Microfluidic three-dimensional hydrodynamic flow focusing for the rapid protein concentration analysis

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A simple microfluidic 3D hydrodynamic flow focusing device has been developed and demonstrated quantitative determinations of quantum dot 525 with antibody (QD525-antibody) and hemagglutinin epitope tagged MAX (HA-MAX) protein concentrations. This device had a step depth cross junction structure at a hydrodynamic flow focusing point at which the analyte stream was flowed into a main detection channel and pinched not only horizontally but also vertically by two sheath streams. As a result, a triangular cross-sectional flow profile of the analyte stream was formed and the laser was focused on the top of the triangular shaped analyte stream. Since the detection volume was smaller than the radius of laser spot, a photon burst histogram showed Gaussian distribution, which was necessary for the quantitative analysis of protein concentration. By using this approach, a linear concentration curve of QD525-antibody down to 10 pM was demonstrated. In addition, the concentration of HA-MAX protein in HEK293 cell lysate was determined as 0.283 ± 0.015 nM. This approach requires for only 1 min determining protein concentration. As the best of our knowledge, this is the first time to determinate protein concentration by using single molecule detection techniques.

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INTRODUCTION

Over the past decade, single molecule detection (SMD) techniques have been incorporated with microfluidics systems^{1–3} and studied for single molecule detections for DNA and fluorescent dyes.^{4–6} For instance, DNA fragment sizing was demonstrated by using fluorescent signal detection in fused silica microfluidic channels and a high numerical aperture (NA) microscope objective lens.^{7–9} DNAs were stained with the dimeric fluorescent intercalating dyes and electrokinetically flowed into the detection channel. Whenever DNA molecules passed through the detection spot, fluorescent photon was detected by the avalanche photo diode (APD). Since long DNA fragments were able to carry more fluorescent tags, length of DNAs could be identified by their fluorescent intensities from Gaussian photon histogram. Recently, these microfluidic SMD techniques have been expanded to the protein detection in cell lysate solutions.^{10,11} Although there were some successful single protein detections, fused silica based microfluidic device had difficulty to characterize protein concentration because of the fluctuation of electroosmotic flow (EOF) velocity. Proteins tend to adsorb on the surface of fused silica that caused surface charge instability.

The conventional 2D capillary flow focusing system with 50–200 μm width of pinched analyte flow has been developed for single molecular detection of fluorescent dye molecules.^{12–14}

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In these approaches, low NA objective lens and high power laser were used to form a large laser spot to cover the width of capillary. On the other hand, larger laser spot would be a cause of the target molecules bleaching because of the longer residual time in the laser spot. In case of cell lysate sample solution, the target molecule signals would be inhibited by the auto-fluorescent from other molecules contained in a large detection volume.

As a result, the photon burst histogram was disturbed from normal Gaussian profile and provided inaccurate concentration measurements. Recently, the capillary flow focusing technique was implemented to microfluidics and has detected single molecules and fluorescent dyes.^{15–19} The dimensions of analyte flow in microfluidic device were reduced to 20–50 μm , but detection volumes were not significantly reduced. Therefore, the auto-fluorescent was influenced to photon burst histograms.

So far, there have been no reports of protein concentration determination by using single molecule detection system. Accurate determination of protein concentration is important in cancer research to understand the complex process of development and progression. Also, the Myc/MAX protein complex is a transcription regulator that controls various physiological functions such as cell proliferation, differentiation, metabolism, and apoptosis.²⁰ More importantly, Myc/MAX complex is frequently upregulated in human cancer and plays a critical role in cancer development and progression.²⁰ Myc and MAX form a heterodimer and directly bind to specific DNA sequences to activate or repress gene transcription.²¹ The expression levels of Myc and MAX in cells are critical determinants for multiple cellular functions. Therefore, in the present study, we chose the MAX protein as a model to further develop a novel protein detection system. This is the first time to determine the protein concentration by counting each protein. In order to detect accurate protein concentration, normal Gaussian distribution of photon burst distribution is necessary.²² In this paper, we have developed simple 3D hydrodynamic focusing devices to overcome the current problems and improve the SMD accuracy for the protein concentration analysis. Our design can provide Gaussian photon burst distributions to determine the protein concentration by reducing significantly the detection volume.

