

Chapter 2

Nanomaterial-Enhanced Fluorescence Polarization and Its Application

2.1 Principles of Fluorescence Polarization

Fluorescence polarization (FP) was first applied in biochemistry in the 1950s, when Gregorio Weber reported his studies on bovine serum albumin and ovalbumin conjugated with 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride) [1]. FP methods have become increasingly popular following Weber's work during the past few decades. The increase in the number and diversity of fluorescence polarization studies has been astonishing and the method is an intrinsically powerful technique for the rapid and homogeneous analysis of molecular interactions in biological/chemical systems. The technique has been successfully employed in many diagnostic fields including the detection and quantitation of antigens or antibodies based upon antibody–antigen interactions, protein-DNA interactions, protein-RNA interactions, DNA detection and the monitoring of therapeutic drug levels. It has also been used in monitoring enzyme-catalyzed hydrolysis, protein–protein interactions, and high-throughput screening during the course of drug discovery.

The fluorophore rotation can be calculated in a more quantitative manner [2]. Depolarization of the emission occurs if the fluorophore, and hence the emission dipole, rotates through an angle ω between the moment of excitation and emission. In fact,

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3} \right) \left(\frac{2}{3 \cos^2 \omega - 1} \right) \quad (2.1)$$

where P is the observed polarization. The total depolarization is determined by an intrinsic factor (P_0) and an extrinsic factor (ω). Perrin related the observed polarization to the excited state lifetime and the rotational diffusion of a fluorophore to derive the famous equation [3],

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3} \right) \left(1 + \frac{RT}{\eta T} \tau \right) \quad (2.2)$$

where P is the observed polarization, P_0 is the limiting or intrinsic polarization, V is the effective molar volume of the rotating unit, R is the universal gas constant, T is the absolute temperature, η is the viscosity, and τ is the excited state lifetime.

For a spherical molecule, it follows that,

$$\rho_0 = \frac{3\eta V}{RT} \quad (2.3)$$

For a spherical protein, it follows that,

$$\rho_0 = \frac{3\eta M(v + h)}{RT} \quad (2.4)$$

where M is the molecular weight, v is the partial specific volume, and h is the degree of hydration.

Combined with the Eq. 2.1, the Eq. 2.2 can be rewritten as follows,

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3} \right) \left(1 + \frac{3\tau}{\rho} \right) \quad (2.5)$$

where ρ is the Debye rotational relaxation time, which is the time for a given orientation to rotate through an angle given by the $\arccos e^{-1}$, which is 68.42° .

From Perrin equation (2.5), one can obtain that the FP value P is sensitive to a change in any of the three parameters ρ , τ , or P_0 . Experimentally, τ is depending on the probe environment, with changes often associated with a shift from a hydrophilic to hydrophobic environment, or vice versa. Changes in the P_0 at a fixed excitation wavelength are uncommon, but changes in the effective P_0 can occur when energy transfer is present, resulting in a measured P_0 that is lower than the true P_0 . Therefore, the FP value of a fluorophore is proportional to its rotational relaxation time, which in turn depends upon its molecular volume (molecular weight) at the constant temperature and solution viscosity. In a system where a random array of molecules is freely rotating, the FP value will be between 0 and 500 mP depending upon the rotation rate of the molecules. If a molecule is small, it will rotate faster and hence will have a smaller FP value. Conversely, the larger molecules will have bigger FP values due to their slow rotation.

Another term frequently used in the context of polarized emission is anisotropy, designated as r , which is calculated as

$$r = \frac{2P}{3 - P} \quad (2.6)$$

By analogy to polarization, the limits of anisotropy in completely oriented systems are +1 to -0.5 [2].

2.2 Sensitive Detection of Hg^{2+} by Fluorescence Polarization Enhanced by Gold Nanoparticle

Colloidal gold is a suspension (or colloid) of sub-micrometer-sized particles of gold in water, usually. The liquid displays either an intense red color (for particles less than 100 nm), or a dirty yellowish color (for larger particles). Gold nanoparticles (AuNPs) are colloidal gold particles with dimensions ranging from 1 to 100 nm. AuNPs possess unique optical, electronic, catalytic, and molecular-recognition properties, which make them as the research subject in a wide variety of areas, including electron microscopy, electronics, nanotechnology, and materials science. Especially, enhancement functions of AuNPs have been employed for signal generation and transduction in developing novel biosensing systems, in order to substantially improve the performance and sensitivity. For example, the enhanced effects were implemented by the use of AuNPs as labels for the amplified quartz crystal microbalance (QCM) detection and electrochemical detection. AuNPs can be used as electron relays to facilitate the interfacial electron transfer on electrode, or as fluorescence enhancer and quencher. AuNP-enhanced effects in a responsive polymer gel and in network field-effect transistors have also been reported.

