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Peptide-Functionalized Spherical Polyelectrolyte Nanobrushes for Real-Time Sensing of Protease Activity

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Proteases play vital roles in physiological processes from simple protein catabolism to highly regulated cascades, such as blood coagulation, cell regulation and signaling and immune defense, as well as pathological processes, including inflammation and tumor growth.^[1] Consequently, interest in the activity of proteases, as well as their inhibition mechanism, is increasingly directed toward biochemical research, diagnosis of protease-related diseases, and the development of potential therapeutic drugs. Over the past decade, many methods for detecting protease activity have been reported, such as high-performance liquid chromatography (HPLC),^[2] polyacrylamide gel electrophoresis,^[3] capillary electrophoresis,^[4] and FRET-based methods (fluorescence resonance energy transfer),^[5] but most of them are time-consuming and impractical for a real-time, multiplex or high-throughput format. Therefore, there is a high demand for a simple, rapid, sensitive, and high-throughput routine assay to detect protease activity. Recently, some inorganic nanomaterials, such as quantum dots and gold nanoparticles, have been widely explored for use in protease assays.^[6] Herein, we demonstrate that peptide-functionalized spherical polyelectrolyte brushes (SPB), as a new type of biocompatible colloidal nanostructure with good water-solubility, can be exploited to generate a highly sensitive fluorescence polarization (FP) assay for protease activity. SPB consists of a solid polystyrene core with a diameter of 50–100 nm onto which linear polyelectrolyte poly(acrylic acid) chains (PAA) are grafted.^[7] By its excellent dispersibility and good retention, PAA has been shown to be an attractive carrier for drug delivery systems, biosensors, immunoassays, and biocatalysts. Ballauff group have done a systematic work of nanobrushes (SPB) in the biological application,^[8] especially in the protein immobilization onto the SPB. Scheme 1 illustrates the principle of the SPB-based FP assay proposed in this work. Briefly, fluorophore-labeled peptide substrates are rapidly assembled onto the thin polyelectrolyte brushes to form Peptide-SPB composite probes, which, in turn, are capable of displaying a high fluorescence polarization value. The FP value of a fluorophore is sensitive to changes in the rotational motion of fluorescently labeled molecule. Fluorophore-labeled peptides confined in SPB nanobrushes will have bigger anisotropy values due to their slow rotation. In the presence of protease, the Peptide-SPB composite probes are recognized and specifically cleaved to release the fluorophore-labeled peptide segments, resulting in depolarization of the system. This SPB-

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peptide hydrolysis event is then translated into measurable FP decrease by free cleaved dye-labeled peptide segments, which, in turn, results in the sensing of protease activity.

The most important feature of polyelectrolyte (PE) brushes is the strong confinement of counterions within the brush and their distribution along the polymer backbone. We investigated the adsorption of different biomolecules (nucleic acids, peptides, and protein) from the solution onto the brush shell chains of SPB. As expected, more than 90% of the positively charged peptides were adsorbed onto the SPB particles in a quick equilibration within 10 min, which also showed the excellent stability of Peptide-SPB composite probes against coagulation and leaching out of the peptides. By titrating peptide to SPB solution, the number of peptides per SPB nanoparticle was estimated to be 250 (Figure S1).

