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FRET-based homogeneous immunoassay on nanoparticle-based photonic crystal

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Abstract

Fluorescence resonance energy transfer (FRET) was tested in a photonics crystal (PC) nano structured array to the speed and sensitivity of a protein-based immunoassay. Forty nm carboxylated particles, conjugated with donor-labeled capture antibodies, were trapped by electrophoresis and used as a FRET energy-donor. The PC array can enhance the fluorescent excitation and emission by phase matching. To demonstrate a proof-of-concept of a FRET-homogenous assay on a PC chip, an immunoassay using a simple immunoglobulin G (IgG)-based reaction was performed. A standard curve was generated by testing two different antibody reactions time: 20 minutes and 1 minute. The results were compared directly to a FRET assay that used a modern, high sensitivity plate reader with a 96-well plate with 1 hour-reaction time. The limit of rabbit-IgG detection was 0.001 μg/mL and 0.1 μg/mL for 20 minute and 1 minute incubation times respectively. The sensitivities were 10^3 and 10 times better than a 96-wells plate-reader detection. The FRET on a PC-immuno-platform demonstrated its potential for implementing a facile, but effective, rapid and sensitive detection technology.

Keywords

Fluorescent resonance energy transfer; photonics crystal; immunoassay; nanoparticles

Introduction

Fluorescence resonance energy transfer (FRET) is a spectroscopic method involving non-radiative energy transfer from a fluorescent donor molecule to an acceptor molecule due to a dipole-dipole interaction [1]. The efficiency of energy transfer is dominated by the distance between the donor and acceptor [2]. Because the efficiency of the FRET varies sensitively with the change of distance between donor and acceptor, FRET has been widely employed in bioassays that rely on binding between the biological molecules, offering high sensitivity and specificity [3]. In addition, given that there is no need for separation and purification of biological molecules during an assay, FRET has been a favored format for homogeneous immunoassays [4-6] which offer a reduction in the false positive due to reduced background interference from non-specific binding of fluorescent labels to extraneous surfaces.

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To obtain high sensitivity from using FRET-based immunoassay in biosensors, it is critical to improve the efficiency of energy transfer between two different fluorescent dyes attached to the biological molecules by increasing spectral overlap and/or increasing the extinction coefficient of the acceptor [7]. However, in general, fluorescent dyes are susceptible to photobleaching and have wide emission spectra and narrow absorption spectral bands [8]. Quantum dots (QDs) [4,7,8] or up-conversion luminescent nano particles [9-11] have been considered as alternative luminescent labels due to their photochemical stability and high quantum yield, properties that can lead to robustness of a FRET-based biosensor as well as ultrahigh sensitivity once incorporated with the photonic crystal. In this study, we only tested the impact of a photonic crystal nanostructure for enhancement of FRET.

A FRET-based homogeneous immunoassay (HIA) on a photonic crystal (PCs) nanostructured array has been demonstrated for a generic immunoassay to detect immunoglobulin G (IgG). The PC-immunoplatform is able to boost the fluorescent signal from the ensuing immuno-fluoro-complex, leading to a high signal-to-noise ratio [12]. Nanoparticle-based IgG immobilization using an electrophoretic particle entrapment system can minimize the use of expensive biological reagents and improve total assay time compared to other immobilization methods [12-16]. From previous studies, PCs have shown their superiority in various kinds of fluorescence-based immuno- and DNA-assays in terms of sensitivity [14-17]. The novel use of FRET on a PC nanostructured array offsets the inherent disadvantages of fluorescent dyes and simultaneously provides a simple, rapid, but sensitive method for rapid, point-of-use detection of markers of diseases.

