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Peptide cleavage induced assembly enables highly sensitive electrochemiluminescence detection of protease activity

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ABSTRACT

Proteases perform essential functions in a multitude of physiological processes and participate in many human diseases. Probing protease activity sensitively and accurately is critical for both basic research and clinical diagnosis. Herein, a novel optical biosensor for high-sensitive detection of protease was developed based on the specific protease cleavage of synthesized peptide substrate and subsequent assembly between exposed cysteine and $Ru(bpy)_3^{2+}$-2-cyanobenzothiazole ($Ru(bpy)_3^{2+}$-CBT). The assembly complexes can be enriched by streptavidin coated magnetic beads on work electrode for high-sensitive electrochemiluminescence (ECL) analysis. Using trypsase and caspase-3 as the examples, the limit of detection (LOD) of 8.4×10^{-4} U mL^{-1} and 5×10^{-6} U mL^{-1} were obtained respectively. This represents one of most sensitive protease assay reported. Current peptide cleavage induced assembly (PCIA) method can be easily extended to detect other proteases by changing peptide substrate sequence, shows its great potential as versatile biosensor in sensing techniques and clinical diagnosis.

1. Introduction

Proteases are vital enzymes and perform critical functions in a wide range of physiological processes, proteases are also implicated in the initiation and progression of many important diseases such as cancer, inflammation and infection [1,2] and has been recognized as biological markers and therapeutic targets for clinical diagnosis and therapy. Thus, it is of high importance to probe protease activity sensitively and conveniently for research and clinical diagnosis.

Development of optics-based techniques has revolutionized the field of bioanalysis [3–5]. At present, a considerable amount of ingenious approaches based on FRET [6,7], bioluminescence [8], mass spectrometry [9], electrochemical [10,11], colorimetric [12] have been established to effectively profile protease activity. However, various shortcomings have limited their wider application. For example, FRET-based method mainly relies on the efficiency of energy transfer between the fluorescence donor and acceptor, which may limit their sensitivity and applicability. Mass spectrometry technique is labor-intensive, and requires highly technical expertise and sophisticated instrumentation. The colorimetric method is susceptible to the background signal interference which severely troubles the detection sensitivity. Therefore, it is beneficial to develop a sensitive, simple and low-cost detection technology for protease assay.

The condensation reaction between cysteine and 2-cyanobenzothiazole (CBT) (Fig. 1A), which has been used for the synthesis of D-luciferin, could proceed rapidly under mild conditions with a second-order rate constant 9.19 M^{-1} s^{-1} [13,14]. Notably, CBT can react with free cysteine to form stable products, but little or no condensation products are produced while reacting with a compound lacked a 1,2-aminothiol group, such as glutathione [13,14]. More specifically, when peptides containing the cysteine residue in the middle of the sequence are mixed with CBT, no ligation product can be detected, which means that CBT specifically reacts with the N-terminal cysteine residues of peptides [14]. Thus, this fast reaction kinetics and high selectivity make CBT-cysteine condensation a promising bioconjugation method for high specific peptides labelling at N-terminal cysteine residues [15]. Much works have been carried out based on this bioorthogonal reaction, such as site-specific protein labelling [14], intracellular self-assembly [13,16,17] and plant vacuolar processing enzyme assay [18].

ECL technology is a powerful tool with excellent performance and distinct advantages because of the high sensitivity, broad measuring range, controlled reaction system and high signal-to-noise ratio [19–23]. ECL has been widely employed in analytical and clinical fields, such as DNA detection [24,25], enzyme assay [22,26], immunoassay [27]. Herein, we develop a novel system which takes advantage of ECL technique and highly selective reaction between

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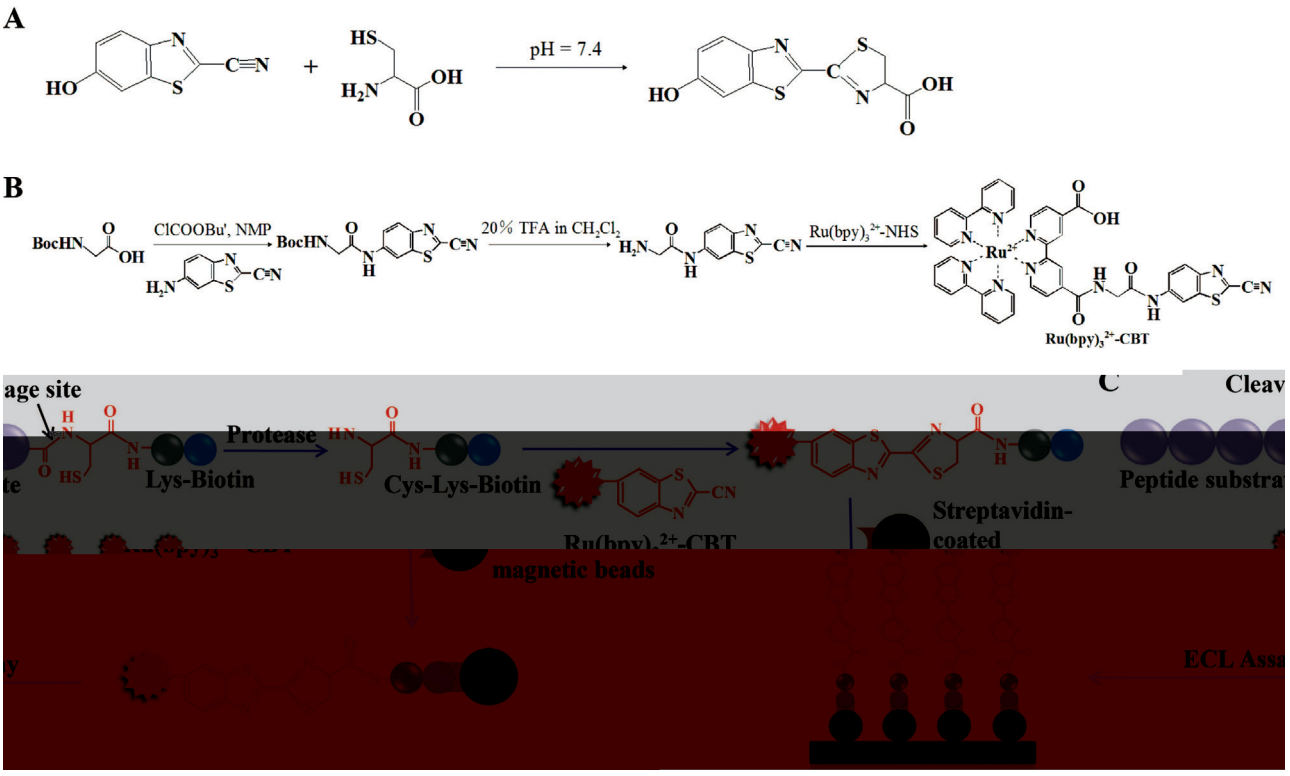