In the published papers, the amplified signals and improved sensitivity of the different biosensing systems by incorporating AuNPs have been revealed. For example, AuNPs were employed to enhance microgravimetric quartz-crystal-microbalance measurements. Willner's group developed a novel method, based on the AuNP-catalyzed deposition of gold, for the amplified microgravimetric QCM detection of DNA [4] and thrombin [5]. The detection sensitivity of QCM for DNA can be significantly improved to 10^{-15} M by using AuNPs as "seeding catalyst" for gold precipitation on the transducer. Yan et al. [6] described the systematic evolution of improved electrical contacting between glucose oxidase and the electrode by the biocatalytic enlargement of AuNPs tethered to the enzyme electrode. They demonstrated that the enlargement of AuNPs facilitated the interfacial electron transfer, thereby enhanced the mediated bioelectrocatalyzed oxidation of glucose, and substantially improved the bioelectrocatalytic performance of the enzyme electrode. Dong et al. [7] reported that the detection sensitivity of single-walled carbon nanotube network field-effect transistors (SNFETs) for DNA can be further improved to ca. 100 fM by using AuNPs, in which the target DNAs were hybridized with probe DNAs on the device, and reporter DNAs labeled with AuNPs flank a segment of the target DNA sequence. Fan et al. [8] developed a "sandwich-type" detection strategy for sequence-specific detection of femtomolar DNA via a AuNPs-mediated amplified chronocoulometric DNA sensor. Minko et al. [9] investigated localized plasmon resonance excited in AuNPs coupled with a responsive polymer gel. The results demonstrated that a specially designed structure (vertically aligned cylindrical pores decorated with gold nanoparticles) of responsive polymer gel thin films were used as a platform for the transduction of external signals/stimuli into a strong optical effect that is enhanced by interactions of AuNPs.

Contamination of the environment with heavy metal ions has been an important concern throughout the world for decades. Mercury accumulated in vital organs and tissues, such as the liver, brain, and heart muscle, is a highly toxic material with lethal effects on living systems. Mercury originates mainly from coal-burning power plants, oceanic and volcanic emissions, gold mining and combustion of waste [10]. Also, microbial biomethylation of Hg^{2+} yields methyl mercury, a potent neurotoxin that concentrates through the food chain in the tissues of fish and marine mammals [11, 12]. Therefore, it should be highly desirable to develop a sensitive and selective mercury detection method that can provide simple, practical, and high-throughput routine determination of Hg^{2+} level for both environmental and food samples. Much effort has been devoted toward the design of Hg^{2+} sensing systems, including sensors based on organic chromophores [13–17] or fluorophores [18–21], conjugated polymers [22], DNAzyme [23], gold nanoparticles [24], semiconductor quantum dots [25], proteins [26], and genetically engineered bacteria [27]. However, most of these methods have either limitations with respect to poor selectivity with interference from closely related metals, or insufficient sensitivity [limit of detection (LOD) > 100 nM], and in certain cases not stable or functional in aqueous media due to low water solubility.

Another emerging approach for the detection of Hg^{2+} ion involves the use of oligonucleotide. Recent studies have demonstrated that Hg^{2+} ions can specifically interact with thymine bases to form strong and stable thymine- Hg^{2+} -thymine complexes (T- Hg^{2+} -T). Various Hg^{2+} ion detection assays based on this property of T- Hg^{2+} -T coordination chemistry have been developed in recent years [28–31]. Ono and Togashi [28] described the simple method based on the Hg^{2+} -induced formation of DNA folding that yields an intramolecular fluorescence resonance energy transfer process to detect Hg^{2+} with high selectivity and sensitivity (up to 40 nM). Mirkin et al. [29] recently reported the colorimetric detection of Hg^{2+} in aqueous media using DNA-functionalized gold nanoparticle probes with deliberately designed T–T mismatches (sensitivity up to 100 nM). Further, Liu et al. [30] developed a one-step, room temperature, colorimetric assay of Hg^{2+} using DNA/nanoparticle conjugates (sensitivity of 1 μM). According to the U.S. Environmental Protection Agency (EPA) standard, the maximum allowable level (MAL) of Hg^{2+} in drinking water is 10 nM (2.0 ppb). This concentration is much lower than the detection limit of most available assays. Thus, the development of facile and practical assay for Hg^{2+} detection with higher sensitivity remains a challenge.

In this section, we apply fluorescence polarization assay (FPA) to analyze Hg^{2+} ion using a “AuNPs enhancement” approach [32]. The large surface area of AuNPs can increase the amount of assembled molecules, effectively raise the complex size of recognition molecule and target molecule, thus reach the aim of signal amplification, which significantly improve the detection sensitivity and stability. Our method to analyze Hg^{2+} ion is based on the formation of T- Hg^{2+} -T complexes. The detection sensitivity can be significantly improved to 0.2 ppb (1.0 nM) by using the “AuNP enhancement” approach. The outline of the method is shown in Fig. 2.1. Two complementary probes (Probe A, AuNP-5'S-A₁₀-GCTTCTGTTCTCT3'; and Probe B, 5'FAM-GTTGTGTTTCAGTTGC3'), which

