Sub-nanomolar detection of thrombin activity on a microfluidic chip

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Bioluminescence resonance energy transfer (BRET) is a form of Förster resonance energy transfer. BRET has been shown to support lower limits of detection than fluorescence resonance energy transfer (FRET) but, unlike FRET, has not been widely implemented on microfluidic devices for bioanalytical sensing. We recently reported a microscope-based microfluidic system for BRET-based biosensing, using a hybrid, high quantum-efficiency, form of BRET chemistry. This paper reports the first optical fiber-based system for BRET detection on a microfluidic chip, capable of quantifying photon emissions from the low quantum-efficiency BRET² system. We investigated the effects of varying core diameter and numerical aperture of optical fibers, as well as varying microfluidic channel design and measurement conditions. We optimized the set-up in order to maximize photon counts and minimize the response time. The optimized conditions supported measurement of thrombin activity, with a limit of detection of 20 pM, which is lower than the microscope-based system and more than 20 times lower than concentrations reported to occur in plasma clots. © 2014 AIP Publishing LLC.

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I. INTRODUCTION

Compared to conventional technologies, microfluidics offers several advantages such as reduced reagent consumption, faster reaction rates, shorter analysis times, and amenability to automation and mass production. Förster resonance energy transfer, which is a ratiometric technique, has been a popular optical detection technique in the microfluidics domain.¹ Advantages of Förster resonance energy transfer over non-ratiometric fluorescence detection techniques include the elimination of signal variations due to sample volume variations, signal decay, or assay conditions.

Bioluminescence-resonance energy transfer (BRET) is a form of Förster resonance energy transfer whereby non-radiative energy is transferred from a bioluminescent donor to a ground-state fluorescent acceptor.² In a BRET system, the donor requires the addition of a substrate to initiate the bioluminescent emission. Energy is transferred to the acceptor if it is close enough to the donor, i.e., typically within 10 nm. Renilla luciferase (RLuc) is probably the most commonly used donor and can be excited by the oxidation of either naturally occurring coelenterazine (native CLZ) or synthetic analogues, for example, coelenterazine h (CLZh) or coelenterazine 400a (CLZ400a). Yellow or green fluorescent proteins (YFP or GFP) are often used as acceptors. Specific combinations of donor, substrate, and acceptor result in different versions of

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BRET with different characteristics. For example, BRET¹ incorporates RLuc, YFP and CLZh and BRET² comprises RLuc and GFP² with CLZ400a.³

We have previously shown that in a static microplate assay, BRET offers lower limits of detection and greater sensitivity than FRET.³ These advantages arise, in part, because BRET does not require an external light source and is not impacted by photo bleaching, autofluorescence, light scattering, photoisomerisation of the donor moiety, or photodamage.⁴ Furthermore, the elimination of an external light source reduces background noise, enabling lower limits of detection. These aspects of BRET make it potentially compatible with microfluidic-based detection technologies but, so far, detection of BRET has generally been limited to either imaging systems or static microplate assays.^{5–10}

BRET² is ideally matched to the scale of many protein interactions and rearrangements due to its relatively long Förster distance but has relatively low luminance arising from the low quantum yield of the substrate oxidation.¹¹ Recently, we reported a microscope-based microfluidic system for BRET-based biosensing,¹² using a hybrid high quantum-efficiency form of BRET chemistry (BRET^H) to detect thrombin activity. While the BRET² system consists of the acceptor GFP² ($\lambda_{em} = \sim 510$ nm) and the donor RLuc, with CLZ400a substrate ($\lambda_{em} = \sim 395$ nm), the BRET^H system combines the acceptor and donor domains of BRET²: i.e., GFP² ($\lambda_{em} = \sim 510$ nm) and RLuc, but with the BRET¹ substrate, native CLZ or CLZh ($\lambda_{em} = \sim 475$ nm). Thus the spectral separation of BRET² is ~ 115 nm while that of the BRET^H is only ~ 35 nm.^{3,12} So although the BRET^H system produces more light, it is inferior to the BRET² system not only in Förster distance but spectral separation. It is expected that BRET² is more sensitive to BRET^H on that basis, even though BRET² produces two orders of magnitude less light than BRET^H does.

