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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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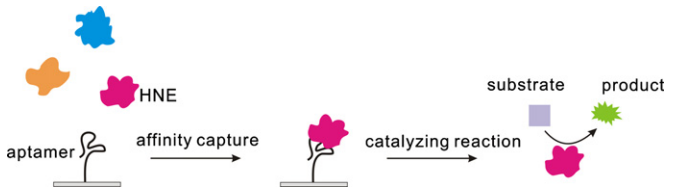

Fig. 1. Scheme of the principle of the aptamer-capture based assay for human neutrophil elastase (HNE). HNE is specifically captured from sample mixture by the aptamers on solid supports (magnetic beads or microplates), and the obtained HNE converts substrates to products. Measurement of the products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

was added into wells to stop the enzyme reaction, and the absorbance of the solution was finally measured at 405 nm by a plate reader (Varioskan Flash, Thermo Fisher Scientific, Inc.).

In the fluorogenic assays for HNE, 100 μL of HNE at different concentrations in buffer B was added in the wells of the aptamer-modified black microplates, and then the solution was incubated at room temperature for 1 h. After that, the wells were washed with 100 μL of buffer B three times, and then 100 μL of enzyme reaction solution (buffer C+20% DMF) containing 0.21 mM $(AAAA)_2$-R110 was added in the wells. After the wells were incubated at 37 °C for 2 h or 24 h, 20 μL of acetic acid was added into wells to stop the enzyme reaction. The fluorescence of the solution emitted at 530 nm (excitation at 495 nm) was measured by a plate reader (Varioskan Flash, Thermo Fisher Scientific, Inc.).

2.4. Specificity analysis

To test the specificity of the assays, other proteins and enzymes were tested by using the aptamer modified magnetic beads and the fluorogenic substrate of $(AAAA)_2$R110 (or the chromogenic substrate Meo–Suc–AAPV–pNA). In the assay for 5 μL of sample, HNE at 10 nM was tested along with thrombin (10 nM), trypsin (10 nM), proteinase K (10 nM), chymotrypsin (10 nM), porcine pancreatic elastase (10 nM), lysozyme (10 nM), proteinase 3 (10 nM), cathepsin G (10 nM), immunoglobulin G (IgG, 10 nM), human serum albumin (HSA, 10 nM), and transferrin (10 nM). 0.21 mM $(AAAA)_2$R110 and 2-h enzyme reaction was applied in the assay using the fluorogenic substrate. 0.84 mM Meo–Suc–AAPV–pNA and 2-h enzyme reaction was applied in the assay using the chromogenic substrate. To test the effect of high concentration of abundant blood proteins on the detection of HNE, HNE (10 nM) was also tested in the presence of IgG, HSA, and transferrin (1 μM for each protein).

2.5. Recovery test and detection of HNE in diluted human serum

We took human serum as an example of complex sample matrix. In the recovery test, HNE was spiked in the serum diluted by the assay buffer (buffer B), and then 250 μL of the serum containing spiked HNE was analyzed in the assay using the aptamer modified magnetic beads and the fluorogenic substrate ($(AAAA)_2$R110) by following the same assay procedure described above. To avoid the effect of the inhibitors in serum on the recovery test, HNE was also spiked in the diluted and heated serum, and then the spiked HNE was analyzed. During the heating treatment, the serum was heated at 65 °C for 30 min to inactivate the inhibitors, and cooled at 4 °C.

To determine the level of HNE in serum, 250 μL of the diluted serum sample was analyzed in the assay using the aptamer modified magnetic beads and the fluorogenic substrate ($(AAAA)_2$R110). The level of HNE in the serum was determined by the calibration equation obtained by the HNE standards in the binding buffer solution.