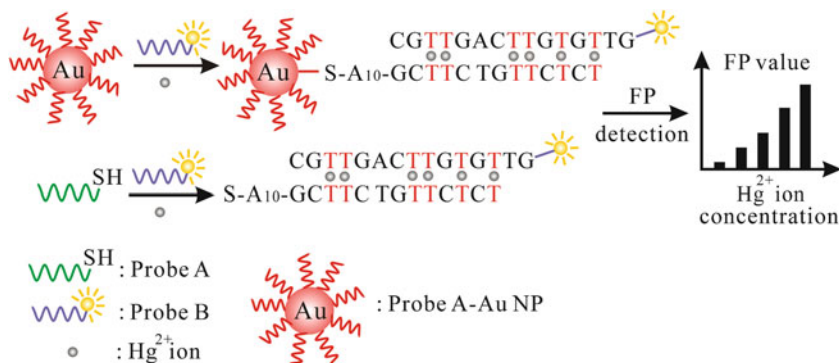


Fig. 2.1 Schematic illustration of the strategy for Hg^{2+} ion detection using fluorescence polarization via gold nanoparticle enhancement. Reproduced from Ref. [32], with the permission of Wiley John and Sons

contain strategically placed six thymine–thymine mismatches complexed with Hg^{2+} ions, were prepared. Probe A was modified with AuNPs (13 nm in diameter). Probe B was labeled with the fluorescence dye, 6-fluorescein-CE phosphoramidite (FAM). The stable hybridization between Probe A and Probe B occurs only when Hg^{2+} ion is present. From Perrin equation (2.5), the *FP* value will significantly increase when a fluorophore-labeled Probe B binds to AuNPs surface in the presence of Hg^{2+} ions.

AuNPs may enhance or quench the dye fluorescence, depending upon the size and the precise distance between the dye and AuNP [33–35]. At the small distances (1–3 nm), AuNPs can serve as ultra-efficient quenchers of molecular excitation energy in fluorophore-AuNP composites [36–39], outranging the quenching efficiency of organic acceptor molecules. Schneider et al. [40] employed the layer-by-layer deposition of oppositely charged polyelectrolytes onto 13 nm-diameter gold colloids to fabricate metal core-polymer shell capsules, in which fluorescent organic dyes fluorescein and lassamine rhodamine B were situated at various distances from the gold core. They found that the gold nanocore quenched the fluorescence of the fluorescein and lassamine dyes significantly with 20 spacer layers. Thus the large fluorescence quenching efficiency of AuNPs ($\text{QE} > 98\%$) has been employed for DNA detection. Li and Rothberg [39] reported a sensitive detection assay of oligonucleotide sequences using the different electrostatic properties of single- and double-stranded DNA (ss-DNA and ds-DNA) to absorb on citrate-coated gold nanoparticles. The unhybridized oligos labeled with fluorescence would efficiently adsorb onto the gold nanoparticles so that their fluorescence was quenched. Dubertret et al. [36] described a novel molecular beacon using a hybrid material composed of 1.4 nm diameter gold nanoparticle, an organic dye, and a 25 bp ss-DNA stem-loop molecule. That was, a nucleic acid probe with-hairpin shaped structure in which the 5' and 3' ends are self-complementary, bringing a fluorophore and a nanoparticle into close proximity to quench fluorescence. The results demonstrated that this composite

Table 2.1 Samples used in this experiment

Samples	Components (Final conc.)
#1	FAM-Probe B (10 nM)
#2	FAM-Probe B (10 nM) + AuNP-Probe A ^a (10 nM)
#3	FAM-Probe B (10 nM) + AuNP-Probe A (10 nM) + Hg ²⁺ (10 nM)
#4	FAM-Probe B (10 nM) + SH-Probe A (10 nM)
#5	FAM-Probe B (10 nM) + SH-Probe A (10 nM) + Hg ²⁺ (10 nM)
#6	FAM-Probe B (10 nM) + TAMRA-Probe A (10 nM)
#7	FAM-Probe B (10 nM) + TAMRA-Probe A (10 nM) + Hg ²⁺ (10 nM)

^a The concentration of AuNP-Probe A represented the oligo concentration, which was 10 nM; while the AuNPs concentration was 0.1 nM

molecule was a different type of molecular beacon with a sensitivity enhanced up to 100-fold. However, in those cases where the fluorophores and the AuNPs are separated by bulky spacers, such as antibodies and DNA, the fluorescence is less quenched. Moreover, enhanced fluorescence is frequently reported if the fluorophores are placed within 3–50 nm above a surface covered with nanostructured noble metals [41–45]. The effect has been called metal-enhanced fluorescence (MEF) or surface-enhanced fluorescence (SEF).

Considering the fluorescence quenching properties of AuNPs, the case that AuNPs would quench the fluorescence of FAM-labeled Probe B (FAM-Probe B, 5'-FAM-GTTGTGTTTCAGTTGC-3') and then result in non-uniformity of the assay, would probably occur. Therefore it is necessary to study the effects of AuNPs and molecular quencher (TAMRA) on fluorescence intensity (FI) and *FP* values in the biosensing system. In this study, we chose molecular quencher (TAMRA) to modify Probe A (TAMRA-Probe A, 5'-TAMRA-A₁₀-GCTTCTGTTCTCTAC-3') to carry out the molecular quencher experiment among DNA–DNA systems to compare the quenching effect performance between the nanoparticles and quencher. The thiol-modified oligonucleotides (SH-Probe A, 5'-SH-A₁₀-GCTTCTGTTCTCTAC-3') were used to functionalize AuNPs (13 nm in diameter) to obtain AuNP-modified DNA (AuNP-Probe A, 5'-AuNP-SH-A₁₀-GCTTCTGTTCTCTAC-3'). All measurements of fluorescence intensities and *FP* values were done in the same condition. Seven samples were prepared in 0.1 M NaNO₃/10 mM MOPS (pH 7.5) as listed in Table 2.1.

