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Nanostructured bioluminescent sensor for rapidly detecting thrombin

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ABSTRACT

Thrombin plays a key role in thrombosis and hemostasis. The abnormal level of thrombin in body fluids may lead to different diseases, such as rheumatoid arthritis, glomerulonephritis, etc. Detection of thrombin level in blood and/or urine is one of important methods for medical diagnosis. Here, a bioluminescent sensor is developed for non-invasively and rapidly detecting thrombin in urine. The sensor is assembled through conjugating gold nanoparticles (Au NPs) and a recombinant protein containing Renilla luciferase (pRluc) by a peptide, which is thrombin specific substrate. The luciferase-catalyzed bioluminescence can be quenched by peptide-conjugating Au NPs. In the presence of thrombin, the short peptide conjugating luciferase and Au NPs is digested and cut off, which results in the recovery of bioluminescence due to the release of luciferase from Au NPs. The bioluminescence intensity at 470 nm is observed, and increases with increasing concentration of thrombin. The bioluminescence intensity of this designed sensor is significantly recovered when the thrombin digestion time lasts for 10 min. In addition, a similar linear relationship between luminescence intensity and the concentration of thrombin is found in the range of 8 nM to 8 μM in both buffer and human urine spiked samples. The limit of detection is as low as 80 pM. It is anticipated that our nanosensor could be a promising tool for clinical diagnosis of thrombin in human urine.

1. Introduction

Thrombin is the key protease involved in hemostasis by converting soluble fibrinogen to fibrin clot (Chang, 1985; Shuman, 1986). However, abnormal level of thrombin in blood can cause coagulopathy, renal failure (Sapsford et al., 2006), rheumatoid arthritis (Bar-Shavit et al., 1990), pulmonary fibrosis (Hernández-Rodríguez et al., 1995) and glomerulonephritis (Kitamoto et al., 1998). A number of strategies have been developed for sensing and monitoring thrombin in blood, including antibody based radioimmunoassay/ELISA (Shuman and Majerus, 1976), fluorophore labeled substrate bound sensor (Jaffer et al., 2002; Lin et al., 2013; Olson et al., 2012; Tung et al., 2002), and nucleic acid probes based aptamersensor (Huang and Murray, 2002; Tennico et al., 2010; Zhao et al., 2009). Among them, peptide substrate-based sensors show the high selectivity as the designed peptide substrate only response to the active thrombin and thus avoid false positive from prothrombin.

Quite recently, urinary thrombin demonstrates its being a specific biomarker for glomerular inflammation for the diagnosis of crescentic glomerulonephritis (Kitamoto et al., 2015) because the existence of thrombin in urine could be caused by the disorder of the formation of prothrombin (or thrombin–antithrombin complex) (Kitamoto et al., 2004; Shuman and Majerus, 1976). Consequently, detection of urinary thrombin can realize a non-invasive diagnosis of glomerulonephritis, or a disease related to rental failure. Unfortunately, very few methods have been used in detecting thrombin in urine. Two major challenges are: (1) a sensor for detecting urinary thrombin should be able to detect very low level of thrombin, less than nanomolar (Kitamoto et al., 2004); (2) As human urine is a complex mixture of proteins and many components with small molecular weight (Edwards et al., 1982), no additional steps for processing urine sample is required for detecting urinary thrombin with a quick manner.

Bioluminescence resonance energy transfer (BRET) technique has been applied in detecting low concentration of biological process through the energy transfer between two light-sensitive molecules; the electronic excited state of a donor chromophore may transfer energy to an acceptor chromophore through non-radiative dipole–dipole coupling (Waud et al., 2001; Kim et al., 2010; Xie et al., 2011). The efficiency of the energy transfer depends on the spatial distance between donor and acceptor, the effective range being less than 100 Å (10 nm). Compared to other sensors by applying electrochemistry, Raman, and/or fluorescence (Branchini et al., 2011; Wang et al., 2013; Wu et al., 2015; Li et al., 2014), BRET sensors do not need external energy device as the

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donor emits light naturally. Unlike fluorescent energy transfer technique, BRET sensors are able to avoid the photobleaching, autofluorescence, and undesirable stimulation of photo-biological processes because no fluorescence excitation is required The current developed BRET systems are able to detect very low concentration of thrombin in buffer media by using bioluminescent protein (aequorin or luciferase) donor and fluorescent protein acceptor (Molinari et al., 2008; Dacres et al., 2009a, 2009b, 2012). However, false-positive signal may arise for those systems made by organic chromosomes due to large overlapped emission peaks between bioluminescent proteins and fluorescent molecules. Sequential BRET–Fluorescent resonance energy transfer (FRET) approach was also reported to diminish this false-positive noise (Branchini et al., 2011), the process is relative more complicated. Recently, nanostructured fullerene has being used as an acceptor in BRET pair to replace organic chromophores to gain more accurate results due to their large surface area to volume ration, and the size-dependent luminescence properties with large absorption and narrow-band emission (Yu et al., 2012). However, overnight incubation is required for detecting thrombin detection.