To enhance the optical signal strength in order to allow use of BRET², we made two changes to the design of the detection system. First, we increased the volume of reaction that is optically sampled by incorporating a cylindrical chamber (“BRET reaction chamber”), perpendicular to the common direction of flow with a total volume of $2\pi \mu\text{l}$. Second, we replaced the microscope, which optically sampled a volume of ≈ 6 nl, with an optical fiber, arranged so as to collect light from the entire BRET reaction chamber. There have been numerous reports describing the use of optical fibres for fluorescence, chemiluminescence, or bioluminescence sensing without or with microfluidic chips.^{13–17} However, there are few reports of research into the use of optical fibres for bioluminescence resonance energy transfer detection on a microfluidic chip. The use of an optical fiber for light harvesting increases the photon collection efficiency compared to a microscopy¹² and markedly simplifies the optics, paving the way for multiplexed detection.

Even with these improvements, it does not warrant that we would be successful in detecting BRET² on a microfluidic chip. So the sensible approach would be to start the optimization with a system that is well known to produce sufficient light, which is the BRET^H in this case. Accordingly, using BRET^H, we studied the effects of optical fiber core diameter and numerical aperture on the BRET signal and optimised, the channel design, flow rate and mixing to give the best combination of signal strength and response time. We then switched to the BRET² system for thrombin protease assays. In the previous work, detection of BRET² on microfluidic chip was not possible due to the combination of low luminescence and low photon capture efficiency. Here, thanks to system optimisation in both optical fiber and microfluidic chip, we were able to detect BRET², which has a quantum yield much smaller than BRET^H, on the chip. To the best of our knowledge, this is the first time BRET² has been realised on a microfluidic chip. We obtained a limit of detection of 20 pM for thrombin, which is lower than for the microscope-based system.

II. EXPERIMENTAL

A. Materials

Coelenterazine h and coelenterazine 400a were purchased from Biosynth, Switzerland. Thrombin was purchased from Amersham Biosciences, USA. Ethanol, phosphate buffer saline (PBS), Tris, and EDTA were all purchased from Sigma Aldrich.

Two BRET systems were used in this study, namely, hybrid BRET (BRET^H)¹² and BRET². The two systems have the same BRET construct. The donor and acceptor of the BRET sensor were linked by a peptide sequence containing the thrombin cleavage site (LQGS^LVPR↓GSLQ (RG)) (GFP²-RG-RLuc) to make the thrombin biosensor.³ They were expressed in *Escherichia coli* and purified as reported previously.³ The BRET^H system, with coelenterazine h, was used for initial characterisation and comparison of the microfluidic chips and the optical detection system.

For subsequent protease assays, we used the BRET² system, which has the same BRET sensor construct but, instead of coelenterazine h, uses coelenterazine 400a as the bioluminescence substrate. The BRET² system is reported to produce much less light than the BRET^H system due to the fact that quantum yield of coelenterazine 400a is two order of magnitude smaller than that of coelenterazine h.¹⁸ However, because of its large Förster distance and spectral separation,¹¹ BRET² is ideally matched to the size of the thrombin biosensor. Accordingly, the BRET²-based thrombin sensor is a good option for validating the microfluidic setup and optical detection system for high sensitivity biosensing.