3. Results and discussion

3.1. Detection of HNE by using chromogenic substrates and aptamer modified magnetic beads

Chromogenic substrates are widely used for enzyme assays because of the simplicity of absorbance measurement and low cost. Magnetic beads are advantageous in large surface area, ease of magnetic separation, and easy immobilization of aptamers [34,35]. By using a chromogenic substrate of HNE, N-Methoxysuccinyl–Ala–Ala–Pro–Val p-nitroanilide (denoted as Meo–Suc–AAPV–pNA) and aptamer modified magnetic beads, we developed a chromogenic assay for HNE to extend the previous work [31,32]. HNE could be captured by the aptamer modified magnetic beads, and the enriched HNE catalyzed the hydrolysis of the substrate of Meo–Suc–AAPV–pNA to release the p-nitroaniline. The generated p-nitroaniline had a maximum absorbance at about 380 nm, and could be measured at 405 nm to reduce the background interference. The measurement of the generated p-nitroaniline allowed the final detection of HNE. By using the optimized condition (see the Supplementary material and Figs. S1 and S2), the assay for HNE was constructed. As Fig. 2 shows, 5 μL of HNE in the range from 0.2 nM to 20 nM was detected with a good linear relationship when 2-h enzyme reaction was applied ($y=0.0567x+0.012$, R^2 0.993). The concentration detection limit was 0.2 nM, as defined by IUPAC (detection limit equals to 3.29 Sb/m, where Sb is the standard deviation of the blank control, and m is the slope of calibration curve). The corresponding lowest detected amount of HNE was 1 femtomole. Triplicate analyses of HNE sample showed the relative standard deviation (RSD) was less than 5%. When the control DNA modified magnetic beads were used, HNE was not captured by the beads, and no significant

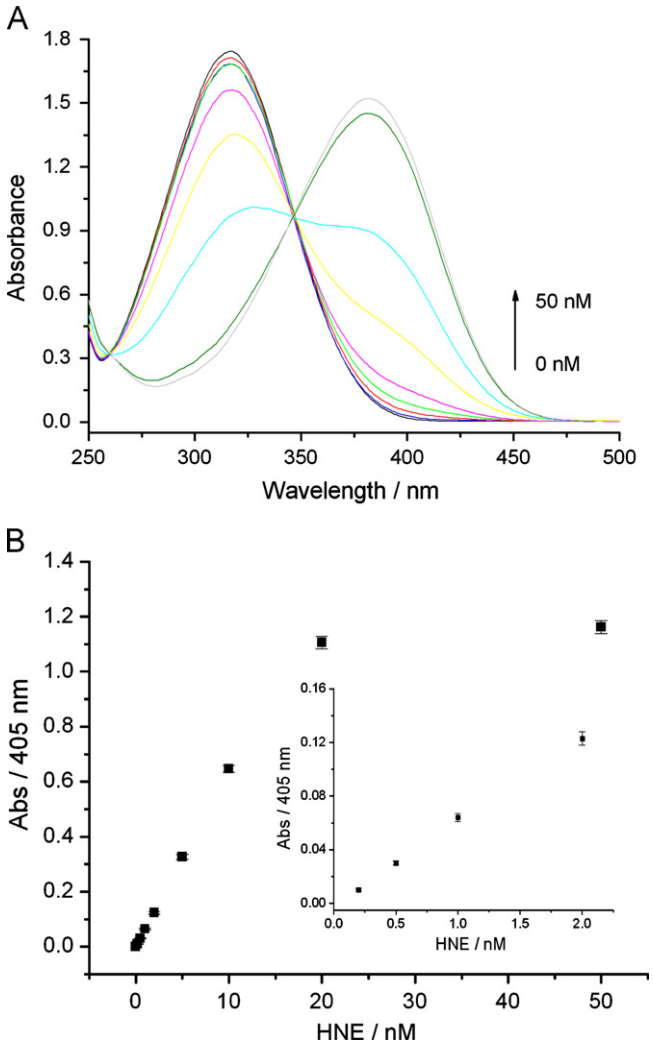

Fig. 2. Detection of HNE by using the chromogenic substrate of Meo-Suc-AAPV-pNA and the aptamer modified magnetic beads. (A) The absorbance spectra obtained by HNE at various concentrations (0, 0.2, 0.5, 1, 2, 5, 10, 20, and 50 nM). (B) The relationship between the absorbance change at 405 nm over the control and the concentration of HNE. The inset shows the absorbance changes at 405 nm over the control, obtained by low concentrations of HNE. 2-h Enzyme reaction was applied, and 5 μL of HNE was detected.

signals from enzyme reaction were obtained. This result shows the binding between aptamers and HNE is dependent on the aptamer sequence, consistent with the previous report [29].

