

Open Sandwich FRET Immunoassay of Estrogen Receptor β in a PDMS Microfluidic Channel

Hyejin Park, Moonkwon Lee, Gi Hun Seong, Jaebum Choo,* Eun Kyu Lee,[†]
Joong Yull Park,[‡] Sanghoon Lee,[‡] Kyeong-Hee Lee,[§] and Young-Wook Choi[§]

Department of Applied Chemistry, Hanyang University, Ansan 426-791, Korea. *E-mail: jbachoo@hanyang.ac.kr

[†]Department of Chemical Engineering, Hanyang University, Ansan 426-791, Korea

[‡]Department of Biomedical Engineering, Korea University, Seoul 136-701, Korea

[§]Fusion Technology Research Laboratory, KERI, Ansan 426-170, Korea

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Immunoassay is a fast and cost-effective protein detection method that can be applied to clinical diagnostics and biological research. Enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR), and protein array chips are the most widely used immunoassay techniques for the detection of a target protein.^{1,2} In all of these methods, however, antibodies or antigens should be immobilized on a solid substrate. As a result, they require a long assay time for complete immunoreaction between antigens and antibodies, being limited by the diffusion kinetics. Moreover, they also require several cycles of consecutive binding and washing steps to separate free reagents from binding reagents. To overcome the problem of slow immunoreactions in conventional immunoassay techniques, we recently reported a fast and sensitive one-step immunoassay of estrogen receptor β (ER β) using quantum dot (QD)-based fluorescence resonance energy transfer (FRET).³ To induce a strong FRET effect, QD was used as a fluorescence donor. FRET occurs when the electronic excitation energy of a donor chromophore is transferred to a nearby acceptor molecule *via* a dipole-dipole interaction between the donor-acceptor pair.^{4,5} Conventional fluorescence dyes, which cause an appreciable overlap between the emission band of the donor and the absorption band of the acceptor, have generally been used as a donor-acceptor set. However, QD provides significant advantages over conventional fluorescence dyes, including brighter fluorescence, resistance to photobleaching, and narrow emission bands, which makes it suitable as a sensitive fluorescence donor.⁶⁻⁸ As a result, QD reaches a longer distance to the acceptor than conventional fluorescence dyes because of their superior optical properties. Furthermore, the QD-based FRET immunoassay of ER β was faster (30-40 min of immunoassay time including incubation) than conventional immunoassay techniques because it does not need any solid-phase carriers and multiple washing steps for free reagent separations.³ In our previous work, the advantages of a QD-based FRET immunoassay in an open space were fully explored. Nonetheless, a faster reaction time and a smaller sample volume are still needed to apply this QD-based FRET immunoanalysis technique to clinical diagnostics.

In the present work, a lab-on-a-chip technique, combined

with the QD-based FRET immunoassay method, was used to develop a more sensitive and faster immunoanalysis technique. The lab-on-a-chip technique has several advantages over conventional bench-top techniques, such as minimal sample requirement, reduced reaction time, ease of use, improved product conversion, and reduced waste generation.⁹⁻¹¹ In particular, the reaction time can be greatly reduced to less than a few minutes if a properly designed channel to obtain highly efficient mixing is adopted. Furthermore, only a small reaction volume is required in the lab-on-a-chip analysis. An alligator-teeth-shaped poly(dimethylsiloxane) (PDMS) microfluidic channel was fabricated to obtain a fast immunoreaction between antibody and antigen under a flow condition. The detailed fabrication process for this PDMS channel has been reported elsewhere.¹²⁻¹⁵

ER β is one of the most important breast cancer markers that are closely related with cell proliferations.¹⁶ The anti-ER β monoclonal antibody (McAb, mouse ascites, IgM isotype; Sigma, St. Louis, MO, USA) was labeled on QD 565 using an antibody conjugation kit (Invitrogen, Eugene, OR, USA). In addition, the anti-ER β polyclonal antibody (PcAb, rabbit immune serum, IgG; Biogenex, San Ramon, CA, USA) was labeled with AF 568 protein labeling kits (Invitrogen, Eugene, OR, USA). The formation of an open sandwich immunocomplex and its energy transfer process from QD-McAb to AF-PcAb are illustrated in Figure 1(a).

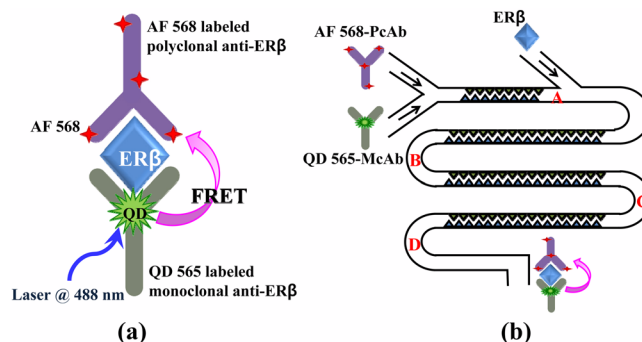


Figure 1. Schematic illustrations of (a) the FRET-based open sandwich immunoassay and (b) an alligator-teeth-shaped PDMS microfluidic channel for the FRET immunoassay. Each position (A to D) indicates a fluorescence measurement point.

