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Aptamer-capture based assays for human neutrophil elastase

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ABSTRACT

Human neutrophil elastase (HNE) is a multifunctional serine protease, involved in infection defense, inflammatory process regulation, and physiopathological processes of several diseases. We developed aptamer-capture based assays for human neutrophil elastase with different substrates and solid supports to meet different demands, such as simplicity, sensitivity, and high throughput. Aptamers against HNE were immobilized on magnetic beads or microplates as affinity ligands to capture HNE, and then the enriched HNE catalyzed the conversion of chromogenic substrates or fluorogenic substrates to products. The measurement of the generated enzymatic products enabled the final detection of HNE. In the assay using chromogenic substrates and aptamer modified magnetic beads, 0.4 pM HNE could be successfully detected. The sensitivity of the assay was further improved by using fluorogenic substrates, and a detection limit of HNE at 20 fM was achieved. The use of aptamer-coated microplates instead of aptamer modified magnetic beads in the assays also allowed the sensitive detection of HNE, offering advantages in fast sample handling and measurement. The established assays for HNE displayed good specificity, and proteins including serum albumin, transferrin, immunoglobulin G, thrombin, porcine pancreatic elastase, trypsin, proteinase K, chymotrypsin, lysozyme, cathepsin G, and proteinase 3 did not cause interference in the detection of HNE.

1. Introduction

Human neutrophil elastase (HNE) is a multifunctional serine protease localized in the azurophilic granules of the neutrophil and prefers to cleave the peptide containing small hydrophobic residues (e.g. Val, Ala, Met). HNE is involved in innate immune defense against invading pathogens and participates in inflammatory process regulation [1–3]. It is secreted by neutrophils during inflammation, and destroys bacteria through the cleavage of the virulence factors and outer membrane proteins of bacteria. The proteolytic activity of HNE is usually regulated by the inhibitors. The high levels of unregulated HNE can disrupt healthy tissues and lead to the development of diseases. HNE plays essential roles in a variety of physiopathological processes, such as acute respiratory distress syndrome, chronic obstructive pulmonary disease, cystic fibrosis, acute lung injury, arthritis, emphysema, and atherosclerosis [1–4]. HNE is a potential therapeutic target of drugs and a biomarker of some diseases [1–7]. Sensitive and specific detection of HNE is favored for therapy and diseases diagnostics. The reported methods for HNE detection include the catalyzing hydrolysis of the chromogenic or fluorogenic peptide substrates, immunoassays, electrochemical sensing, etc. [8–12]. Peptide substrates with fluorescence resonance energy transfer reporters have been applied to sensitive and selective measurement of HNE activity [13–16]. HNE at sub-nanomolar or tens of picomolar levels could be detected in the previous report [8,13–16].

Aptamers, the single stranded oligonucleotides selected from random nucleic acid library, show advantages in assay developments, such as good thermal stability, easy generation, and easy modification for labeling [17–25]. Aptamers against HNE have been selected [26–29]. Lin et al. reported one DNA aptamer that specifically bound to HNE with a dissociation constant (K_d) about 17 nM [28,29]. Fluorescence polarization assay for HNE was demonstrated by using fluorescently labeled aptamers as affinity ligands [23]. In this assay the detection limit of HNE was around nanomolar level [23]. The aptamer-based assay using fluorescent molecular beacon probes was developed, allowing the detection of HNE at sub-nanomolar levels [30]. Aptamer-capture based assays for enzyme molecules have been developed by combination of aptamer-capture and enzymatic reactions [31–33]. In previous work [31], we demonstrated an aptamer-capture based assay for HNE relying on the affinity capture of HNE by aptamer modified magnetic beads and the subsequent catalyzing cleavage of fluorogenic substrates by HNE. In our previous work HNE at 0.1 pM could be detected when 24-h enzyme reaction was applied [31]. On the basis of previous work [31–33], herein we made more investigation and further developed the aptamer-capture assays for HNE by using

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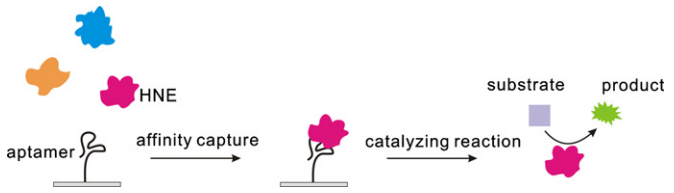

Fig. 1. Scheme of the principle of the aptamer-capture based assay for human neutrophil elastase (HNE). HNE is specifically captured from sample mixture by the aptamers on solid supports (magnetic beads or microplates), and the obtained HNE converts substrates to products. Measurement of the products provides the detection of HNE.

different peptide substrates (chromogenic and fluorogenic substrates) and solid supports (aptamer modified magnetic beads and microplates) to meet different demands of assays in simplicity, sensitivity, specificity, and high throughput analysis. The principle of the assay is that HNE is specifically captured from the mixture by aptamers on a solid support (magnetic beads or microplates), and then the enriched HNE catalyzes the conversion of the peptide substrates to products (Fig. 1). By measuring the generated products, detection of HNE can be achieved. The use of chromogenic substrate allows simple absorbance measurement and colorimetric detection of HNE [32], while the use of fluorogenic substrate enables more sensitive detection of HNE. Aptamer modified magnetic beads show advantages in easy magnetic separation [34,35]. The aptamer modified microplates offer benefits in fast sample handling and analysis of multiple samples. These assays show high sensitivity and good specificity in the detection of HNE. The assays will be helpful for biomarker detection and disease diagnostics.

