Microfluidic chip fabrication

Microfluidic chips shown in Fig. 1 were fabricated by standard semiconductor photolithographic techniques [21–23] with minor modifications. Prior to fabrication, Pyrex glass wafers (Corning Co., USA) were annealed at 520°C for 1 h and 40 min and 500°C for 1 h and 30 min, and remained at room temperature for 1 day. The wafers were then cleaned in H2O2/H2SO4 (1:4 vol/vol) to remove organic materials, rinsed with deionized water, and dehydrated at 200°C for 5 min before processing. Thin films of Cr (adhesion layer) of 300 Å and Au (protection layer) of 2000 Å were deposited on the wafers by means of e-beam evaporation. After an adhesion promoter HMDS and a positive photoresist AZ1512 were consecutively spin-coated onto the metal surface, the channel pattern was photolithographically transferred onto the wafer, and the exposed photoresist was hard-baked and developed. A stripper was then used to remove the exposed metal successively, and the exposed glass was chemically wet-etched in a mixture of HF/HNO3/HNO (1:2:2 vol/vol/vol). Two millimeter-diameter through-holes were etched-out with a sand blaster as reservoirs. Finally, the photoresist and the metal thin films were removed, and the wafer was cleaned, followed by alignment of the top and bottom glass substrates and bonding using a thermal technique.

2.3. Instrumentation

A laboratory-built FP measurement system was constructed on an optical table in a temperature-controlled room (25°C), to minimize any temperature-dependent variations in FP measurements. A 5 mW helium-neon laser (Coherent, USA) with a maximum wavelength of 543.5 nm was used as the excitation source. The light with a beam passed through an interference filter (543.5 nm, Melles Griot, CA, USA) and mirrors (CVI, USA) was polarized with a broadband polarizing beam splitter cube (CVI, USA). The polarized light was focused with a mirror and a lens (Melles Griot, CA, USA) onto a cuvette or the microchan-
nel of a chip, which was placed on the stage using a specially constructed holder. Fluorescence was collected at right angle with respect to the laser beam by using a spectrally neutral cube beam splitter (CVI, USA). To reject scattered laser light, fluorescence was spectrally filtered with a band-pass filter (580 nm, Melles Griot, CA, USA). The fluorescence was then split using a broadband polarizing beam splitter cube to photomultiplier tubes (PMT1 & PMT2, Hamamatsu, Japan) for measuring horizontally and vertically polarized light. Filters (580 nm, Thorlab, USA) for both planes were used, and a polarizer was placed in front of the PMT2 for horizontally polarized light to minimize the vertical component and maximize the horizontal component of the PMT2 signal. In addition, an optical chopper and two lock-in amplifiers were used to improve the signal-to-noise ratio. The outputs from the two PMTs were digitized and processed for FP. The values of FP were calculated according to:

\[ \text{FP} = (I_v - G I_h)/(I_v + G I_h) \]  

where \( I_v \) and \( I_h \) are the fluorescence intensities of the vertically and horizontally polarized components, respectively, and \( G \) is an empirical constant that corrects for the polarization bias introduced by the optics and the detection system. FP values were averaged for the data collected every 0.1 s for 60 s on chip, and presented.

2.4. Interaction assays

Binding of TMR–biotin to streptavidin with concentrations indicated in the figure legends in phosphate-buffered saline (PBS) was measured by FP on an LS50B Perkin-Elmer fluorimeter. For on-chip assays, the fabricated chip was cleaned with deionized water, 1N NaOH, deionized water again, and PBS for 10 min. The chip was filled with PBS followed by addition of TMR–biotin and streptavidin in the inlet reservoirs, placed in the holder, and mounted on the stage. After alignment of the chip detection point for the fluorescence polarization detection, a vacuum was applied at the outlet well, and fluorescence data were obtained by the detection system. A constant vacuum was applied at the outlet well, and fluorescence data were obtained by the detection system. Constant vacuum was applied at the outlet well, and fluorescence data were obtained by the detection system.

2.5. Protease assays

Assays were carried out in a 500-μL cuvette containing TMR-α-casein at a final concentration of 100 nM with each protease at 25°C, and fluorescence polarization values were recorded using an LS50B Perkin-Elmer fluorimeter. Buffers optimized for protease assays were 0.1 M HEPES, 50 mM DTT, 5 mM EDTA, 5% DMSO, pH 7.4 for proteinase K and trypsin, 0.1 M sodium citrate, 1.1 mM EDTA, 0.067 mM 2-mercaptoethanol, 5% DMSO, pH 6.0 for papain, and 0.1 M HEPES, 0.15 M NaCl, 5 mM EDTA, 50 mM DTT, 5% DMSO, pH 7.8 for elastase.