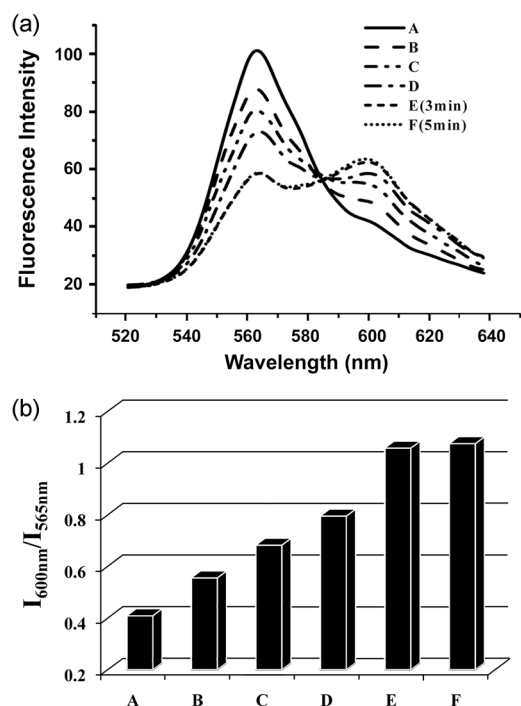


Figure 2. (a) Fluorescence emission spectra and (b) their relative fluorescence intensities measured at four different points along the channel with a constant flow rate of $2 \mu\text{L}/\text{min}$. Positions A, B, C, and D correspond to the fluorescence measurement points denoted in Figure 1. E and F indicate the fluorescence spectra at position D after 3 min and 5 min, respectively under stop-flow conditions.

An on-chip assay process using a lab-on-a-chip device is shown in Figure 1(b). While confluent streams of QD-McAb, AF-PcAb, and antigen traveled along the microfluidic channel, the transverse and vertical dispersions of the fluid occurred simultaneously through the upper and lower teeth. Fluorescence spectra in different channel positions were measured using a Leica TCS SP confocal fluorescence microscope. The QD 565 was excited by an Ar ion laser at 488 nm. The emission band of QD 565 was observed in the 530-590 nm range, and the emission band of AF 568 was simultaneously observed in the 590-640 nm range. All the spectra during the sandwich immunobinding process of the QD-McAb, AF-PcAb, and ER β were measured using the λ -scanning mode of the confocal laser scanning microscope. The emitted fluorescent spectrum was detected in the 520-640 nm range.

Stock solutions of QD-McAb and AF-PcAb ($0.5 \mu\text{M}$) were prepared in a PBS buffer solution (pH = 7.2). 100 nM ER β target antigen was also prepared for the FRET immunoassay in a microfluidic device. Figure 2(a) shows the fluorescence spectra measured at four different positions (A, B, C, and D) along the channel with a constant flow rate of $2 \mu\text{L}/\text{min}$. The fluorescence bands at 565 and 600 nm correspond to the emission bands of QD 565 and AF 568, respectively. The intensity of QD 565 decreased with increasing channel distance, while the intensity of AF 568 increased. This means that the FRET between the donor QD 565 and the

acceptor AF 568 successfully occurred as a result of the formation of the immunocomplex. The changes in the relative fluorescence intensity (I_{600}/I_{565}) along the channel distance for the formation of the sandwich immunocomplex are plotted in Figure 2(b). The continual increase of the relative intensity from A to D indicates that the immunoreaction has not been completed at channel position D because the intensity still increases after channel position D. To find an equilibrium point for the completion of the immunoreaction, a stop-flow measurement was performed. The flow of the solution was stopped and the fluorescence signal was measured at intervals of 1 min at position D. According to our measurements, the relative fluorescence intensity remained constant after 3 min.

In this work, a lab-on-a-chip-based FRET immunoassay technique for ER β , which is known as one of the important breast cancer markers, was developed. This novel immunoanalysis method requires only a tiny volume of sample (about $1.6 \mu\text{L}$) because the immunoreaction occurs in a narrow microfluidic channel. Furthermore, the analysis time is greatly reduced (3-5 min) compared with our previously reported results in open space (30-40 min) due to the high mixing efficiency of the alligator-teeth-shaped PDMS microfluidic channel. This conceptually new immunoassay technique is considered to be a very effective diagnostic tool for specific cancer markers.

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