EXPERIMENT

Microfluidic channel fabrication

Microfluidic devices were fabricated on polydimethylsiloxane (PDMS; Dow Corning, Midland, MI) substrate by standard micro-molding procedures.²³ Two lithographic processes were used to fabricate a mold of the shallow and deep channels on a silicon wafer separately. SU-8 2010 photoresist (Microchem Corp., Newton, MA) was spin-coated with the thickness of 10 μm on a silicon wafer and exposed by EV620 contact aligner. After the development process, the shallow channel was formed. Then, 50 μm thickness of SU-8 2050 (Microchem Corp., Newton, MA) was spin-coated on the smaller channel patterned silicon surface. The second mask was aligned with the alignment markers made by the first lithography process. At this process, the shallow channel was connected to the deep channels to form the step junction structure. After the exposure and development processes, the micro-mold for microfluidic devices was made on the silicon wafer. Then, uncured PDMS mixture was dispensed over the lithographically patterned silicon mold and cured at 65 °C for 2 h. Then, cured PDMS substrate was peeled off from the master silicon mold. The PDMS microchannel substrate was bonded to quartz cover slide glass (170 μm thickness) by oxygen plasma treatment for 30 s. Since most proteins are positively charged at pH 7, the PDMS channel was coated by polyethyleneimine (PEI), which is a positively charged polymer, to prevent protein absorption on channel wall. A schematic diagram of the 3D microfluidic flow focusing device was shown in Figure 1(a). This device mainly consisted of two different depths and widths of microchannels. The shallow channel (10 μm in depth and width) was connected to the deep sheath inlet channels (50 μm in depth and width) at the cross junction. Sheath and analyte streams were delivered into the main detection channel (50 μm in depth and width) by two separate syringe pumps (11 plus, Harvard Apparatus, Cambridge, MA) at various flow rates. A scanning electron microscope

FIG. 1. The schematic diagram of microfluidic channel. (a) Chip-level view of the 3D hydrodynamic focusing device. (b) The SEM image of the cross junction area (scale bar: $10\ \mu\text{m}$). (c) Experimental setup: syringe pumps delivered analyte and sheath flows to the microfluidic device. QDs were excited by 375 nm laser. Emitted fluorescent signals were detected by APD in photon burst detection system.

(SEM) image showed the sheath, the detection, and the analyte channel at the cross junction area (Figure 1(b)).

Photon burst detection system

The microfluidic device was placed on the Nikon 2000 Inverted microscope stage as shown in Figure 1(c). Analyte and sheath streams were delivered through their inlets and 375 nm laser (16 mW) was focused through $60\times$ water immersion objective lens at the top of the triangular shaped analyte stream, which was about $15\ \mu\text{m}$ above the bottom of the main channel. The radius of laser spot was calibrated to $1.1\ \mu\text{m}$, whenever analyte molecules passed through the detection spot; they were excited and emitted fluorescent photon bursts. For all detections, five sets of raw photon burst data were recorded for 10 s each. The recorded raw photon burst data were smoothened by fast Fourier transform (FFT) filter²⁴ and the individual photon burst peaks higher than the noise level, which was determined by the blank solution, were defined as single molecule peaks. These peaks were plotted on the photon burst histogram which indicated the number of target molecules detected.