B. Setup and device

The complete system consists of a microfluidic chip, a syringe pump, and a fiber-based photon collection unit (Figure 1). The photon collection unit interfaces to the microfluidic chip with a flat-cleaved multimode optical fiber. We tested five optical fibers with different combinations of core diameter and numerical aperture (Thorlabs, USA). The flat-cleaved end of the fiber was fixed by an x, y, z micromanipulator. The other end of the fiber was terminated by an FC-connector and plugged into a fiber adaptor block (A10037, Hamamatsu, Japan) connected to a dichroic block (A10034-01, Hamamatsu, Japan). The dichroic block splits the emission into a band with wavelength less than 505 nm (blue band) and a band with wavelength greater than 505 nm (green band). The blue and green bands were narrowed with two band pass filter blocks (A10033-90, Hamamatsu, Japan and Omega filters, USA) to retain bands with wavelength (475 nm, 30 nm) or (410 nm, 80 nm) and (515 nm, 30 nm) for blue and green, respectively. The blue and green channels were sampled independently using a pair of dedicated photomultiplier tubes (PMT) (H7421-40, Hamamatsu, Japan) with associated counting units and power supply units. Data acquisition from both PMTs was controlled by the same counting software so as to present simultaneous blue and green light traces. A computer-controlled syringe pump (Harvard Apparatus, Boston, USA) actuated two syringes, one delivering coelenterazine h substrate and the other the thrombin biosensor, with or without pre-incubation with thrombin, to the inlets of the microfluidic chip. The syringe pump was controlled by Elite Method Manager (Harvard Apparatus, Boston, USA). To avoid background light interference, the setup was enclosed in a light shielding box during data acquisition.

FIG. 1. Optical setup for bioluminescent detection on a microfluidic chip with a multimode optical fiber. Five types of optical fibers, differing in core diameter and NA, were used. Movement of the optical fiber was controlled by a micromanipulator, (a) schematic diagram of the cross section of reaction chamber and (b) photo of the microfluidic chip in operation.

For an anisotropic source like a fluorophore, the dimensionless collection efficiency of fiber η depends on the numerical aperture (NA) of the fiber and the refractive index of the medium in front of the fiber (water), i.e., $\eta = f(\text{NA}, n)$ (Figure 1, inset (a)),¹⁹

$$\text{NA} = n \times \sin(\alpha) \quad \text{or} \quad \alpha = \sin^{-1}\left(\frac{\text{NA}}{n}\right), \quad (1)$$

where NA is the numerical aperture of the fiber and n is refractive index of medium of sampling in front of the fiber

$$n = 1.33 \text{ for water.} \quad (2)$$

Solid angle equivalent to a cone with apex angle of 2α ,

$$\Omega = 2\pi(1 - \cos \alpha). \quad (3)$$

A BRET sensor emits light anisotropically, i.e., with a solid angle of 4π .

The collection efficiency is

$$\eta = \frac{1}{2}(1 - \cos \alpha) = \frac{1}{2} \left[1 - \sqrt{1 - \left(\frac{\text{NA}}{n}\right)^2} \right]. \quad (4)$$

As an example, from Eq. (4), when a fiber with a NA of 0.48 is used, the corresponding collection efficiency would be 3.37%. To prevent heavy Fresnel loss at the glass/air/fiber interface, which is about 4% of total signal transmission,²⁰ index matching oil was applied between the microchannel and the fiber tip. The use of index matching oil not only compensates for the Fresnel loss but also prevents physical contact between the polished fiber tip and the cover glass which could scratch the surface of the fiber tip.

Four microfluidic chip designs were used in this study (Figure 2). All channels were $200 \mu\text{m}$ wide $\times$ $35 \mu\text{m}$ high and were fabricated from polydimethylsiloxane (PDMS) using standard photolithography. The chip design was completed in a commercial drawing package (Adobe Illustrator CS4), and the design pattern was printed on a transparency mask (5080 dpi, Allardice). Master patterns of the microfluidic devices were fabricated using a laminar dry film resist (Shipley 5038). Multiple layers of resist were laminated at 113°C onto a substrate of polished stainless steel. The channels were lithographically patterned using a collimated UV source ($\lambda = 350\text{--}450 \text{ nm}$) operated at 20 mJ/cm^2 and a transparency film mask. After exposure, the test pattern was developed in a 20% Na_2CO_3 solution. The pattern in the resist was replicated as a nickel shim using an initial sputter deposition of 100 nm Ni followed by electroplating to a thickness of $150 \mu\text{m}$. A 10/1 (w/w) mixture of PDMS and curing agent was poured over the