When 250 μL of HNE was analyzed instead of 5 μL of HNE, the concentration detection limit of HNE was lowered by preconcentration of HNE with the aptamer-modified beads. HNE (250 μL) at concentrations ranging from 4 pM to 0.4 nM could be detected in the assay applying 2-h enzyme reaction. The sensitivity of the assay could be further improved about 10 fold by extending the enzyme reaction time to 24 h, and 0.4 pM HNE (250 μL, corresponding to 0.1 femtomole HNE) could be detected (Table 1).

As the generated product is yellow at high concentration, a simple visual detection of HNE can be achieved. 4 pM HNE (250 μL) was successfully detected by observing the color change of the collected product solution when 24-h enzyme reaction was applied (Fig. 3). When 2-h enzyme reaction was applied, 40 pM HNE could be detected by the visual detection method. This colorimetric detection provides a simple way to measure the HNE with high sensitivity without using the spectrometer, and it is useful for the detection on field or point of care.

3.2. Detection of HNE by using chromogenic substrates and aptamer modified microplates

To adapt the aptamer-capture based assay for the commonly used ELISA platform in high throughput analysis and automatic analysis, we also developed the assay for HNE by using the aptamer-modified clear 96-well microplates and the chromogenic substrates. HNE was captured by the aptamers on microplates, and then the captured HNE catalyzed the cleavage of the chromogenic substrates to products. The absorbance of the generated products was directly measured by a plate reader. By using this method the concentration detection limit of HNE (100 μL) reached 0.02 nM (corresponding to 2 femtomole HNE) when 2-h enzyme reaction was applied, and the linear range of concentration of HNE was from 0.02 nM to 5 nM ($y=0.372x+0.026$, $R^2=0.994$) (Fig. S3 of Supplementary material). When the enzyme reaction time was extended to 24 h, HNE at concentrations ranging from 2 pM to 0.5 nM could be detected with a good linear relationship (Table 1).

Table 1 summarizes the results of the detection of HNE in the assay using the chromogenic substrate of Meo–Suc–AAPV–pNA and the aptamer modified microplates (the aptamer modified magnetic beads). The assay using aptamer-modified microplates is faster in washing procedure than that using the aptamer-modified magnetic beads because multiple wells can be simultaneously handled. The absorbance measurement of multiple wells by a plate reader is also faster. The path length of wells on the clear microplate is shorter than the standard quartz sample well (1 cm) for absorbance spectrometer, causing lower sensitivity. The microplate is suitable for multiple sample handling and analysis. The assay using aptamer modified microplates can share the commonly used ELISA platform.

3.3. Aptamer-based assay for HNE using fluorogenic substrates

Taking advantage of the high sensitivity of fluorescence measurement, we also developed aptamer-capture based fluorogenic assay for HNE by using the fluorogenic substrates. Previously, we demonstrated aptamer-capture based fluorogenic assay for HNE by using one fluorogenic substrate of HNE, (N-Methoxysuccinyl–Ala–Ala–Pro–Val–7-amido-4-methylcoumarin, Meo–Suc–AAPV–AMC) and aptamer modified magnetic beads [31]. Here we used another fluorogenic substrate of HNE, $(AAAA)_2$-R110, and the aptamer modified magnetic beads to further improve the sensitivity of the assays for HNE and to show different substrates can be applied in the assays. This fluorogenic assay using aptamer modified magnetic beads exhibited a higher sensitivity than the chromogenic assay using aptamer modified magnetic beads, and 0.2 pM HNE (250 μL, corresponding to 0.05 femtomole HNE) could be detected by applying this fluorogenic substrate and 2-h enzyme reaction. The linear concentration range for HNE detection was from 0.2 pM to 0.2 nM. (Fig. 4) As low as 20 fM HNE (250 μL, corresponding to 5 attomole HNE) was successfully detected when 24-h enzyme reaction was applied. Comparing with our previous work for HNE detection using the fluorogenic substrate (Meo–Suc–AAPV–AMC) and the aptamer modified magnetic beads [31], the detection limit was lowered by five-fold because low background signal was obtained by using the fluorogenic substrate($(AAAA)_2$-R110).