Materials and methods

Materials

The 40 nm-fluorescent carboxylated polystyrene (PS) nanoparticles (F-8789; ex: 660 nm/em: 680 nm) were purchased from Invitrogen (Carlsbad, CA). In our case, the fluorescence of the particles did not play a role in the FRET assay as discussed later; these particular particles were simply a suitable size and readily available. Goat-anti-Rabbit IgG-Alexa 555 (the donor molecule with excitation: 555 nm and emission : 565 nm; A21428) was purchased from Invitrogen. Rabbit IgG-Alexa 647 (the acceptor molecule with excitation: 650 nm and emission: 668 nm; SC-24647) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 3-PBA hapten was synthesized. The detailed method was described previously [18]. Alexa 647 was conjugated to 3-PBA hapten by using a commercial protein labeling kit (A-20173, Invitrogen).

FRET immunoassay using a 96-well plate

A 96-well plate (Maxisorp, Nunc) was coated with goat-anti-rabbit IgG (RIgG)-Alexa 555 at 128 μg/mL in phosphate buffered saline (PBS) during 4 hours-incubation at 37°C. The wells were then washed five times with PBS. Other nonspecific sites of the well in the plate were blocked by using 300 μL of 2% bovine serum albumin (BSA) for 1 hour at room temperature. After blocking solution was removed, 100 μL of rabbit IgG (RIgG)-Alexa 647 at 10^{-5}, 10^{-4}, 10^{-3}, 0.01, 0.1, 1, and 10 μg/mL were added to wells. After 1 hour incubation at room temperature, the fluorescent intensities were obtained at 670 nm by using a plate

reader (Tecan infinite M1000Pro , San Jose, CA). As a negative control, hapten-Alexa 647 of 3-phenoxy benzoic acid (3-PBA) was added as a target to anti-rabbit IgG-Alexa 555 coated wells. The 3-PBA hapten was chosen because the reagent had already been conjugated with Alexa 647 in our lab and was readily available to us.

FRET immunoassay using a photonic crystal nanostructured array

One milliliter of 0.05 % fluorescent carboxylated PS particles was coated with goat-anti-RIgG-Alexa 555 in deionized (DI) water by passive adsorption for 2 hours at room temperature with gentle shaking. The amount of the antibody was estimated to provide full coverage of the surface of the particle, following a protocol provided by the vendor (Bangs Laboratories, TechNote 205). Particles with anti-RIgG-Alexa 555 were trapped by using an electrophoretic particle entrapment system (EPES) followed by removal of the solution [19]. After trapping of the particles was completed, 10 μL of RIgG-Alexa 647 was dropped onto the photonic crystal array and then incubated for either 20 minutes or 1 minute followed by removal of the solution. Either particle or IgG solutions from the surface of the array was removed by using Couette-flow that we have described earlier [19]. The array was excited by a 532 nm-laser and signals were collected at 670 nm by using a single photon counting detection system. The materials and detailed procedures for fabricating a PC nanostructured array with EPES for nanoparticle-in-well assembly and a single photon counting detection system have been described previously [14,17].

Results and discussion

FRET assay using a 96-well plate

We performed a FRET-based immunoassay by using a plate reader on a 96-well plate, which is a conventional laboratory-based method. Seven different concentrations of RIgG-Alexa 647 were applied to the wells of the plate where anti-RIgG-Alexa 555 had been already coated: 10^{-5}, 10^{-4}, 10^{-3}, 0.01, 0.1, 1, and 10 μg/mL. To obtain a datum point on the standard curve (Fig. 1), three replications were performed. To determine the blank noise, PBS buffer without RIgG was used. The LOD was defined as the concentration of the RIgG that produced three standard deviations higher than the signal of background noise (dashed line), leading to 1 μg/mL. To test cross reactivity of the anti-RIgG-Alexa 555 to the other target molecule, 3-PBA-hapten-Alexa 647 was used. The signal was within the interval of the background noise.