Sample protein preparation

Quantum dot (QD)-conjugated anti-rabbit IgG secondary antibody was purchased from Invitrogen, Inc. In this solution, $1\ \mu\text{M}$ concentration of QD525-antibody was suspended in 1 M

betaine, 50 mM borate, pH 8.3 with 0.05% sodium azide solution. This solution was diluted with phosphate buffered saline (PBS) to a concentrations of 10 pM, 100 pM, and 1 nM. Hemagglutinin epitope tagged MAX (HA-MAX) expression plasmid or empty vector was transiently transfected in human embryonic kidney 293 (HEK-293) cells by using liposome. 24 h after transfection, the cells with or without HA-MAX expression were harvested and lysed in RIPA buffer (50 mM Tris-HCl, 1% NP-40, 150 mM NaCl, 1 mM EDTA plus protease inhibitors). After measuring protein concentration, the cell lysates containing 100 μ g total proteins were mixed in IP binding buffer (20 mM Hepes-KOH, 0.1 mM KCl, 2 mM MgCl₂, 15 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 10% glycerol). The HA-MAX protein was labeled with QD525-antibody through anti-HA rabbit polyclonal primary antibody and QD525-conjugated anti-rabbit IgG secondary antibody. The cell lysate from empty vector-transfected 293 cells that did not express HA-MAX was mixed with anti-HA polyclonal primary antibody plus QD525-conjugated anti-rabbit IgG secondary antibody and used as a control sample.

Confocal microscope imaging for characterization of 3D hydrodynamic flow focusing

The confocal fluorescent microscopy (Olympus FV1000) equipped with air-cooled 543 nm HeNe laser was used to characterize the 3D hydrodynamic flow focusing. Rhodamine B resolved in PBS solution was injected through the analyte inlet and deionized (DI) water was pumped through the sheath inlet to form the 3D flow focusing. The cross-sectional images were obtained at the 100 μ m apart from the cross junction with different analytes and sheath flow conditions. The confocal microscope had 60 $\times$ water immersion objective (NA = 1.2) and Z-stacked series of fluorescent images were taken at 0.53 μ m intervals. In addition, a top view and a side view of the analyte stream in the detection channel were also characterized.

RESULTS AND DISCUSSION

Characterization of analyte flow profile

In order to characterize the 3D hydrodynamic flow focusing of the analyte stream, the cross-sectional images were obtained by using the confocal microscope. 1 μ M concentration of Rhodamine B dissolved in PBS solution was induced into the analyte inlet and DI water was pumped through the sheath stream inlet. The analyte flow rate (V_{Analyte}) and the sheath flow rate (V_{Sheath}) were independently controlled by two syringe pumps. The V_{Analyte} was set to 1 μ l/min and the V_{Sheath} was also controlled from 10 μ l/min to 160 μ l/min. Also, the cross sectional profiles were characterized at the different combinations of V_{Analyte} and V_{Sheath} (data were not shown). Figure 2(a) showed the cross sectional view, top view, and side view of fluorescent images of the analyte stream at the flow condition of V_{Analyte} : 1 μ l/min and to V_{sheath} : 60 μ l/min. Same as other flow conditions, the analyte stream was straight and uniform for 0.1 mm from the cross junction. Cross sectional images of the analyte stream with different flow conditions of analyte and sheath streams were shown at Figure 2(b). From these images, it was obvious that the height of the analyte stream was reduced by increasing V_{sheath} , and the cross section of the analyte stream was generally deformed to a triangular shape. Because the depth of sheath channels was deeper than that of the analyte outlet channel, the analyte stream was pressured from three directions, i.e., top to down, left to right, and right to left as shown in Figure 2(a). As shown in Figure 2(b), the analyte stream showed the large cross sectional area at the lower sheath flow rate. The cross section with low sheath flow rate caused the distortion of photon burst histograms because the analyte stream was larger than that of the laser spot as described earlier. Also, there would be high possibility that multiple analytes pass through the detection spot simultaneously. In addition, the flat shaped analyte streams were also observed at the higher sheath flow rate. Although the vertical resolution was improved, the analyte stream was extended to x -direction and the width of analyte stream was still larger than laser spot. Moreover, higher flow velocities were avoided due to the limitation of sampling frequency of APD. By considering these two factors, the flow rate was optimized $V_{\text{Analyte}}:V_{\text{Sheath}} = 1:60$ (μ l/min) and used for the single molecule photon detections. Then, QD525-antibody in DI water was pumped through the analyte inlet and the laser was