For on-chip assays, the designed chip in Fig. 1 was first verified for bulk fluidic behavior using simulation software Coventorware (Coventor Inc.), while the mixing of the inlet flows was analyzed using an analytical solution of diffusion equations \([24,25]\). The fabricated chip was cleaned as described above. The chip was filled with the assay buffer followed by addition of protease and TMR-α-casein in the inlet reservoirs, placed in the holder, and mounted on the stage. After alignment of the chip detection point for the fluorescence polarization detection, a vacuum was applied at the outlet well, and fluorescence data were obtained by the detection system. A constant vacuum was checked to be maintained for the duration of each experiment.

3. Results and discussion

3.1. Microfluidic chips and FP detection system

Fig. 1 shows the layout of the glass microfluidic chip suitable for both binding experiment and protease assay. The chip consists of two inlet reservoirs, microchannel networks with the mixing intersection, and an outlet waste reservoir. The depth of the fabricated microchannel was ∼12 μm, and the widths of the inlet and main flow were ∼29 and ∼74 μm, respectively, confirmed by scanning electron microscopy. The chip was designed to mix the fluorescently labeled-substrate TMR–biotin or TMR-α-casein into the solution containing streptavidin or protease in the main channel. Therefore, when the solutions of these interacting/reacting molecules placed on the inlet reservoirs of the chip are induced to flow into the main channel by application of vacuum on the outlet waste reservoir, binding events or reactions would be expected to result in changes of FP that could be monitored at the detection point in Fig. 1. In addition, this design with which the inlet flow from the protease reservoir meets with the main channel prior to inflow of the substrate would provide a potential interface site to the micro-well plates, for example capillary insertion, thereby adding functional versatility of the chip.

In order to measure emission signals of the vertically and horizontally polarized components at the detection point of the microchannel, an FP detection unit was configured with a polarized 543.5 nm laser for excitation of TMR-labeled molecules, laser focusing and fluorescence collection optics, and polarizing beam splitters. The vertically and horizontally polarized fluorescence emitted from the sample in the microchannel could then be simultaneously detected by two separate PMTs (Fig. 1), allowing the determination of FP. Ideally, PMT1 and PMT2 should receive 100% vertical and horizontal fluorescence signals, respectively. However, ∼10% vertical fluorescence signal was mixed in the signal received by PMT2 while >99% vertical fluorescence signal was received by PMT1. Accordingly, another polarizer was placed in front of PMT2 to minimize the vertical component of the fluorescence. In addition, an optical chopper was placed between a light source filter and a mirror in the path of excitation light, which improved signal-to-noise ratio dramatically and made FP detection in the narrow main channel possible (see below).

Optical performance of the constructed FP detector was obtained by aligning a tightly focused laser beam with a small-diameter (<74 μm) sample stream and by balancing signals from two PMTs. An aqueous solution of TMR was filled in the main channel hydrodynamically, and the laser beam was focused onto a spot 32 mm away from the junction of two inlet reservoirs.
The angle and position of the optical path were adjusted so that roughly equal signals with maximum outputs from both PMTs were observed. To correct for the polarization bias introduced by the emission optics and the PMT detectors, an empirical constant \( (G \text{ value}) \) was determined as the intensity ratio of vertical to horizontal polarization components of a dilute solution of the small fluorescent TMR dye, and incorporated in the calculation of FP as in the Eq. (1). The detector was then calibrated by measuring FP as a function of the TMR, which showed independence of the dye concentration up to 100 \( \mu \)M and with a lower detection limit of \( \sim 10 \) nM (data not shown). Since the previously reported CE-based FP detection[9] had demonstrated a nanomolar sensitivity by using a 0.2 mm \( \times \) 0.2 mm cuvette, which is \( \sim 17 \) fold larger than our microchannel depth, it appeared that our detection set-up was well optimized. In addition, the pressure controller was tested for reliable performance of driving the fluids through the microchannel hydrodynamically by applying a vacuum to the waste reservoir of up to \( -69 \) kPa with a resolution of 0.34 kPa as described previously[23].