FRET assay on a PC nanostructured array

To perform FRET immunoassays on PC nanostructured arrays (Fig. 2), carboxylated fluorescent PS particles conjugated with anti-RIgG-Alexa 555 were trapped by using a EPES. Five different concentrations of RIgG-Alexa 647 were used: 10^{-3}, 0.01, 0.1, 1, and 10 μg/mL. There were six arrays patterned with 5 mm-separation on a 37.5 × 25 mm PC chip. The arrays could be used independently for measuring the signals from all five concentrations of the target on a chip. Fig. 3 shows the standard curve that quantified the concentrations of the RIgG-Alexa 647. Background noise (primarily backscattered laser light that was not rejected by a notch filter) from the 532 m laser was measured by shining the laser on the arrays in the absence of particles and immunoassay reagents. The signal was

114±10 photons/second. Non-specific binding of a different target molecule to the PC systems was measured with the same target molecule used in 96-well plate reader detection. The signal arising from the test was 137±29 photons/second which was not significant in comparison to the background signal.

The fluorescent particles were chosen because the size of the particle was compatible with the size of the wells (diameter of the well: 60nm, depth: 240 nm) that was engineered to form intensified electromagnetic waves in the PC. The fluorescence of the particles was not used in this assay but we assessed any potential for the particle fluorescence to play an extrinsic role. The background FRET signal generated from the fluorescent particles-anti-RIgG-Alexa 555 was measured by exciting with the 532 nm-laser in the absence of RIgG-Alexa 647. The measured signal of energy transfer from the anti-RigG-Alexa 555 to the fluorescent particle was insignificant compared to the background noise.

To demonstrate the performance of FRET-based PC detection for rapid point-of-care, two different incubation times (20 minutes and 1 minute) were used for antibody-antibody binding (Fig. 3). The limit of detection with 20 minutes of incubation was 0.001 μg/mL which was a hundred times better than the LOD obtained with 1 minute incubation. However, the sensitivity obtained from 1 minute of incubation was still ten times better than the detection limit using a plate reader (1 μg/mL, Fig. 2). In comparison to the plate reader, the PC array showed superiority in terms of sensitivity. As a result of the enhanced sensitivity of the PC immunoplatform, a 1 minute-incubation could be enough to obtain the sensitivity equivalent to the plate reader system – leading to a dramatic reduction of assay time. However, the PC array may face some limitation in application because the choices of the luminescent dyes and the source of incident light are limited by a phase matching requirement of the array – limiting the choice of dyes and excitation sources.

Conclusions

A FRET-based homogeneous immunoassay obviates the need for washing and simplifies an immunoassay. Signal strength in a conventional FRET-based assay may limit the sensitivity of the method. Migration of the FRET format to a photonic crystal array offers greatly improved sensitivity compared to a conventional plater reader, with no loss of specificity. In addition, the FRET-PC array system can achieve detection levels commensurate with much more expensive and complex laboratory instruments in a time as short as one minute.

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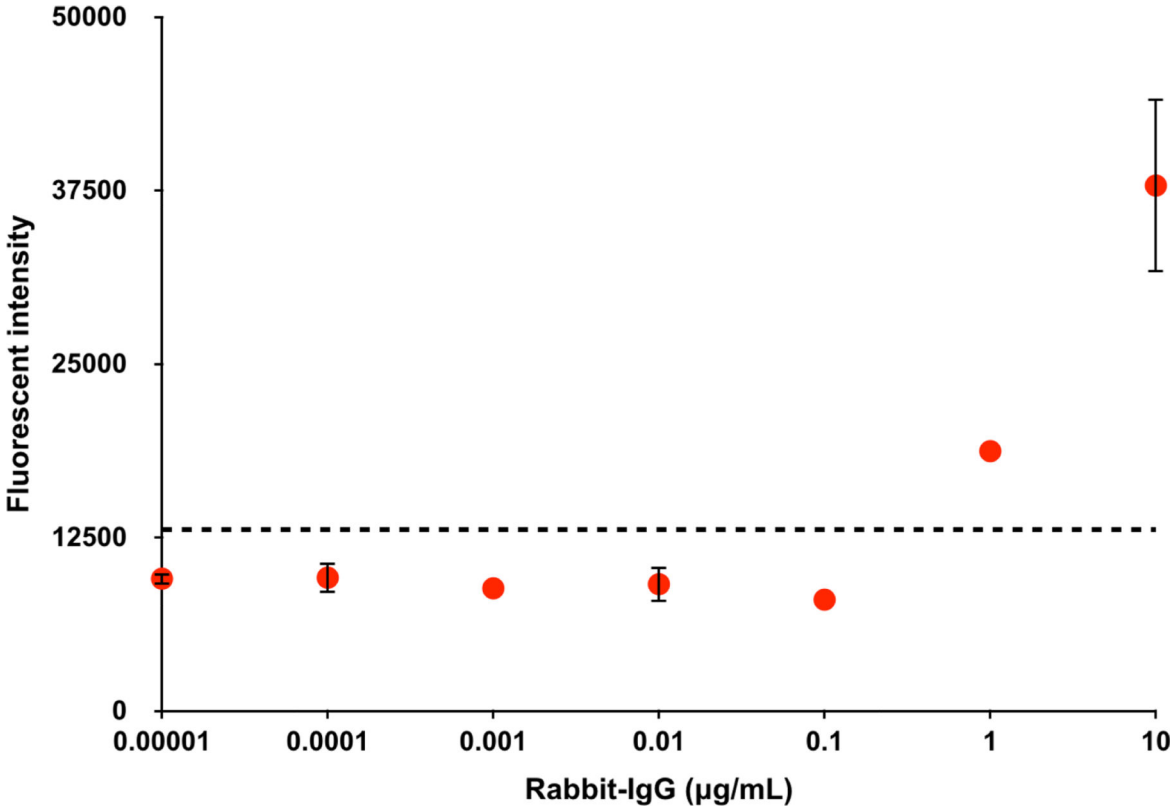