As shown in Fig. 2.2a, Samples #1, #2, #3, #4, and #5 had the similar FI (*F*₁, corresponding to FI of free FAM-Probe B of 10 nM), while the FI of Samples #6 and #7 was a little lower than *F*₁ due to the quenching property of TAMRA. When detecting the *FP*, there were almost the same *FP* values (*FP*₁, corresponding to *FP* value of free FAM-Probe B of 10 nM) among the samples #1, #2, #4, #5, #6 and #7, there was increased *FP* in sample #3 (a substantial increasing because of AuNP enhancement (Fig. 2.2b). However, the reasonable polarization changes of Samples #5 and #7 to Samples #4 and #6 were not observed at a low concentration of 10 nM Hg²⁺. This is because FPA has insufficient sensitivity without AuNP enhancement. These experiment results demonstrated that the gold nanoparticle has no quenching effect on the FI of FAM-labeled Probe B.

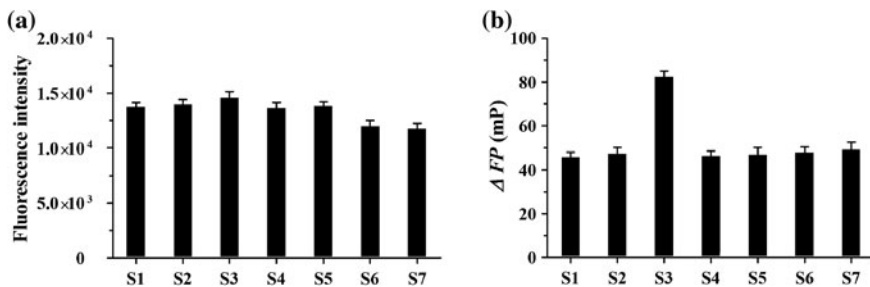


Fig. 2.2 The effects of AuNPs and TAMRA on the fluorescence intensity and FP value of dye-labeled Probe B. Reproduced from Ref. [32], with the permission of Wiley John and Sons

In the study conducted by Malicka et al. [43], a dense matrix of data for the dye-DNA-AuNP hybrid diameters versus ss-DNA length has been provided for many different surface coverages. The extrapolation of these data allows us to estimate the diameters of Probe A-AuNP hybrids used in this work. From the hybrid diameters $h \approx 32$ nm (oligo of 23 bp) we can derive the distance $d = (h - 13 \text{ nm}) / 2 = 9.5$ nm between the 3'-end part of Probe A and the surface of the AuNPs. The effective diameter depends on the conformation of oligos adsorbed on the AuNP. The stable hybridization of Probe A on the surface of the AuNP with FAM-labeled Probe B occurs when Hg^{2+} ion is present. For high surface coverage and ds-DNA, the DNA would be shown to be oriented perpendicular to the surface and fully stretched. The diameter between the conjugated fluorophoric part of the FAM molecules and the surface of the AuNPs should be larger than 9.5 nm. Our experimental results showed that the fluorescence was less quenched by AuNPs in the case where the fluorophores and the AuNPs were separated at above 9.5 nm by DNA oligos. Therefore, we believe the fluorescence quenching or enhancing properties of the gold nanoparticles have no effect on the AuNP-enhanced FPA of Hg^{2+} in this work.

To evaluate the sensitivity of the FPA enhanced by AuNPs, different concentrations of Hg^{2+} from one stock solution were tested. The mP value was sensitive to Hg^{2+} ions and increased as Hg^{2+} increased (Fig. 2.3). The detection limit of this method is approximately 1.0 nM (0.2 ppb) Hg^{2+} . It is lower than the MAL of Hg^{2+} ion in the drinking water of 10 nM (2 ppb) in the U.S. EPA. The result illustrated that the method has a wide range of detection of Hg^{2+} ion from 1.0 nM to 1.0 mM. The selectivity of the method was also investigated by testing the response of the assay to other metal ions, including Mg^{2+} , Pb^{2+} , Cd^{2+} , Mg^{2+} , Ca^{2+} , K^+ , Na^+ , Zn^{2+} , Fe^{2+} at a concentration of 10.0 μM , which is 1,000-fold in excess as compared to that for Hg^{2+} . The results demonstrated excellent selectivity over alkali, alkaline earth, and transition heavy metal ions. There was very little mP value increase observed in the presence of other metal ions. The specific detection for Hg^{2+} is mainly attributed to its chelating ability of T-T mismatches, resulting in the formation of stable T- Hg^{2+} -T complexes.