FIG. 2. Four microfluidic geometries used in this study. Device 1 does not have a common channel while devices 2 and 3 have a 9 and 18 mm common channel length, respectively. Device 4 is equipped with a serpentine common channel with a total length of 28 mm. The microfluidic channels have the same cross section of $35 \mu\text{m} \times 200 \mu\text{m}$. The reaction chambers are formed at the end of the channels and have a diameter of 2 mm and height of 2 mm.

shim and cured on a hot plate at 100 °C for 15 min. The thickness of the chip was controlled by 2 mm-high posts. The device was peeled off the shim and cut to the desired size. A reaction chamber (diameter 2 mm $\times$ height 2 mm) was formed at the end of the channel using a punch and sealed with a lid containing a drainage channel to allow escape of reacted product (Figure 1, inset (b)). The PDMS chip was exposed to air plasma for two minutes and immediately sealed with a glass slide (170 μ m, Matsunami, Japan). The residence times for the four devices based on flow rates ranging from 50 μ l h⁻¹ to 400 μ l h⁻¹ are summarised in Table I.

C. Methods

1. Optical fiber optimisation

Device number 1 (Figure 2) was used to compare the light collection properties of five different optical fibers. The BRET signal collected in the presence of a mixture of coelenterazine h and TE (10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA) buffer was defined as the background noise level. The BRET signal was defined as the photon count rate, when coelenterazine h substrate and the thrombin biosensor were pumped simultaneously onto the chip. The purified thrombin sensor was prepared in TE buffer at a concentration of 1 μ M. Coelenterazine h was used at a concentration of 5 μ M. 100 μ l each of the TE buffer, with or without biosensor and/or thrombin, and the substrate solution were loaded into two syringes (Insulin syringes, Japan) and connected to the two inlets of the chip by silicone tubes (Gecko optical, Australia). The flow rate was fixed at 200 μ l h⁻¹ for each syringe and the PMT gate time was fixed at 500 ms.

2. Microfluidic geometry and flow rate

To study the effects of device design on signal stabilisation time, we tested four different chip geometries (Figure 2). For these experiments, we used the 1 mm diameter, NA = 0.48 fiber, which gives the highest optical signal collection. Protein and substrate solutions were the same as for optic fiber optimisation and flow rates were varied from 50 to 400 μ l h⁻¹.

3. Thrombin assay

A 1 unit (U)/ μ l stock solution of thrombin was prepared in 1 $\times$ phosphate buffer saline (PBS). The thrombin biosensor was used with coelenterazine 400a substrate, i.e., in BRET² mode. The concentrations of the thrombin biosensor and substrate were 1 μ M and 12.5 μ M, respectively. The rationale for choosing 1 μ M for the thrombin biosensor was twofold. First, it enabled direct comparison with our previous studies using a plate reader to detect thrombin with the same biosensors³ and thence comparison to Zhang's studies using a FRET based probe.²¹ Second, we have found 1 μ M to be a convenient compromise between generating sufficient luminance and efficient use of the bioprobe, with excellent sensitivity. Increasing concentrations of thrombin were added to the purified thrombin biosensor and incubated at 30 °C for 90 min.

D. Data acquisition and analysis

Signals from the blue and green channels were recorded simultaneously using control software C8855-01 (Hamamatsu, Japan). The baseline was recorded for 30 s then the syringe pump

TABLE I. Residence times for the four devices used in this study at different flow rates from 50 to 400 μ l h⁻¹.

Flow rate (μ l h ⁻¹)	Residence times (s)				
	50	100	200	300	400
Device 1	226.2	113.1	56.5	37.7	28.3
Device 2	228.4	114.2	57.1	38.1	28.6
Device 3	230.7	115.4	57.7	38.5	28.8
Device 4	233.3	116.6	58.3	38.9	29.2

was turned on, resulting in a total acquisition time of 150–500 s. The last 50 s of the signal trace from each channel was averaged and the background noise subtracted, resulting in the effective signals of RLuc and GFP² for both BRET^H and BRET², respectively. For the thrombin assay, the BRET² ratio was defined as the ratio of the GFP² to the RLuc effective signal. The normalized BRET² ratio was obtained by normalizing the BRET² ratio recorded with thrombin against the BRET² ratio in the absence of thrombin. The normalized BRET² ratio was plotted against thrombin concentration, and limits of detection were calculated using these data (blank signal + 3 × standard deviation of the blank). For optimization assay with BRET^H, data are plotted as average ± standard deviation (SD) from the last 50 s of signal from each channel from a single measurement. For thrombin assay, data are reported as mean ± SD from three measurements. All data analysis was carried out using GraphPad Prism (version 6 for Windows, Graphpad Software, California, USA).