Fig. 1.

Detection of RIgG on a 96-well pate using a plate reader. Seven different concentrations of RIgG-Alexa 647 in PBS were detected using FRET: 10^{-5}, 10^{-4}, 10^{-3}, 0.01, 0.1, 1, and 10 μg/mL. LODs was determined from the mean plus three standard deviations of the background noise (dashed line). Error bars were based on the standard deviations of three replicates.

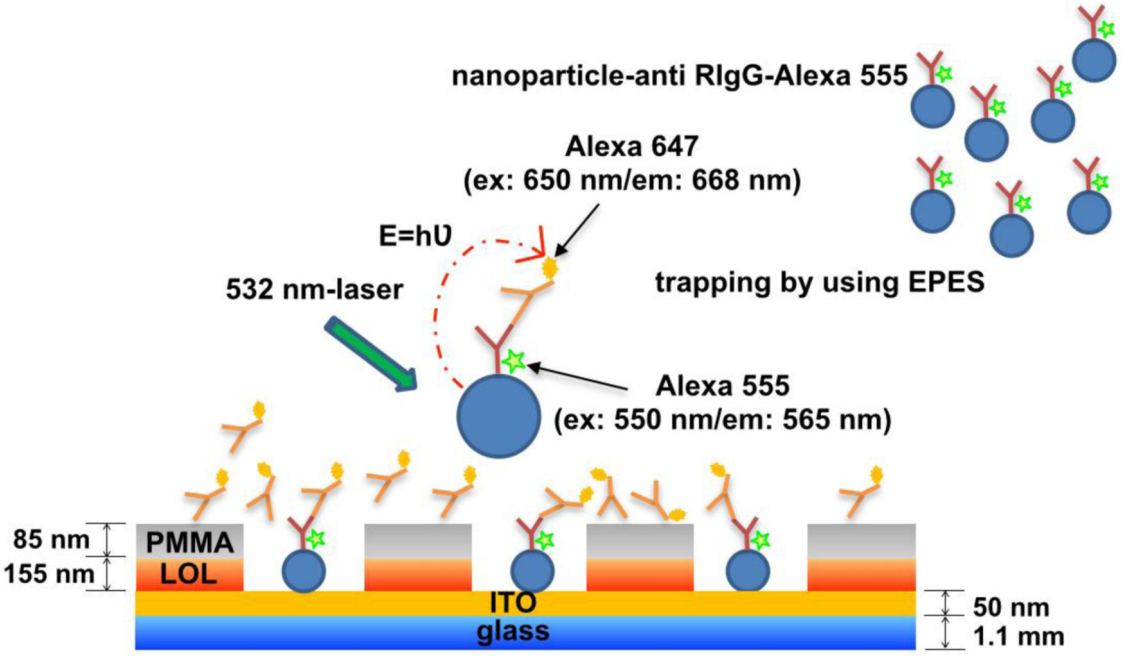

Fig. 2.
Schematic of the FRET-homogeneous immunoassay on a photonic crystal array.