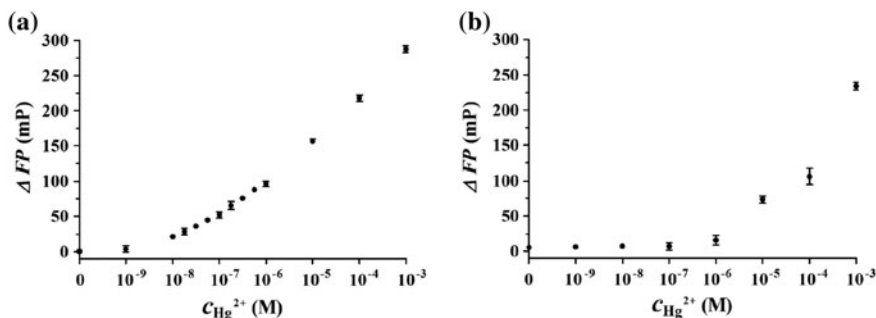


Fig. 2.3 Plots of fluorescence polarization changes as function of Hg^{2+} ion concentrations (*left*: AuNP-DNA; *right*: DNA-DNA). Reproduced from Ref. [32], with the permission of Wiley John and Sons

To assess the applicability of the method, the real samples and the spiked samples of river water were analyzed at two different concentrations (0.4 and 4 μ M). The method revealed the good recovery rates from 97.6 to 102.5%. No interference in river water was observed.

In summary, we developed a highly sensitive and selective Hg^{2+} determination method at room temperature using thymine- Hg^{2+} -thymine coordination chemistry and FP via gold nanoparticle enhancement. Our method demonstrated several analytical advantages. First, it has high sensitivity with 0.2 ppb, which can be 2–3 orders of magnitude more sensitive than other techniques. Second, it is highly selective, which allows detection of Hg^{2+} in the presence of an excess (1,000-fold) of other metal ions in samples. Third, it takes only approximately 10 min to determine the concentration of mercury in aqueous media. Last, the assay can be carried out in 96- or 384-well plates, can render it suitable for routine high-throughput applications. We believe that the method has enormous potential for application of mercury monitoring in environment, water, and food samples.

2.3 DNzyme-Self-Assembled Gold Nanoparticles for Determination of Cu^{2+} and Pb^{2+} Using Fluorescence Polarization Assay

For a long time it was believed that all enzymes are proteins and this thought has been changed with the discovery of the first natural ribozymes by Cech and Altman [46, 47]. A ribozyme is an RNA molecule with a well defined tertiary structure that enables it to catalyze chemical reactions, including RNA cleavage [48], splicing [49], and peptide bond formation in ribosomes [50]. The first deoxyribozyme was discovered in 1994-by Breaker [51]. Since then, intensive research has focused on the identification of the new nucleic acid enzymes, elucidation of mechanism of catalytic activity, and development of practical application. So far, all known

DNAzymes, obtained by a combinatorial method called *in vitro* selection or systematic evolution of ligands by exponential enrichment (SELEX) [51–55], are not only the static depository of genetic information, but also can catalyze a surprising variety of biochemical reactions, including RNA cleavage, DNA cleavage, RNA ligation, DNA ligation, RNA branching and lariat RNA formation, DNA phosphorylation, capping, deglycosylation, cleavage of the phosphoramidate bond, photocleavage of thymine dimers, porphyrin metalation, and other enzymatic activities as peroxidases [56]. DNAzymes are preferentially selected to develop optical or electronic sensor due to their impressive chemical stability compared with RNAzymes and other enzymes.

Similar to protein enzymes, one kind of DNAzyme formed by G-quadruplexes (also called G-quadruplex-based DNAzymes), is capable of recruiting hemin as a cofactor, mimics horseradish peroxidase (HRP) and catalyzes the H_2O_2 -mediated oxidation of 2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) or luminol. The G-quadruplex-based DNAzyme binding with hemin has peroxidase-like activity and has been extensively studied as colorimetric and/or chemiluminescence biosensors for the sensitive and specific detection of proteins, metal ions, and other biomolecules, indicating a great potential in biological applications. Willner's group pioneered the application of this peroxidase DNAzyme for signal amplification in analytical applications. For example, they developed DNAzyme-functionalized AuNPs as catalytic labels for the generation of biochemiluminescence [57]. The system was applied to detect DNA or telomerase activity. The DNAzyme stimulates, in the presence of hemin, H_2O_2 , and luminol, the generation of chemiluminescence. The method had a detection limit for the detection of DNA of 1×10^{-10} M, and enabled the detection of telomerase activity originating from 1,000 HeLa cells. The researchers also developed a number of applications of this DNAzyme. They introduced a new paradigm for the sensitive analysis of DNA by using a DNA-based machine that consists of a DNA template, polymerization/nicking enzymes, and strand displacement of the peroxidase-mimicking DNAzyme [58]. The isothermal and rapid (approximately 90 min) analysis of the target DNA, together with the quantitative detection of the analyte, demonstrate the appealing bioanalytical features of the method. They also employed a circular DNA as a template for the amplified detection of M13 phage ssDNA by a rolling circle amplification (RCA) process that synthesizes peroxidase-mimicking DNAzyme chains, thus enabling the colorimetric or chemiluminescent detection of the analyte [59]. This method had two amplification steps: the RCA synthesis of numerous DNAzyme units, as a result of a single recognition (hybridization) event, and the catalytic activities of the synthesized labels that lead to the colorimetric or chemiluminescent detection of DNA. A detection limit of 1×10^{-14} M was reported for this method.