The method was tested with thrombin, a serine protease responsible for playing a pivotal role in hemostasis and blood clotting by selectively cleaving Arg-Gly bonds in fibrinogen to form fibrin and platelet activation.^[9] The FITC-labeled peptide 1 (Lys-Cys-Ala-Leu-Asn-Asn-Gly-Ser-Gly-dPhe-Pro-Arg-Gly-Arg-Ala-Lys(FITC)-OH), of which the core peptide chain dPhe-Pro-Arg-Gly was designed according to the reported material,^[10] was determined to be one of the best thrombin substrates. Therefore, the prepared peptide 1-functionalized SPB (Peptide-SPB composite probes), corresponding to an initial FP value of ~310 mP, were utilized for thrombin activity detection. Addition of thrombin (2.5 units) to the Peptide-SPB composite probes solutions with the different concentrations resulted in time-dependent decreases in fluorescence polarization (Figure 1A). Specifically, the peptides of Peptide-SPB composite probes were selectively cleaved at the Arg-Gly bonds gradually, and then the cleaved FITC-labeled peptide was released from the SPB to the solution with faster rotation, resulting in depolarizing the system and lowering the FP value. From these data, the kinetic parameters for hydrolysis of Peptide-SPB composite probes by thrombin were determined (Figure 1B). The $V_{\max}$ was calculated to be $0.15 \pm 0.03 \mu\text{M}/\text{min}$, and the k_{cat} was 0.30 min^{-1} , which were comparable to the literature values of $0.2 \pm 0.01 \mu\text{M}/\text{min}$ and 0.20 min^{-1} .^[6c] The K_M value is $3.45 \pm 1.95 \mu\text{M}$, which was 7.5-fold greater than the reported value of $0.46 \pm 0.12 \mu\text{M}$.^[6c] The high K_M value may have resulted from the phenomenon in which some peptides of the innermost SPB brushes are inaccessible for thrombin. The SPB-based FP assay is also suited to a quantitative determination of the amount of thrombin present in solution. Figure 1C illustrates the real-time change in FP value with different concentrations of thrombin. The assay allowed for the detection of thrombin activity at concentrations as low as 1.0×10^{-3} units (1.85~5.55 nM) based on the three times of standard deviation (3σ). At a thrombin concentration of 1.0×10^{-3} units (1.85~5.55 nM), the rate of the hydrolysis of peptide was $0.0245 \mu\text{M}/\text{min}$ (Figure 1D). This detection limit was better than the recently reported gold nanoparticle methods of 20 nM^[11] and 5 nM,^[6a] as well as electrochemical sensors of 1 nM^[12] and 0.5 nM,^[13] considering the low enzyme activity used in this work (50-150 NIH units/mg protein). The substantial improvement of our method in the sensitivity is mainly attributed to the slower rotation of fluorescence unit when dye-labeled peptides were confined in the nanobrushes shell of SPB.

Interest in the screening of thrombin inhibitors is rapidly increasing for anticoagulant drug development. Therefore, the inhibition of thrombin activity by the two known anti-thrombin aptamers was investigated using the SPB-based FP assay. One is 15-mer ssDNA (aptA: 5'GGTTGGTGTGGTTGG3'), which forms an intramolecular quadruplex structure and primarily binds to thrombin at the fibrinogen-recognition exosite.^[14] The other one is 29-mer ssDNA (aptB: 5'AGTCCGTGGTAGGGCAGGTTGGGGTGACT3') binding at the heparin-binding exosite with a higher affinity.^[15] Figure 2A shows representative plots of velocity derived from monitoring changes in the fluorescence polarization value for increasing concentrations of Peptide-SPB composite probes exposed to the thrombin (2.5 units) and the aptamers (10 μM). The inhibition constant (K_i) values for aptA and aptB were

estimated to be $40 \pm 15 \mu\text{M}$ and $20 \pm 14 \mu\text{M}$, respectively (Figure 2B). When the two aptamers were exposed to the thrombin simultaneously, the K_i value was reduced to $11 \pm 4 \mu\text{M}$, which was much smaller than any one of the individual aptamers alone. This result clearly indicated that the two aptamers have different binding sites on the thrombin. At the same time, we observed that the random nucleic acid of DNA 4 had no effect on thrombin, while aptB displayed a stronger inhibitory function of thrombin than aptA, which was in a good agreement with the reported result. [15]

Following the successful demonstration using the SPB-based FP method to study the inhibition of two anti-thrombin aptamers, we further investigated the applicability of the method for the high-throughput screening of a library of inhibitors. To achieve this, four known thrombin inhibitors, including α -iodoacetamide, sodium fluoride, and two aptamers, together with a set of 103 organic compounds (terpenoids, neonicotinoids, benzothiazinoids), were used to screen the potential inhibitors of thrombin. The experiment was conveniently carried out on a 384-well plate with all samples in duplicate. Because the organic compounds are insoluble in water, all compounds were dissolved in DMSO, which is commonly used as a cosolvent. First, we assessed the influence of DMSO concentration on the FP signal in this work. The experimental results demonstrated that DMSO concentrations up to 2% had no effect on FP measurement and were also well tolerated by thrombin and trypsinase (data not shown). The thrombin (2.5 units) was preincubated with the known inhibitors or the compound (20 μM) before adding the Peptide-SPB composites probes solution (6 μM). Then the mixture was incubated for 2 h before FP measurement. In the screening experiment, we selected the negative-control reactions without added compounds and positive-control reactions with known thrombin inhibitor to set low and high boundaries for FP signals, respectively. In this work, a “hit” was defined as compounds that reduced the FP signal by 50% relative to control reactions. As expected, the α -iodoacetamide (51.8%), sodium fluoride (60.1%), aptA (55.9%), and aptB (65.2%) all showed obvious inhibitory effect on the thrombin, while none of the organic compounds exhibited a significant inhibitory effect on thrombin (Figure 3). These results, in turn, show that the method is suitable for high-throughput applications in the drug discovery of specific protease inhibitors.