Here, luciferase is used as a donor in BRET sensor, and gold nanoparticles (Au NPs) is chosen as a fluorescence quenchers (acceptor) because of their extraordinary molar extinction coefficients and broad energy absorption in the visible range (Jain et al., 2007; Sapsford et al., 2006). In addition, the unique chemical reactivity of gold allows Au NPs can play a suitable platform to selectively react with a wide range of organic or biological fluorescent ligands for detecting small molecules and biological targets. In the last decades, a number of innovative approaches using Au NPs based resonance energy transfer systems have been developed for the detection of metal ions (He et al., 2005; Huang and Chang, 2006; Huang and Murray, 2002), small molecule (Chen and Chang, 2004; Zhang et al., 2008), nucleic acids (Dyadyusha et al., 2005) and enzymes (Lee et al., 2008; Xia et al., 2011). Fig. 1 is the illustration of the designed sensor, a recombinant protein, which is composed of Renilla luciferase (Rluc) used as a donor and a short thrombin substrate peptide, is conjugated to Au NPs. The bioluminescence from Rluc-catalyzed oxidation of coelenterate (CTZ) is quenched by Au NPs through the BRET mechanism. In the presence of thrombin, substrate peptide linking Rluc (pRluc) and Au NPs is digested. As a result, pRluc is released from Au NPs and bioluminescence intensity is recovered. In this paper, the nanostructured BRET sensor has been used for detecting thrombin in both buffer and urine samples. We have studied the effects of linkers and the ratio of Au NPs to pRluc on the sensor efficiency to be able to have optimal results for detecting urinary thrombin.

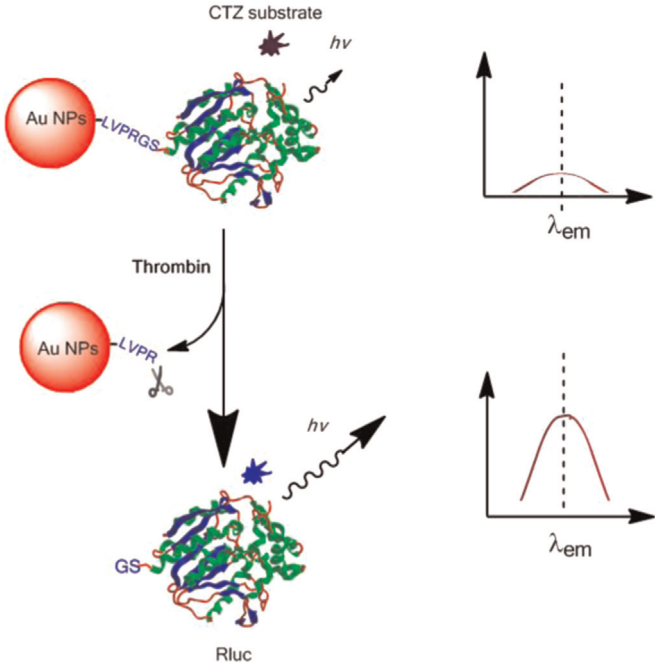

Fig. 1. Illustration of detection of thrombin via pRluc conjugated gold nanoparticles.

2. Experimental

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich. Only NANOpure water ($\rho = 18.2$ MΩ) was used.

2.1. Plasmid constructions

A recombinant protein (pRluc) containing *Renilla* luciferase mutant protein (Rluc) and a short peptide sequence, e.g. the thrombin substrate, was constructed as following. Firstly, Rluc gene from the plasmid pRL-null (Promega, Inc) was cloned into the MCS site of pET 32-a (EMD Millipore Inc.) plasmid under two restriction sites (BamH I and Xho I).

Two primers were designed for the cloning (forward: 5′ AAAGGATCCAGCGGTGGTGGTGGTAGCATGACTTCGAAAGTTTATGATCCAG; reverse: 5′ TGTGCTCGAGTTGTTCATTTTTGAGAACTCGCTC 3′). Thrombin substrate originally from pET 32-a plasmid is thus located at upstream (N-terminal) of Rluc gene. A trx region from pET 32-a coding for thioredoxin protein is kept to maintain high level of recombinant protein expression (LaVallie et al., 1993). The bold underline in the forward primer indicates a six amino acid linker (SGGGGS) was inserted after BamH I site to leave a flexible space for proper folding of pRluc protein.

The PCR products and the plasmid were digested with relating restriction enzyme and purified through agarose gel, respectively. The digested DNA insert was ligated into the relating MCS site at the plasmid. The ligation product was transformed into *E. coli* BL21 cells. The successful construction of the plasmid was confirmed by DNA sequencing (Robarts Institute, Western University, London, ON).

2.2. Protein expression and purification

The above bacterial cells with recombinant plasmid were grown overnight at 37 °C in 5 mL of Luria Bertani (LB) broth containing 100 μg/mL ampicillin. This culture was used to further inoculate 500 mL of broth containing 100 μg/ml of ampicillin, and this was grown at 37 °C. When the culture reached an OD_{600} of 0.375, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to 1 mM final concentration to induce the expression of recombinant protein (pRluc) and the bacteria were left to grow for 4 h at room temperature. The cells were harvested by centrifugation at 12,000 rpm for 5 min at 4 °C. The pellet was re-suspended in a binding solution (BS) of 20 mM Tris/HCl, pH 7.4, 500 mM NaCl and 5 mM imidazole and sonicated on ice using 15-s bursts followed by 30-s rest for 30 cycles using a Mandel Scientific Q500 sonicator. The suspension was centrifuged at 10,000 rpm at 4 °C for 30 min to collect the supernatant from bacterial cell pellet. The protein was purified via His-trap HP columns (GE lifescience, Inc.) by a syringe pump. The column was first equilibrated with BS. The supernatant containing the protein was loaded on the column, and the column was washed with 10 column volumes of the BS. The protein was eluted using BS with a gradient of imidazole from 20 mM to 200 mM) over 10 column volumes. Five milliliters fractions were collected. SDS-PAGE (10% SDS-PAGE under reducing conditions and stained with Coomassie Blue) was used to verify