Another kind of DNAzyme is a divalent metal ion cofactor-specific. Consequently, the development of DNAzyme-based sensors with that characteristic has begun to attract much attention to detect a variety of environmentally relevant metal ions, such as Cu^{2+} [60–63], Zn^{2+} [64–66], Pb^{2+} [67–70], Hg^{2+} [23, 71], UO_2^{2+}

[72] and Ca^{2+} [73]. Heavy metals are ubiquitous environmental contaminants, and their high toxicity makes their presence undesirable. Generally, metal ions are classified into two categories: essential and nonessential. Nonessential heavy metals like lead (Pb) and mercury (Hg) can cause a number of adverse health effects even at low-level of exposure. Essential metals such as copper (Cu) and zinc (Zn) are required to support biological activities. However, even these essential metals are toxic in excess [74]. A large number of heavy metals can cause disease or even death, constituting a serious threat to human health. Cu^{2+} and Pb^{2+} also pose serious environmental problems and are potentially toxic for all living organisms like other heavy metal ions. Due to their toxicity, the U.S. EPA has set the MAL-of copper and lead in drinking water as 20 μM (1.3 ppm) and 72 nM (15 ppb), respectively. Therefore, accurate determination of these metals at the trace level in environmental and biological samples has become increasingly important. Correspondingly, a number of analysis methods for the detection of heavy metal ions have been developed over the last few years, including not only atomic absorption spectroscopy [75], voltammetric detection [76], and inductively coupled plasma mass spectrometry [77] as classical detection methods, but also some other strategies such as sensors [78], surface plasmon resonance (SPR) [79], peptide [80], and peptide coated quantum dots [81]. Lu's group developed fluorescent sensors using DNAzyme for the detection of a number of metal ions. These studies demonstrated high sensitivity and selectivity. For example, they reported nanoparticle-based colorimetric sensors using DNAzyme-catalyzed ligation reaction for Cu^{2+} with a detection limit of $\sim 5 \mu\text{M}$ [63]. Furthermore, they developed fluorescent metal sensors using a Cu^{2+} -dependent DNA-cleaving DNAzyme with a detection limit of 35 nM (2.3 ppb) [82].

In this section, we present a novel highly sensitive and selective FPA for detecting Cu^{2+} and Pb^{2+} ion based on metal ion-dependent DNAzyme [83]. The detection sensitivity of Cu^{2+} and Pb^{2+} can be significantly improved to $\sim 1 \text{ nM}$ (Ca. 65 and 200 ppt) by using a "nanoparticle enhancement" approach. Figure 2.4 outlines the principle to use fluorescence anisotropy assay enhanced by AuNPs for the Cu^{2+} ion or Pb^{2+} ion detection. From Perrin equation, the fluorescence anisotropy (r) is sensitive to changes in the rotational motion of fluorescently labeled molecules. The r value of a fluorophore is proportional to its rotational relaxation time, which in turn depends upon its molecular volume (molecular weight). On the basis of Cu^{2+} -dependent DNA-cleaving DNAzyme, we first designed and prepared two DNA strands: DNAzyme named as Cu-Enz and its substrate DNA strand named as Cu-Sub. Cu-Sub DNA labeled with 6-fluorescein-CE phosphoramidite (FAM) at the 3' end and thiol group at the 5' end (Cu-Sub, 5' SH-T₂₀AGCTTCTTTCTAATACGGCTTACC-FAM3') was immobilized onto the surface of gold nanoparticle (13 nm) via a thiol-Au interaction. The Cu-Enz and Cu-Subs on AuNPs form the complexes through two base-pairing regions; the 5' portion of the DNAzyme binds the substrate via Watson-Crick base pairs and the 3'-region through formation of a DNA triplex (Fig. 2.4d). The FAM fluorophore in the "big" AuNPs-mediated Cu-Sub and Cu-Enz complexes displays the high anisotropy (Fig. 2.4a, b). In the presence of Cu^{2+} , the Cu-Enz as a recognition