III. RESULTS AND DISCUSSION

A. Effects of optical fibers on photon collection

The BRET signals emitted from the reaction chamber are transmitted to the PMTs by an optical fiber. We compared background and signal intensities for different optical fibers to find out which type gives the best signal to noise ratio in our setup. Small core fibers have good bending strength and can support small radii of curvature, which makes for easy integration with microfluidic systems. Larger core, high NA fibers are generally stiff, making it more difficult to integrate them into compact devices. On the other hand, they support more efficient light transfer and are better for lower level light sources. Consistent with these generalisations, the larger core diameter fibers collected up to three orders of magnitude more light than the smaller core fibers. Figure 3(a) plots the RLuc and GFP² collection power of five types of optical fiber for both background and BRET^H signals. The interaction of coelenterazine h with TE buffers gives very little background noise, i.e., less than 5 counts/gate. Considering the typical BRET^H signals when measured with thrombin sensor (e.g.), this level of background is very low. This leads to very high signal to background ratios for both RLuc and GFP² channels, especially for the larger core fibers (Figure 3(b)).

Apart from the collection efficiency, which is dictated by the NA, the core area of a fiber is also important to determine the light collection capacity of a fiber. Thus the larger the core and NA of an optical fibre, the more light the fiber can collect. Theoretically, a fiber of core diameter = 200 μm , NA = 0.48 can collect 8.7 times more light than a 100 μm , 0.22 NA fiber. For an optical fiber with the same numerical aperture, the core diameter decides the optical collection capacity. Thus, a 400 μm , 0.48 NA fiber should collect four times more light than the

FIG. 3. (a) Background and BRET^H signal of device 1 when varying optical fiber diameters. (b) Corresponding signal to background ratios calculated from (a). The same flow rate 200 $\mu\text{l h}^{-1}$ and integration gate time 500 ms were used for all experiments. For experiments to measure background signal, coelenterazine h at 5 μM and TE buffer were pumped into device 1. For experiments to measure BRET^H signal, thrombin biosensor at 1 μM prepared in TE buffer and coelenterazine h at 5 μM were used.

200 μm , 0.48 NA fiber. It is obvious that photon count rapidly increase with fiber core diameter and NA. However, the data do not precisely follow our theoretical calculation. The discrepancy could be due to possible non-uniform light emission from the reaction chamber. Alternatively, there might be minor angular or translational misalignments of the fiber surface with the reaction chamber, with more severe effects on the smaller core fibers. There are also different levels of bending losses with different fiber core diameters. Nonetheless, these results show that, in order to collect more photons and improve the signal to background ratios, fibers having the largest possible core diameters that can be integrated with the required microfluidic system should be used.

B. Effects of device design and flow rates on photon collection

Device design can affect the efficiency of substrate-sensor mixing and therefore photon generation. We compared photon collection power of the four device designs in Figure 2 at the same optical and integration time conditions. All devices rely entirely on diffusion between substrate and thrombin biosensor for bioluminescent reaction because, even at the highest flow rates, these devices still operate in the laminar flow regime with $\text{Re} \ll 100$.²² Here, diffusion happens both along channel as well as inside the reaction chamber. At the same time, the rate of the reaction mix being pushed out of the reaction chamber and the rate of decaying of the BRET signal of that mix also come into play. For device 1, there was a monotonic decrease in signal with increasing flow rate (Figure 4). We postulate at higher flow rates, the reaction mix is expelled from the device before mixing is complete since this device does not have the common channel. For devices 2 and 3, the strongest signal occurred at 100 $\mu\text{l h}^{-1}$ and decreased as the flow rate increases further. Again, we suggest that in this case, mixing was not complete by the time the reactants were expelled from the device.