The use of $(AAAA)_2$-R110 and the aptamer-coated 96-well microplates also allows the sensitive detection of HNE. The biotinylated aptamers were immobilized on a streptavidin coated black 96-well plate. Following the affinity capture of HNE by the aptamers on the microplates and the subsequent cleavage of the fluorogenic peptide substrate by HNE, HNE was detected on the aptamer-coated microplates. When 2-h enzyme reaction was applied in the assay, HNE (100 μL) in the concentration range of 0.5 pM–0.5 nM could be detected. When 24-h enzyme reaction was applied, as low as 0.05 pM HNE (100 μL, corresponding to 5 attomole HNE) could be detected. The sensitivity of the assay

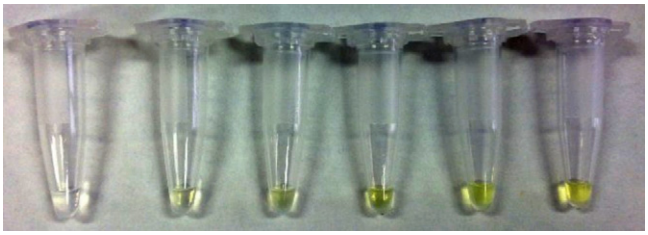

Fig. 3. Color change of the collected enzymatic product solution (20 μL) corresponding to various concentrations of HNE (250 μL) in the aptamer-capture based assay using the chromogenic substrate and the aptamer modified magnetic beads. 24-h Enzyme reaction was applied. The corresponding concentrations of HNE were 0, 4 pM, 10 pM, 20 pM, 40 pM, and 0.1 nM (from the left to the right). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Detection of HNE by using the chromogenic substrate of Meo-Suc-AAPV-pNA with respect to aptamer modified solid support, sample volume of HNE, the time of enzyme reaction, the linear range, the calibration equation, and the concentration limit of detection (CLOD).

Solid support	Volume (μL)	Time (h)	Linear range	Calibration equation[a]	CLOD
Magnetic beads	5	2	0.2 nM–20 nM	$y=0.0567x+0.012$, R^2 0.993	0.2 nM
Magnetic beads	250	2	4 pM–0.4 nM	$y=2.900x+0.007$, R^2 0.999	4 pM
Magnetic beads	250	24	0.4 pM–40 pM	$y=28.495x+0.020$, R^2 0.992	0.4 pM
Microplates	100	2	20 pM–5 nM	$y=0.372x+0.026$, R^2 0.994	20 pM
Microplates	100	24	2 pM–0.5 nM	$y=3.466x+0.025$, R^2 0.990	2 pM

[a] The concentration unit for x in the calibration equation was nanomolar (nM).

using the aptamer-coated 96-well microplates is comparable to that obtained in the assay using aptamer modified magnetic beads. Table 2 summarizes the results of detection of HNE by using the fluorogenic substrate $(AAAA)_2$-R110 and the aptamer modified magnetic beads (the aptamer modified microplates). In addition, the fluorogenic substrate (Meo–Suc–AAPV–AMC) could also be applied in the assay for HNE using aptamer modified microplates (data not shown), and the obtained sensitivity was comparable to our previous work using aptamer-modified beads [31]. Comparing with the previous aptamer-based assays for HNE [23,30] and enzymatic assays for HNE [8,13,14], our method allowed for higher sensitivity and lower detection limit. The high sensitivity results from the amplification of enzyme reaction and the sample preconcentration by affinity capture on solid phase. In our assays, faster analysis for HNE with high concentration can be obtained by applying short-time enzyme reaction of cleavage of substrates to meet the demand of rapidity.

3.4. Specificity analysis

To investigate the specificity of the aptamer-capture based assays for HNE, HNE was tested along with other proteins. The tested proteins include the abundant proteins in blood (e.g. IgG, HSA, and transferrin) and enzyme molecules (e.g. trypsin, proteinase K, chymotrypsin, thrombin, lysozyme, porcine pancreatic elastase (PPE), proteinase 3, and cathepsin G). These tested proteins and enzymes did not cause any interference in the assays (Fig. 5 and Fig. S4 in the Supplementary material). In addition, detection of HNE was not interfered by the presence of high concentration of IgG, HSA, and transferrin. The results show the aptamer-capture based assay has good specificity in the detection of HNE. This high specificity can be attributed to the introduction of two recognition events in the assay procedures, the selective affinity binding by aptamers in the capture procedure and the specific recognition of substrates in the enzyme reactions. Our assays can overcome the possible limitation of enzymatic assays only using chromogenic or fluorogenic substrates in selectivity [8]. The combination of two recognition events in our assays can enable the high selectivity similar to that is obtained by the sandwich format assays, which require two affinity ligands for the same analyte.