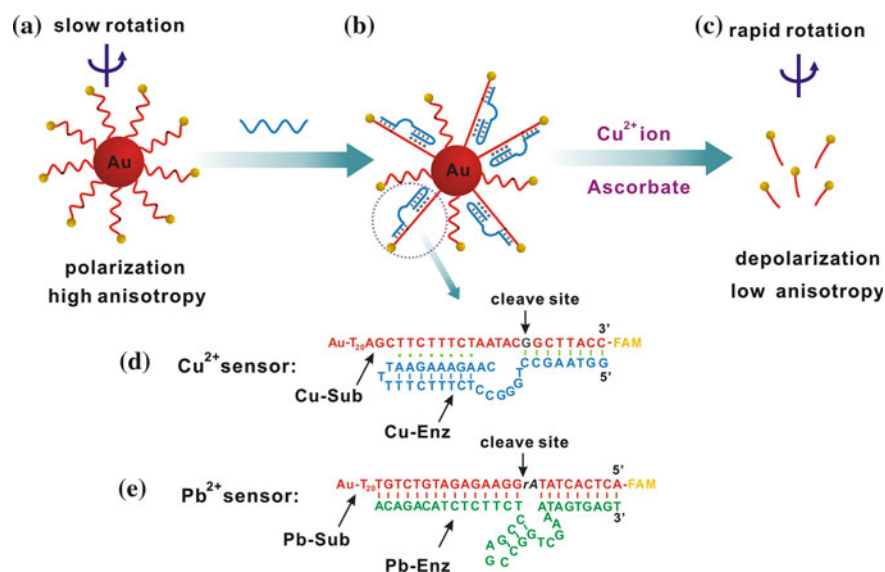


Fig. 2.4 Schematic illustration of the strategy of Cu^{2+} ion or Pb^{2+} ion detection using Cu^{2+} -dependent DNA-cleaving DNAzyme or Pb^{2+} -dependent DNA-cleaving DNAzyme fluorescence anisotropy via gold nanoparticles (AuNPs) enhancement. The point of scission is indicated by black arrow. Structural model and sequences for bimolecular DNA complexes of DNAzyme and its substrate for Cu^{2+} ion and Pb^{2+} ion are presented. The point of scission is indicated by black arrow. Reproduced from Ref. [83], with the permission of Elsevier Inc

element specifically cleaves AuNPs-mediated Cu-Sub at the guanine base (marked in black). The AuNPs-mediated Cu-Sub and Cu-Enz complexes are separated due to lacking thermal stability. The FAM-labeled cleaved products are released in the solution with rapid rotation and depolarize the light (Fig. 2.4c). Thus the anisotropy of the system will significantly decrease correspondingly. In the absence of Cu^{2+} , the AuNPs-mediated Cu-Sub and Cu-Enz associates would have stable high anisotropy. Therefore via the free fluorescent cleavage products, this cleave event is translated into a measurable fluorescence anisotropy decrease proportional to Cu^{2+} concentration. This design can also be easily expanded to Pb^{2+} ion detection, just displace the Cu-Sub and Cu-Enz with Pb-Sub and Pb-Enz. The structural model and sequences for bimolecular DNA complexes of Pb^{2+} -dependent DNA-cleaving DNAzyme and its substrate are also presented in Fig. 2.4e.

In order to verify the specificity of DNA-cleaving DNAzyme relied on the Cu^{2+} ion, we performed the control experiments to investigate if the DNA molecules (Cu-Sub) undergo cleavage in the absence of corresponding DNAzyme (Cu-Enz) and if the Cu^{2+} ion is used as the only cofactor. Three group experiments were carried out as follow: titrating Cu^{2+} ion, Hg^{2+} ion, or Pb^{2+} ion to the Cu-Sub functionalized AuNPs solution (Cu-Enz added), titrating Cu^{2+} ion, Hg^{2+} ion or Pb^{2+} ion to the Cu-Sub functionalized AuNPs solution (no Cu-Enz added), and titrating Cu^{2+} ion, Hg^{2+} ion, or Pb^{2+} ion to Cu-Sub functionalized AuNPs solution

with fully complementary DNA (Cu-Sub-Comp, 5'GGTAAGCCGTATT AGA-AAGAAGCT 3'). There was little anisotropy change when titrating Cu^{2+} , Hg^{2+} or Pb^{2+} ion to the Cu-Sub functionalized AuNPs (Cu-Enz added) solution. The results were the same when the tested metal ions were added to the solution containing Cu-Sub functionalized AuNPs and their complementary DNA. In contrast, when Cu-Enz coexisted with Cu-Sub, there would be a significant anisotropy change as Cu^{2+} ion was added.

As a proof-of-concept experiment, a novel and practical method relying on metal ion-dependent DNAzyme-based fluorescence anisotropy via AuNPs enhancement was presented for detecting Cu^{2+} and Pb^{2+} ions in aqueous media at room temperature. The FAM-labeled DNA substrates in the “big” AuNPs-mediated complexes were cleaved and released. The AuNPs-enhancement function results in a significant decrease. This effect can be used for the Cu^{2+} and Pb^{2+} detection as low as 1 nM. This method opens up a new possibility of rapid and easy detection of toxic metal ions in environmental samples. Indeed, for a practical application purpose, preliminary experiments were performed on the real river water samples and the spiked river samples. The results revealed good recoveries. This method shows distinct advantages over conventional methods in terms of its potential sensitivity, specificity, and ability for rapid response.

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

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 [84–86]. 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) [87], polyacrylamide gel electrophoresis [88], capillary electrophoresis [89], and FRET-based methods (fluorescence resonance energy transfer) [90, 91], 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 [92–94].