The effect of channel length on diffusion can be easily observed in devices 1, 2, and 3 (Figure 4). In this regard, channel length of device 3 is longest thus the signal generated is highest due to better diffusion. Device 4 is unique in that signal strength rose strongly with increasing flow rate and, at flow rates of 100–400 $\mu\text{l h}^{-1}$ was the highest of device/flow rate conditions. It is postulated that device 4 has both the optimal channel length for diffusion and, at the same time, suitable flow rates so that decay in BRET signal can catch up with the rate the mix is expelled from the reaction chamber. As we were focusing on obtaining the highest signal possible with our system, we selected flow rate 400 $\mu\text{l h}^{-1}$ for further study.

C. Effects of device design and flow rates on stabilization time

The “settling” time of the device is the time it takes for the light signal to stabilise following the start of pumping. Settling time estimates the upper bound of the time it would take to achieve a new equilibrium, following introduction of a changed sample into the device.²³ For

FIG. 4. BRET^H signal collection, (a) for RLuc channel and (b) for GFP² channel, of four devices as in Figure 2, operating at five different flow rates when optical fiber with $d = 1 \text{ mm}$, $\text{NA} = 0.48$ was used. For these experiments, thrombin biosensor at 1 μM prepared in TE buffer and coelenterazine h at 5 μM were used. The integration gate time was kept constant at 500 ms.

some applications, it is an advantage for this time to be as short as possible. Here, we define the settling time as the time elapsed from initiation of pumping to when the raw RLuc and GFP² signals first fall within $\pm 5\%$ of their final values. In general, for all devices, the larger the flow rates, the shorter were the settling times (Figure 5). The stabilisation time was dependent on device design at small flow rates (below $200 \mu\text{l h}^{-1}$) but less so at higher flow rates. While reducing flow rate is cost-effective in terms of sparing reagents, it is not ideal for making rapid measurements. In order to have both high signals and fast-responses, we chose device 4 flowing at $400 \mu\text{l h}^{-1}$, for further experiments because it combines a short settling time (Figure 5) with the highest signal (Figure 4) and signal to noise ratios (Figure 3(b)).

D. On-chip comparisons of BRET^H and BRET² luminance

Since device 4 operating at $400 \mu\text{l h}^{-1}$ with, an optical fiber core diameter of 1 mm and NA of 0.48 gave us the best combination of signal strength and response time, we used this configuration to compare the light emissions using the same thrombin biosensor with both BRET^H and BRET² substrates (Figure 6). Using exactly the same system configuration, BRET^H generated 2 order of magnitude more light than BRET². As mentioned previously, even though BRET² produces much less light than BRET^H, as long as the amplitude of the signal is high enough to be recorded, BRET² offers much better spectral separation and Förster distance and therefore better limits of detection and sensitivity for the analyte. Additional options for increasing the signal strength would be to further enlarge the reaction chamber but this would also increase the residence time. Other possible approaches include: integrating a mirror on top of the reaction chamber to capture photons escaping from that surface, which would theoretically increase the light collected by 30%, albeit at the expense of a more complicated fabrication process; or replacing the native RLuc sequence with more stable RLuc2 or RLuc8 variants,²⁴ which have been shown to increase luminance by approximately 30–100 fold with only minor other effects on the system.²⁵

E. Thrombin protease assay

Having optimised the microfluidic system to obtain a reasonable BRET² signal, we tested its ability to support on-chip measurements of an end-point thrombin assay. Thrombin cleavage resulted in a separation of the RLuc donor and GFP² acceptor (Figure 7(b)), thus the GFP² signal is reduced dramatically while the RLuc signal increased marginally. While the donor RLuc still reacts with coelenterazine 400 a to produce luminescence, energy is no longer transferred to the GFP² acceptor. When 27–270 pM of thrombin was pre-incubated with the biosensor for 90

FIG. 5. Effects of device designs and flow rates on stabilisation time, i.e., time it takes for the photon signal to stabilise following the start of pumping. Similar to experiments in Figure 4, optical fiber with $d = 1 \text{ mm}$, $\text{NA} = 0.48$, and integration gate time 500 ms were also used. The BRET^H reaction was also employed in these experiments where thrombin biosensor at $1 \mu\text{M}$ prepared in TE buffer and coelenterazine h at $5 \mu\text{M}$ were pumped into the chips.