3.2. Optimization of FP detection in microchannels

Binding of biotin to streptavidin was chosen to optimize the detection system. When FP changes of TMR–biotin upon complex formation were first examined using a conventional Perkin-Elmer fluorescence spectrometer, addition of streptavidin to TMR–biotin exhibited significant FP, resulted from a substantial increase in the molecular size and a slower rotation of the complex molecule, as compared to little FP of TMR–biotin only (Fig. 2A). It should be noted that the fluorescence intensity of TMR–biotin complexed with streptavidin tends to decrease dramatically owing to quenching. Thus, to minimize variations caused by changes in the fluorescence intensity if any, TMR–biotin was used at an eight-fold higher concentration for the complex formation, which yielded comparable intensities as TMR–biotin only. With the initial set-up for the detection system assembled without an optical chopper, similar measurements were performed in a cuvette. As expected, FP values increased for the sample containing TMR–biotin and streptavidin compared to the one containing TMR–biotin only (Fig. 2B). Therefore, the detection system appeared to be appropriate for measuring FP in bulk.

Experiments were then conducted using a fabricated microfluidic chip with the same detection system without an optical chopper. When TMR–biotin and streptavidin were added to the inlet reservoirs of the chip, and allowed to flow and interact within the main channel by applying a pressure drop of 13.8 kPa to the waste reservoir, the increase in FP upon complex formation could not be observed at the detection point (Fig. 2C). However, when these data were recalculated using the fluorescence intensities obtained by subtracting offset values measured for the microchannel filled with buffer only, the increase in FP was reproducibly observed upon complex formation between TMR–biotin and streptavidin (Fig. 2D).

Accordingly, the detection system was modified with an optical chopper to remove the offset fluorescence, and similar experiments were performed. As shown in Fig. 3A, the on-chip formation of the complex of TMR–biotin with streptavidin was readily detected by an increase of FP to 0.269 from 0.087 for the sample containing TMR–biotin only. Since a mean FP of the complex in the microchannel was found to be constant
Fig. 3. FP measurements in the microfluidic channel by the setup in Fig. 2 with an optical chopper inserted (A) for the binding of 4 μM TMR–biotin with 1 μM streptavidin compared with 4 μM TMR–biotin only, and (B) for competitive inhibitions of the TMR–biotin interaction with streptavidin by D-biotin.

regardless of the concentration of TMR–biotin as long as the biotin-to-streptavidin ratio was kept constant, indicating independence of FP on the fluorescence intensity, TMR–biotin was used at a fixed concentration of 4 μM. The developed system was further explored for applicability by performing competition experiments on chip. The complex formation of TMR–biotin and streptavidin was competitively inhibited by D-biotin supplied as mixed with TMR–biotin from the inlet reservoir of the chip, indicated by the decrease in FP with increasing concentrations of D-biotin (Fig. 3B). These results indicated that the insertion of an optical chopper in the course of excitation apparently improved the signal-to-noise ratio, leading to successful achievement of FP detection for the sample flowing through the very narrow microchannel.

3.3. Development of FP-based protease assays

For the development of protease assays based on FP that can be transferred to an on-chip format, α-casein was selected as a protein substrate since 95% of known proteases have been estimated to cleave casein. The conditions for derivatizing α-casein with TMR were adjusted to generate a lightly labeled probe protein; determination of the molar ratio of TMR to protein yielded 0.18. It has been previously shown that the protein lightly labeled with fluorophores serves as an excellent substrate for protease assays in FP methodology [19], whereas the protein substrate highly derivatized with fluorophores whose fluorescence is completely quenched has been utilized for homogeneous fluorimetric assays by measuring the increase in fluorescence emission upon its cleavage with proteases [17,18,26]. Initial FP values of the synthesized TMR-α-casein were always about 0.35, and not changed over 1 h at room temperature in the absence of protease, demonstrating the stability of the TMR-α-casein under the reaction conditions and the absence of extraneous proteases.

Employing the TMR-α-casein conjugate as a substrate, four different enzymes including proteinase K, trypsin, papain, and elastase were utilized for activity assays. When TMR-α-casein was mixed with proteases, the gradual drop in the FP values was observed by measurements with a spectrofluorometer (Fig. 4), indicating that the TMR-α-casein was being cleaved over time.

Fig. 4. Time courses of protease activity measured by FP changes on a conventional fluorescence spectrometer using 560 nM TMR-α-casein with varying concentrations of (A) proteinase K (0.1 unit mL⁻¹, open circles; 0.01 unit mL⁻¹, closed triangles; 0.001 unit mL⁻¹, closed circles), (B) trypsin (4 unit mL⁻¹, open circles; 0.8 unit mL⁻¹, closed triangles; 0.1 unit mL⁻¹, closed circles), (C) papain (4 unit mL⁻¹, open circles; 0.8 unit mL⁻¹, closed triangles; 0.1 unit mL⁻¹, closed circles), and (D) elastase (8 unit mL⁻¹, open circles; 4 unit mL⁻¹, closed triangles; 0.8 unit mL⁻¹, closed circles).
In addition, the FP approached a unique value of the mixture of TMR-peptides hydrolyzed by the particular protease, which was much higher than the value of TMR itself of −0.03. On the other hand, any significant changes in fluorescence intensity were not observed at the end of the proteolysis reactions (data not shown), which reconfirmed appropriate labeling of the TMR-α-casein for the FP-based assays.