FIG. 2. Analyzing the rhodamine B flow profile using confocal microscope. (a) The concept of 3D focusing mechanism. (b) The cross-sectional profiles of rhodamine B flow with different analytes and sheath flow rate (white dotted line: the wall of the channel).

focused at the top of the triangular shaped analyte stream. Figure 3(a) showed that the photon bursts which were obtained from QD525-antibody sample solution for 10 s period (data shown in 20 μs interval). Since the detection time was 10 s, constant velocities were obtained.²⁵ These photon bursts were correlated to obtain auto-correlation curve that was fitted by using non-linear fitting method,^{26–28} as shown in Figure 3(b). The width of analyte stream and effective detection volume were calculated from the correlation curve fitting. The calculated width of analyte was 0.3 μm , and it was smaller than calibrated laser beam diameter (1.1 μm). This result indicated that the analyte flow was completely filled inside the laser beam path. The effective detection volume was estimated as 1.7 nl and this condition was used for all detections (Figure 3(c)).

Quantitative detection of QD525-antibody

QD525-antibody analyte samples with different concentrations were examined using the 3D hydrodynamic flow focusing microfluidic device. The original QD525-antibody solution with 1 μM was diluted $1/10^3$ (1 nM), $1/10^4$ (100 pM), and $1/10^5$ (10 pM) times via PBS solution and these three samples were characterized with the flow condition of 1 $\mu\text{l/min}$ (V_{Analyte}) and

FIG. 3. The effective volume of analyte stream. (a) Photon bursts graph as a function of time obtained from QD525 in 20 μ s interval. (b) Fluorescent correlation fitting. (c) The effective volume area compared with calibrated laser spot (green square: the calibrated laser spot, white dotted line: the wall of the channel).

60 μ l/min (V_{Sheath}). Photon bursts for each QD525-antibody concentration were shown in Figures 4(a), 4(c), and 4(e) in 20 μ s interval. It is obvious that more photon bursts were detected by increasing analyte concentrations. After the FFT filtering with 1 μ s bin time, photon counts were normalized by 100 counts to 1.0. The histogram of normalized photon bursts were plotted as shown in Figures 4(b), 4(d), and 4(f). The 1 μ s bin time was determined by analyte residual time calculated from how long it took for analyte to pass through the detection volume. In these histograms, there were two distribution peaks. Because the first peaks showed Poisson distribution, these were defined as noise distributions from detection equipment and background. The second peaks in the histograms were defined as single molecule signals from QD525-antibodies due to their Gaussian distribution. In this histogram, the positions of secondary peaks were shifted because the background noise levels increased as following QD525-antibody concentration increase. The numbers of QD525-antibodies in the second peaks were counted and averaged for each result, then plotted as bar graphs with standard deviation as shown in Figure 4(g). It clearly showed that the numbers of detected QD525s were linearly increased with small standard deviation. Based on detected numbers of QD525-antibody, detection volume, and flow rate, the concentrations of QD525-antibody were calculated as 0.971 ± 0.024 nM, 131 ± 40 pM and 11 ± 5 pM for 1 nM, 100 pM, and 10 pM QD525-antibody solutions, respectively. These results indicate that our system works properly to determine the protein concentrations since the calculated concentrations from obtained data are closed to original concentrations.