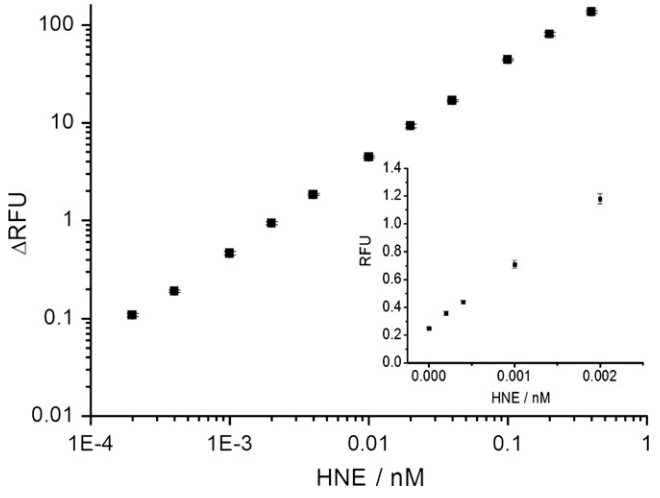

Fig. 4. Detection of HNE by using the aptamer modified magnetic beads and the fluorogenic substrate of $(AAAA)_2$-R110. 250 μL of HNE was detected, and 2-h enzyme reaction was applied. Δ RFU was the net increase of fluorescence over the control, obtained by subtracting the background fluorescence from the control that did not contain HNE. The inset shows the fluorescence signals obtained from the control and low concentrations of HNE.

3.5. Recovery test

To test the applicability of the assay for complex samples, we took dilute human serum as sample matrix. HNE at various concentrations was spiked into 100-fold diluted human serum, and was analyzed in the assay using the fluorogenic substrate $(AAAA)_2$-R110 and the aptamer modified magnetic beads because of the high sensitivity of this assay. The recovery of HNE spiked in the diluted serum was found to be below 7%. (Fig. 6) The low recovery of the spiked HNE was caused by the inhibitors (e.g. alpha-1-antitrypsin) of HNE in human serum, which inactivated the spiked HNE by forming complex of inhibitors and HNE, as previously reported [1–3,36,37]. To avoid the effect of inhibitors

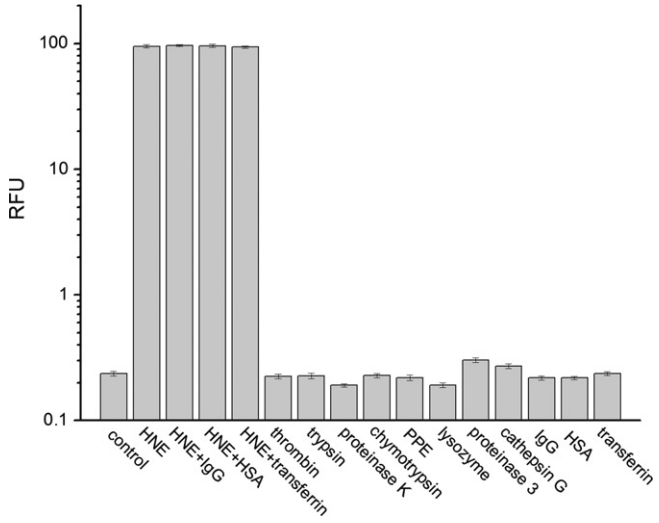

Fig. 5. Specificity of the aptamer-capture based fluorogenic assay for HNE. HNE (10 nM) and other proteins were tested by using the fluorogenic substrate of $(AAAA)_2$-R110 and the aptamer-modified magnetic beads. The concentrations of thrombin, trypsin, proteinase K, chymotrypsin, porcine pancreatic elastase (PPE), lysozyme, proteinase 3, cathepsin G, IgG, HSA, and transferrin were 10 nM. HNE (10 nM) was also tested in the presence of IgG (1 μM), HSA (1 μM), or transferrin (1 μM). 5 μL of protein sample was analyzed, and 2-h enzyme reaction was applied.