Fig. 1. (A) The condensation reaction between CBT and free cysteine. (B) Synthetic route for ECL probe $Ru(bpy)_3^{2+}$-CBT. (C) Schematic representation of protease detection based on ECL probes.

cysteine and CBT, and integrated in magnetic microparticle enrichment technology for highly sensitive protease detection. As shown in Fig. 1B, we synthesized a novel ECL probe $Ru(bpy)_3^{2+}$-CBT (Figs. S1 and S2), which possesses a CBT group as the recognition moiety to react with exposed cysteine. The principle of the experimental design is illustrated in Fig. 1C and explained as follows. (1) Firstly, a peptide substrate containing a C-terminal biotin was synthesized according to the recognition sequence of target protease. A cysteine residue was designed at the N-terminal side of cleavage site. (2) Secondly, CBT moiety enables the ligation of $Ru(bpy)_3^{2+}$-CBT probe specifically to the N-terminal cysteine residue. Thus, cleavage of peptide substrate induced the assembly between severed peptide CK-Biotin and $Ru(bpy)_3^{2+}$-CBT to form $Ru(bpy)_3^{2+}$-CBT-CK-Biotin compound. (3) Finally, The $Ru(bpy)_3^{2+}$-CBT-CK-Biotin compound was enriched and separated from reaction solution by streptavidin coated magnetic beads for ECL analysis. Taken as a whole, the couple of ECL and specific CBT-cysteine condensation provides the possibility for highly sensitive protease assay. Such a peptide cleavage induced assembly (PCIA) reaction provides a versatile protease sensing platform by designing a cysteine residue at the C-terminal side of cleavage site in artificial peptide substrate. Thus, this biosensor can easily be extended to detect a series of proteases by synthesizing corresponding peptide substrates for different biological or biophysical applications, shows its great potential as versatile biosensor in sensing techniques and clinical diagnosis.

2. Materials and methods

2.1. Materials

Peptides were synthesized by ChinaPeptides Co., Ltd (Shanghai, China). 2-cyano-6-amino-benzothiazole (CABT) was purchased from AOKchem (Shanghai, China). Trypsase was purchased from Aladdin. Caspase-3 was purchased from BioVision Incorporated. Thrombin, lysozyme, pepsin, the tripropylamine (TPA) and other chemicals to synthesize the $Ru(bpy)_3^{2+}$-NHS were purchased from Sigma (St. Louis, MO).

2.2. Synthesis of $Ru(bpy)_3^{2+}$-CBT probes

Glycine-CBT was synthesized as described in literature [13]. The amino compound glycine-CBT was obtained by preparative HPLC and confirmed by MS (Fig. S1). $Ru(bpy)_3^{2+}$-NHS was synthesized by our laboratory in term of previously published papers [21]. To synthesize $Ru(bpy)_3^{2+}$-CBT, a solution of glycine-CBT (23.3 mg, 0.1 mmol) in anhydrous dimethyl formamide (DMF) (1 mL) was added slowly to $Ru(bpy)_3^{2+}$-NHS reagent solution (3 mL) and stirred at room temperature for 12 h. The mixture was diluted with 200 mL double-deionized water (ddH_2O), then extracted with 10×100 mL ethyl acetate (EtOAc) followed by drying over Na_2SO_4. After removal of the solvent, the residue was purified by normal flash chromatography (MeOH: CH_2Cl_2 1:5–1:1) to obtain $Ru(bpy)_3^{2+}$-CBT (Fig. S2).

2.3. Validation of PCIA method using synthetic substrate

The solution of Cys-Lys-biotin (CK-Biotin) at different concentration (200, 400, 600, 800, 1000 nM) in PBS buffer were incubated with 5 μM $Ru(bpy)_3^{2+}$-CBT probe and incubated with vortex shaking at room temperature for 30 min. Next, reaction solution was added to streptavidin coated magnetic beads solution and incubated at room temperature (25 °C) for 15 min. The mixture was then washed four times with 100 μL PBS buffer (pH 7.4) and resuspended in 100 μL PBS (pH 7.4) for ECL analysis.

The ECL experiment was performed on a fully automated Elecsys 2010 immunoanalyzer. The washed mixture was aspirated into a thermostated measuring cell (28 °C). The microparticles are magnetically immobilized onto the surface of platinum work-