Quantitative detection of HA-MAX

Subsequently, the concentration of HA-MAX proteins in cell lysate solutions was also determined. 1 nM QD525-antibodies were hybridized to anti-HA rabbit polyclonal antibody

FIG. 4. QD detection with different concentrations. (a) Photon burst signal of QD at 10 pM in 20 μ s interval. (b) Histogram of QD at 10 pM. (c) Photon burst signal of QD at 100 pM in 20 μ s interval. (d) Histogram of QD at 100 pM. (e) Photon burst signal of QD at 1 nM in 20 μ s interval. (f) Histogram of QD at 1 nM. (g) The quantified graph of the number of detected QD with different concentrations.

FIG. 5. Results of HA-MAX detection. (a) Photon burst signal profile of vector in $20\ \mu\text{s}$ interval. (b) Histogram of Vector. (c) Photon burst signal profile of HA-MAX in $20\ \mu\text{s}$ interval. (d) Histogram of HA-MAX. (e) Western blot result of the same samples.

specifically binds to HA tag of HA-MAX proteins. Thus, QD525-conjugated rabbit IgG secondary antibody recognize HA-MAX by binding anti-HA antibody. As a result, multiple QD525s could bind to HA-MAX proteins. As a first step, HEK293 cells without HA-MAX expression were lysed and characterized as a control. In this sample, there was no HA-MAX protein available for anti-HA antibody to recognize. Cell lysate solution mixed with QD525s-conjugated secondary antibody and PBS solution were pumped through the analyte and sheath inlets, respectively. Photon bursts for each sample were detected 5 times for 10 s. The typical photon bursts obtained from the control sample were shown in Figure 5(a) in $20\ \mu\text{s}$ interval. These peaks were from single QD525-antibodies and the photon burst histogram was plotted as shown in Figure 5(b). This histogram showed similar distribution compared with the one in Figure 4(f) that contained a single Gaussian distribution. The number of detected QD525-antibodies in the Gaussian distribution was 958. HA-MAX expressed HEK293 lysate were hybridized with anti-HA-antibody and QD525s-conjugated secondary antibody for labeling. Figure 5(c) showed the photon bursts of the HA-MAX expressed sample as a function of time (in $20\ \mu\text{s}$ interval). Obviously, there was the photon burst that had higher photon counts than those in Figure 5(a) because HA-MAX protein carried more than single QD525s. Individual photon bursts were plotted in the photon burst histogram as shown in Figure 5(d). In this histogram, two obvious Gaussian peaks were obtained. The first Gaussian peak was concluded as unbound QD525-antibodies as proven in Figures 5(b) and 4. The second peak concluded as HA-MAX protein because HA-MAX protein had more than one QD525s. In this histogram, numbers of HA-MAX and unbound QD525 were counted as 347 and 674, respectively. The concentration of HA-MAX was determined as $0.283 \pm 0.015\ \text{nM}$ by considering detected events, detection volume, and flow rate. This process only requires for 1 min. The expression of HA-MAX was also characterized by the conventional western blot, which required for 24 h of biochemical processes, as shown in Figure 5(e). From the result of western blot, HA-MAX expression could be observed, but it did not provide quantitative information of HA-MAX concentration.

CONCLUSION

We have developed the microfluidic 3D hydrodynamic flow focusing device and determined the concentrations of QD525-antibodies and QD525/HA-MAX by using the confocal single molecule detection system. The 3D hydrodynamic flow focusing could compress an analyte stream and formed a triangular cross sectional profile with sharp top angle, which could not be achieved by the conventional flow focusing methods. Since the width of the analyte flow was compressed three dimensionally to $0.3\ \mu\text{m}$ at the top of triangular shaped analyte stream by the sheath flow, it could be filled inside of laser beam path. As a result, Gaussian photon burst histograms were obtained for each analyte. The number of analytes obtained from Gaussian photon burst histograms, detection volume and flow rate, and the concentration of analyte was

determined. Currently, it is very difficult to determine the concentration of proteins directly from biological sample such as cancer cell lysate. This approach provided a novel method to determine the concentration of target proteins that requires only 1 min analysis time for each sample, which is greatly improved by comparing with conventional IP/western blot or liquid chromatography.

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