FIG. 6. BRET signal comparisons of BRET^H and BRET², (a) for RLuc channel and (b) for GFP² channel, of device 4. Optical fiber with $d = 1$ mm, $NA = 0.48$, integration gate time 500 ms and flow rate $400 \mu\text{l h}^{-1}$ were used in all experiments. Note the break on the y-axis. For BRET^H reaction, thrombin biosensor at $1 \mu\text{M}$ prepared in TE buffer and coelenterazine h at $5 \mu\text{M}$ were pumped into the chip. For BRET², thrombin biosensor and coelenterazine 400a substrate concentrations were $1 \mu\text{M}$ and $12.5 \mu\text{M}$, respectively.

min, the BRET² ratio decreased with increasing thrombin concentration, (Figures 7(a) and 7(b)) reflects increasing levels of thrombin biosensor cleavage. Based on the normalized BRET² ratio with thrombin concentration (Figure 7(c)), we calculated the detection limit (blank signal $+3 \times$ standard deviation of the blank) for thrombin as 19.8 pM . This is approximately ten-fold lower than the limit of detection (LOD) of 220 pM obtained using the BRET² reaction read in a commercial static microplate reader.³ We believe that the higher sensitivity observed here is probably due to the smaller detection volume ($6.28 \mu\text{l}$ versus $100 \mu\text{l}$ in the microplate), which minimises self-absorption effects, and the constant refreshment of the thrombin biosensor and substrate with the continuous removal of potentially inhibiting reaction products. The LOD is also approximately 1.5 fold lower than that recently obtained using the BRET^H system with a microscope¹² despite the lower luminance of the BRET² system and with the considerable

FIG. 7. (a) Time dependent BRET² ratio calculated for one set of thrombin assay from 0 pM (control) to 270 pM of thrombin. (b) BRET² ratio calibration curve, error bar represents standard deviation of three measurements. Inset show the mechanism of thrombin cleavage. (c) Normalized BRET² ratio, error bar represents standard deviation of three measurements.

advantages of simplicity and reduced cost of fiber-optics compared with a compound microscope.

We also note that the LOD of 19.8 pM for the fiber-based microfluidic setup used here is approximately 500 fold lower than reported in literature using comparable approaches. For example, an LOD of 10 nM LOD was reported for a microfluidic protease activity assay using fluorescence polarisation,²⁶ 6.2 nM on an electroluminescent-CCD microchip platform in conjunction with quantum dots²⁷ and 4.5 nM with a BODIPY fluorescence microplate assay.²⁸ The long pre-incubation time of 90 min, used here was designed to minimise experimental variation in the extent of cleavage among experimental in order to focus on the light generation and detection aspects. In the future, it would be interesting to perform the thrombin cleavage reaction, as well as light generation, in real-time on-chip.

IV. CONCLUSIONS

We have demonstrated the first ever detection and measurement of a BRET² signal in a continuous flow microfluidic chip. By varying fiber types, microchip designs, and other system parameters such as flow rates, we optimised the BRET signal for a system dead time of less than 75 s. The microfluidic chip was able to detect thrombin using the BRET² thrombin biosensor system with limit of detection less than 20 pM, several orders of magnitude better than unrelated methods. The sensitivity and LOD are also well within the range needed to measure physiological concentrations of thrombin in clots, as recently noted and eliminate the need for complex optics. This work opens the way to developing simple apparatus for BRET-based detection of a range of analytes. We believe it will enable further advances in speed, convenience, and sensitivity for point-of-care/point-of-use applications.

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