2. Experimental

2.1. Chemicals

Human neutrophil elastase (HNE, 22 units/mg) was ordered from Athens Research and Technology. N-Methoxysuccinyl–Ala–Ala–Pro–Val p-nitroanilide (denoted as Meo–Suc–AAPV–pNA), was obtained from Sigma. The fluorogenic substrate of HNE, bis (benzyloxycarbonyl–Ala–Ala–Ala–Ala) derivative of Rhodamine 110, denoted as $(AAAA)_2$-R110, was ordered from Invitrogen. Pooled human serum was obtained from Zhongke Chenyu Biotechnology (Beijing). Bovine serum albumin (BSA), human serum albumin (HSA), human immunoglobulin G (IgG), human transferrin, lysozyme from chicken egg, and trypsin (from bovine pancreas) were purchased from Sigma. Human alpha thrombin was ordered from Haematologic Technologies Inc. (Essex Junction, VT). Porcine pancreatic elastase (PPE) and chymotrypsin were purchased from Ruibio. Human cathepsin G and human proteinase 3 were ordered from Athens Research and Technology. Proteinase K was ordered from Merck. Streptavidin was obtained from Cortex Biochem (San Leandro, CA). Solvents and other reagents were supplied by Amresco and Sangon Biotech (Shanghai, China).

Streptavidin coated magnetic beads (1 μm in diameter, Dynabeads MyOneTM Streptavidin C1; 10 mg/mL, about $7–12 \times 10^9$ beads per mL) were purchased from Invitrogen Dynal. The magnetic beads had large binding capacity for biotin labeled molecules and slow sedimentation rate as the company demonstrated. The DNA aptamer recognizing HNE had the following sequence: 5′-biotin–TAG CGA TAC TGC GTG GGT TGG GGC GGG TAG GGC CAG CAG TCT CGT-3′) [29]. The control scramble DNA oligo had the following sequence: 5′-biotin–TTT TTT TGC TTA GCT CTT ATG AAC CCG ATT CTA AGA CCT TTT GGC-3′. The biotinylated aptamers were synthesized and purified by Sangon Biotech (Shanghai, China). The black 96-well NUNC Maxisorp plates were purchased from Thermo Scientific. The clear 96-well plates were purchased from Corning (Costar 3590). The following buffer solutions were used. Buffer A contained 50 mM Tris–HCl, 2 M NaCl, and 0.1% Tween 20 (pH 7.4). Buffer B contained 150 mM NaCl, 100 mM Tris–HCl, 2 mM $MgCl_2$, 6 mM KCl, and 0.1% Tween 20 (pH 7.0). Buffer C contained 50 mM Tris–HCl, 10 mM $CaCl_2$, 1 M NaCl, and 0.1% Tween 20 (pH 8.0).

2.2. Aptamer-capture based assay using aptamer modified magnetic beads

Biotinylated aptamers were attached on the streptavidin coated magnetic beads through the interaction between biotin and streptavidin (see the Supplementary material). In the chromogenic assay for 5 μL sample, 45 μL of buffer B, 5 μL of HNE at various concentrations, and 1 μL of aptamer modified magnetic beads suspension were pipetted into a 0.6-mL centrifuge tube, and the mixture was incubated at room temperature for 30 min. After magnetic separation, the magnetic beads were rinsed three times with 50 μL of buffer B. The magnetic beads were redispersed in 20 μL of buffer C containing the chromogenic substrate of Meo–Suc–AAPV–pNA (0.84 mM). After incubation at 37 °C for 2 h or 24 h, the reaction solution was separated from the magnetic beads and added to 100 μL of 20% acetic acid solution to stop the enzyme reaction. The collected solution was transferred into a quartz cuvette (working volume: 100 μL; path length: 1 cm), and then the absorbance spectra were recorded by the UV–visible spectrophotometer (HITACHI U3010). The absorbance at 405 nm was measured to achieve the final detection of HNE.

In the chromogenic assay for the detection of 250 μL of sample, the aptamer modified magnetic beads (1 μL) were added into 250 μL of HNE at different concentrations in buffer B, and the mixture was incubated at room temperature for 30 min. Then the same procedure as described above for the analysis of 5-μL of HNE was performed. In the visual colorimetric assay, the collected 20 μL of enzymatic product solution was directly observed with naked eyes and recorded by a digital camera.

In the fluorogenic assay for HNE, the fluorogenic substrate of $(AAAA)_2$-R110 was used. HNE was captured on the aptamer modified magnetic beads and washed by following the same procedures described above. The magnetic beads were dispersed in 20 μL of reaction solution (buffer C+20% DMF) containing 0.21 mM $(AAAA)_2$-R110 for enzyme reaction. After incubation at 37 °C for 2 h or 24 h, the reaction solution was separated from the magnetic beads and was added to 85 μL of 20% acetic acid solution. 100 μL of the collected solution was transferred into wells on microplates, and then the fluorescence emitted at 530 nm (excitation at 495 nm) was measured by a plate reader (Varioskan Flash, Thermo Fisher Scientific, Inc.).

2.3. Aptamer-capture based assay using aptamer modified microplates

The microplates were first coated with streptavidin, and then the biotinylated aptamers were conjugated on the microplates through the interaction between biotin and streptavidin (see the Supplementary material). In the chromogenic assays for HNE, 100 μL of HNE at different concentrations in buffer B was added in the wells of the aptamer-modified clear microplates, and the solution was incubated for 1 h at room temperature. After that, the wells were washed with 100 μL of buffer B three times, and then 100 μL of buffer C containing 0.84 mM Meo–Suc–AAPV–pNA was added into the wells to initiate the enzyme reaction. After the wells were incubated at 37 °C for 2 h or 24 h, 20 μL of acetic acid