In summary, we have developed a simple and sensitive protease assay that utilizes the self-assembled Peptide-SPB probe to enhance the fluorescence polarization signal, which enables the real-time detection of protease activity. This novel SPB-peptide probe design offers many advantages, including simplicity and rapidity of preparation and manipulation compared to methods employing specific synthetic strategies. The SPB-based FP assay demonstrates superior sensitivity. The limit of detection of thrombin activity could be significantly improved to 1.0×10^{-3} units (1.85~5.55 nM), indicating that our system is one of the most sensitive protease detection systems. Moreover, our Peptide-SPB probe design can be extended to develop a variety of probes by simply changing the peptide sequence for measuring the activity of other proteolytic enzymes and kinases. The assay can be carried out in 96- or 384-well plates, making it suitable for routine high-throughput applications in drug development and biotechnology. Overall, our results demonstrate that the colloidal spherical polyelectrolyte brush, as a new class of nanostructures, can provide an excellent platform for the development of rapid and sensitive assays for sensing protease activity and high-throughput screening of inhibitors.

Experimental Section

Preparation of Peptide-SPB composite probes

Spherical polyelectrolyte brushes were synthesized by photo-emulsion polymerization according to the method reported in the literature. [16] The Peptide-SPB composite probes

were freshly prepared as follows. A solution of SPB (1 mL, 10 nM) was centrifuged at 13,000 rpm at room temperature (RT) for 20 min, and the supernatant was removed. The white precipitate was suspended in 99 μ L buffer (20 mM Tris-HCl/0.1 M NaCl/2.5 mM CaCl₂, pH 8.4), and 1.0 μ L peptide solution (5 mM) was added. Then the mixture was incubated for 10 min at RT with gentle shaking.

Monitoring of thrombin activity

The substrate solutions of 98 μ L (peptide 1 or Peptide-SPB composite probes) in the buffer (20 mM Tris-HCl/0.1 M NaCl/2.5 mM CaCl₂, pH 8.4) were added into a 384-well microtiter plate. Then, 1.0 μ L aptA and/or aptB or H₂O was added to the corresponding wells. Finally, 1.0 μ L thrombin was added to the wells, and the fluorescence reading was begun immediately (excitation 485 nm, emission 535 nm), following a 60-min period and a 4-min interval at RT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Thrombin proteolytic monitoring assay. A) Progress curves at different concentrations of Peptide-SPB composite probes (1-18 μM , corresponding to peptide concentrations). Thrombin of 2.5 units was used. B) Plot of the initial velocities for an increasing concentration of Peptide-SPB composite probes at a constant amount of thrombin (2.5 units), and the data were fit by nonlinear least-squares to the Michaelis-Menten equation. C) Progress curves at different concentrations of thrombin. D) Plots of the initial velocities for the studied concentrations of thrombin. Peptide-SPB composite probes of 6 μM were used.

Figure 2.

Thrombin inhibition assay using thrombin aptamers. A) Plots of the initial velocities against Peptide-SPB composite probes in the absence and presence of aptA, aptB or DNA 4, and the data were fit by nonlinear least-squares to the Michaelis-Menten equation. B) Lineweaver-Burk (L-B) double reciprocal plot of the same data for determination of K_i values.

Figure 3. Screening for thrombin inhibitors. A screening of 107 compounds identified 4 hits (1: aptB, 2: aptA, 3: α -iodoacetamide, 4: sodium fluoride) that reduced the FP signal by 50% relative to control reactions in the absence of added compound.

Scheme 1.

Schematic illustration of the strategy using Peptide-SPB composite probes as substrates to monitor the proteolytic activity of thrombin. The colloidal particles consist of a solid polystyrene (PS) core onto which cationic polyelectrolyte poly(acrylic acid) (PAA) chains are grafted. The peptide was labeled with fluorescein isothiocyanate (FITC). The cleavage point at Arg-Gly bonds are indicated by dashed line. The chemical structural formula of FITC and its coupling site with peptide are provided at the bottom right. FITC is the base fluorescein molecule functionalized with an isothiocyanate reactive group ($-N=C=S$) on the bottom ring of the structure, which is reactive towards amine group of the lysine.