Table 2
Detection of HNE by using the fluorogenic substrate of $(AAAA)_2$-R110 with respect to the aptamer modified solid support, the sample volume of HNE, the time of enzyme reaction, the linear range, the calibration equation, and the concentration limit of detection (CLOD).

Solid support	Volume (μL)	Time (h)	Linear range	Calibration equation[a]	CLOD
Magnetic beads	5	2	10 pM–10 nM	$y=7.674x+0.926$, R^2 0.997	10 pM
Magnetic beads	250	2	0.2 pM–0.2 nM	$y=408.9x+0.403$, R^2 0.998	0.2 pM
Magnetic beads	250	24	0.02 pM–0.02 nM	$y=4287.9x+0.708$, R^2 0.997	0.02 pM
Microplates	100	2	0.5 pM–0.5 nM	$y=198.4x+0.606$, R^2 0.997	0.5 pM
Microplates	100	24	0.05 pM–0.05 nM	$y=1913.2x+0.741$, R^2 0.998	0.05 pM

[a] The concentration unit for x in the calibration equations was nanomolar (nM).

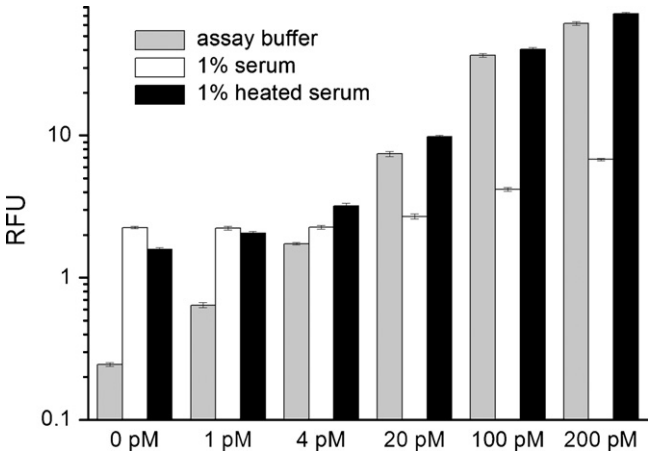

Fig. 6. Recovery test of HNE spiked in the 100-fold diluted human serum and recovery test of HNE spiked in the 100-fold diluted human serum that was heated. 250 μL of HNE was tested by using the aptamer-modified beads and the fluorogenic substrate of $(AAAA)_2$-R110. 2-h Enzyme reaction was applied.

on the recovery test, we heated the serum sample at 65 °C for 30 min to inactivate the inhibitors according to the previous report [38], and then HNE was spiked in the diluted heated serum. In this case an average recovery of the spiked HNE was obtained to be about 110% (Fig. 6). It suggests that serum sample matrix does not affect the detection of the free HNE of serum.

We successfully detected the free HNE in human serum by measuring the diluted serum. According to the calibration equation obtained by the standard HNE, the level of the free active HNE in serum was determined to be about 230 pM (Fig. S5 in the Supplementary material). The result was not validated by other methods for HNE detection. It shows that this assay is applicable to the detection of active HNE in complex samples, and has potentials in the biomarker detection for disease diagnosis.

4. Conclusion

In summary, we developed aptamer-capture based assays for HNE by using the aptamer modified magnetic beads or the aptamer-coated 96-well microplates. HNE was captured by the aptamers on solid supports, and then HNE catalyzed the conversion of substrates (chromogenic or fluorogenic substrates) to products. HNE was detected by measuring the generated products. The assays exhibited high sensitivity through the sample preconcentration by aptamers and the signal amplification by enzyme reaction. The chromogenic assays offer benefits in simplicity and low cost, and the fluorogenic assays show higher sensitivity. The assays exhibited good specificity, and enzyme molecules sharing similar structure of HNE did not cause any interference in the analysis of HNE. The use of 96-well microplates in the assay shows advantages in fast sample handling and rapid analysis. The aptamer-based assay for HNE is applicable to the detection of biomarkers and shows potential in disease diagnostics.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.11.016.

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