3.4. On-chip protease assay

Prior to the assay transfer to the microfluidic chip format, attempts were made to reduce or eliminate nonspecific binding of the proteins, since the microchannel presents sticking problems due to the high ratio of its surface area to volume. Indeed, when TMR-α-casein and enzymes in regular assay buffers were allowed to flow from the inlet reservoirs through the main channel, the molecules appeared to stick to the surface, causing clogging of the microchannel, as determined by no fluorescence observed at the detection point. Therefore, various components including detergents such as Brij-35 and Triton X-100, and surface coating reagents such as BSA and PEG were added to the assay buffer but did not show any effect on the clogging problem. On the other hand, 5% DMSO was found to be effective in preventing clogging of the microchannel. Since 5% DMSO had no effect on protease activities (data not shown), the assay buffers were supplemented with 5% DMSO. In addition, there was no significant drift but <2% fluctuation in fluorescence intensity when data were collected every 0.1 s for at least 600 s for TMR-α-casein alone flowing through the microchannel.

For the on-chip protease assays, changes in FP would be observed with the microfluidic chip as shown in Fig. 1, when TMR-α-casein and protease added in the inlet reservoirs flow through the main channel, with the assumption that the two molecules mix and react before reaching to the detection point. Based on the chip dimensions, the on-chip mixing of the inlet flows perpendicular to the flow direction was first analyzed. Since the fluid flow in microfluidic channels is typically characterized as laminar, fluid streams parallel to each other would only mix through convective and molecular diffusion. The analytical solutions of diffusion equations were thus employed to perform mixing simulations [24]. The diffusion coefficient for casein was approximated to be 80 μm² s⁻¹ according to the report on the linear relationship of the diffusion coefficient to the molecular weight [27]. When the concentration profile was analyzed with respect to time, the casein flow diffused completely across the channel in 7.4 s, which was confirmed by examining fluorescence images at the detection point using the fluorescence microscope unit of the assembled microfluidic fluorescence analyzer reported previously [23]. The fluid flow rate required for protease reaction detectable with our optical set-up was then determined by monitoring FP changes of TMR-α-casein treated with proteinase K on chip. When 5 units mL⁻¹ of the protease and 2 μM TMR-α-casein added to the inlet reservoirs were driven to the main channel, the degree of TMR-α-casein degradation monitored by changes in FP (ΔFP) at the detection point increased dramatically at −20.7 kPa at which the flow rate was 4.1 mm s⁻¹ and the delivery time to the detection point was 7.8 s.

Fig. 5. Relationship between the fluid flow rates and proteolysis on chip. The flow rates in the microchannel were measured by monitoring fluorescence intensity of TMR-α-casein and plotted as a function of pressure. Proteolysis of TMR-α-casein was monitored by detecting FP of TMR-α-casein in the continuously flowing state on chip, where 2 μM of substrate and 5 units mL⁻¹ of proteinase K were added to the inlet reservoirs. The FP values were obtained at the detection point of the main channel by the setup shown in Fig. 2 with an optical chopper inserted, subtracted from that of the substrate only, and these ΔFP values (open circles) in addition to the corresponding flow rates (closed circles) are presented as a function of pressure.

The developed protease assay was finally transferred on chip by adding TMR-α-casein to the substrate reservoir and proteases to the enzyme reservoir. Since each protease was assayed in a specific buffer, the offset was determined prior to each experiment. FP values of the TMR-α-casein were initially between 0.28 and 0.30 in the absence of proteases, and decreased with increasing enzyme concentrations for all four proteases with both 17.2 (Fig. 6) and 2 μM (Fig. 7) of TMR-α-casein. The relat-
tionship between the amount of enzyme added and the change in the FP value was linear at low enzyme levels, indicating that the assay could be used to quantitatively estimate protease activity on unknown samples, provided that one stays within this linear range. As expected, a lower concentration of the substrate concentrations were closely similar for both substrate concentrations, thus facilitating calibration, and the microfluidic device is well-suited for analyzing minute samples very rapidly, our proposed microfluidic device based on FP would serve as an ideal tool for time-sensitive enzymatic assays and inhibitor screening with little modifications.

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