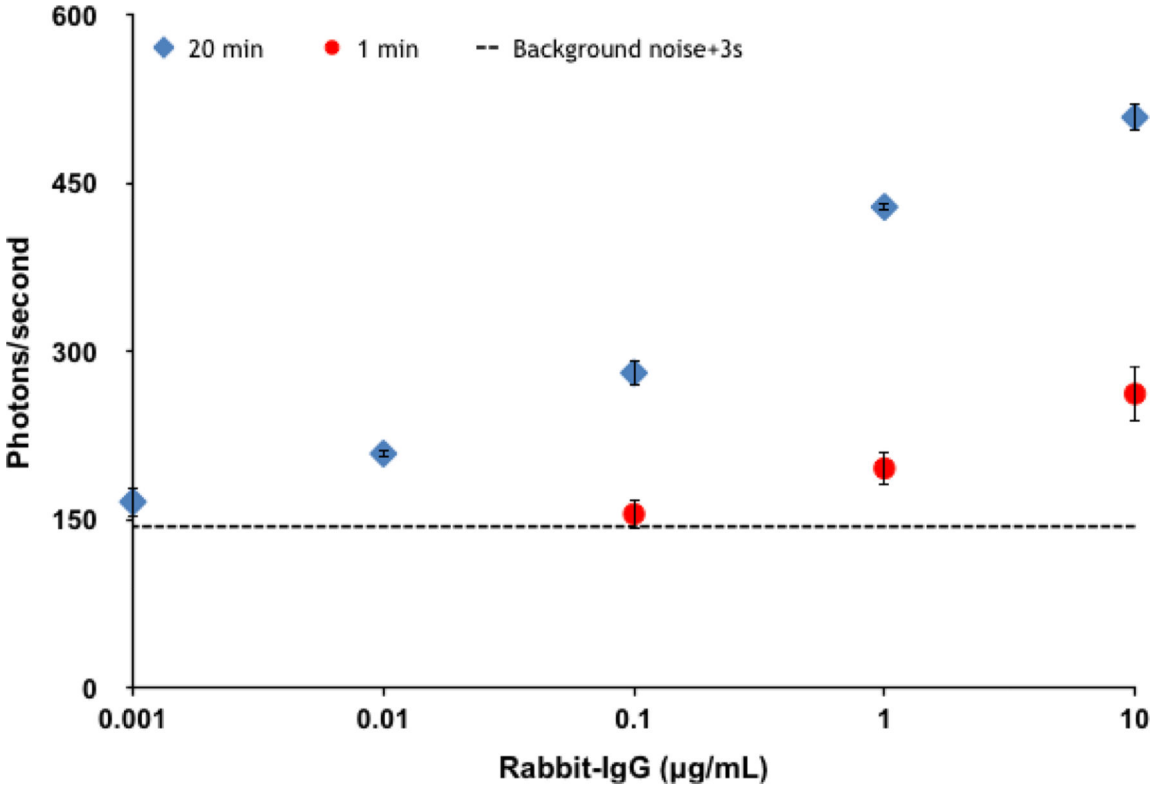

Fig. 3.

Detection of RigG using a photonic crystal array. Incubation times, of 20 minutes (◆) and 1 minute (●) were used for the immune reaction of the RIgG and anti-RIgG. Concentrations of 10^{-3}, 0.01, 0.1, 1, 10 μg/mL were measured. LODs were 10^{-3} and 0.1 μg/mL respectively for 20 minutes and 1 minute. Error bars represent a standard deviation based on three replicates.