In this section, we demonstrate the 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 FPA for protease activity sensing [95]. SPB consists of a solid polystyrene core with a diameter of 50–100 nm onto which linear polyelectrolyte poly(acrylic acid) chains

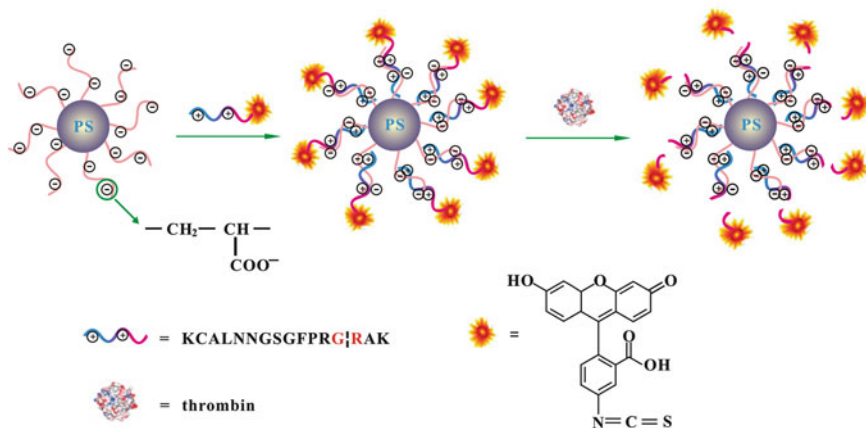


Fig. 2.5 Schematic illustration of the strategy using Peptide-SPB composite probes as substrates to monitor the proteolytic activity of thrombin. The colloidal particles consist of a solid polystyrene (PS) core onto which cationic polyelectrolyte poly(acrylic acid) (PAA) chains are grafted. The peptide 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 is provided at the bottom right. FITC is the base fluorescein molecule functionalized with an isothiocyanate reactive group ($\text{—N}=\text{C}=\text{S}$) on the bottom ring of the structure, which is reactive toward amine group of the lysine. Reproduced from Ref. [95], with the permission of Wiley John and Sons

(PAA) are grafted [96]. Due to its excellent dispersibility and good retention, PAA has been shown to be an attractive carrier for drug delivery systems, biosensors, immunoassays, and biocatalysts. Ballauff's group has done a systematic work of nanobrushes (SPB) in the biological applications [97–100], especially in the protein immobilization onto the SPB. Fig. 2.5 illustrates the principle of the SPB-based FPA 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 FP 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-peptide hydrolysis event is then translated into measurable FP value decrease by free cleaved dye-labeled peptide segments, which 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

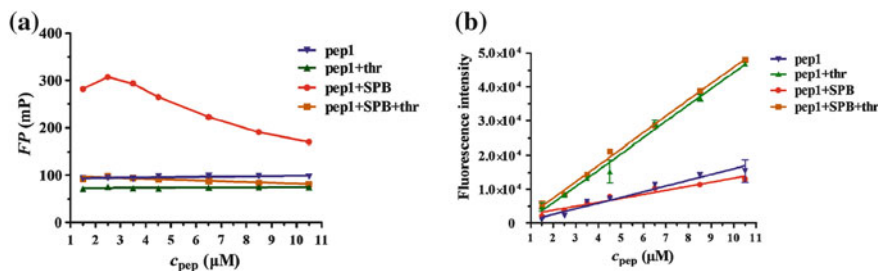


Fig. 2.6 Investigation of binding stoichiometry and fluorescence intensity (FI) for Peptide-SPB composite probes and free peptide1. **a** The change of FP value obtained from the increased peptide 1 in the presence or absence of SPB (10 nM) and in the presence or absence of thrombin (2.5 units). **b** The change of fluorescence intensity (FI) obtained from the increased peptide1 in the presence or absence of SPB (10 nM) and in the presence or absence of thrombin (2.5 units). The data were from the time point of 4 h after the proteolytic reaction. Reproduced from Ref. [95], with the permission of Wiley

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. Next, we investigated the binding stoichiometry of Peptide-SPB composite probes using FITC-labeled peptide 1 (Lys-Cys-Ala-Leu-Asn-Asn-Gly-Ser-Gly-dPhe-Pro-Arg-Gly-Arg-Ala-Lys(FITC)-OH), which was found to be one of the best thrombin substrates. As shown in Fig. 2.6, the binding stoichiometry of Peptide-SPB composites probes was estimated by evaluating the FP value responses of the Peptide-SPB composite probes to different concentration ratios of SPB and peptide 1. The number of peptides per SPB nanoparticle was estimated to be 250. In addition, it is worth noting that we observed the pronounced increasing FI of the mixture caused by more and smaller fluorogenic peptide segments released as the proteolytic reaction proceeded.

The method was tested with thrombin, a serine protease playing a pivotal role in hemostasis and blood clotting by selectively cleaving Arg-Gly bonds in fibrinogen to form fibrin and platelet activation [101]. 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 [102], 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 different concentrations resulted in time-dependent decreases in FP (Fig. 2.7a). 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 (Fig. 2.7b). The V_{max} was calculated to be

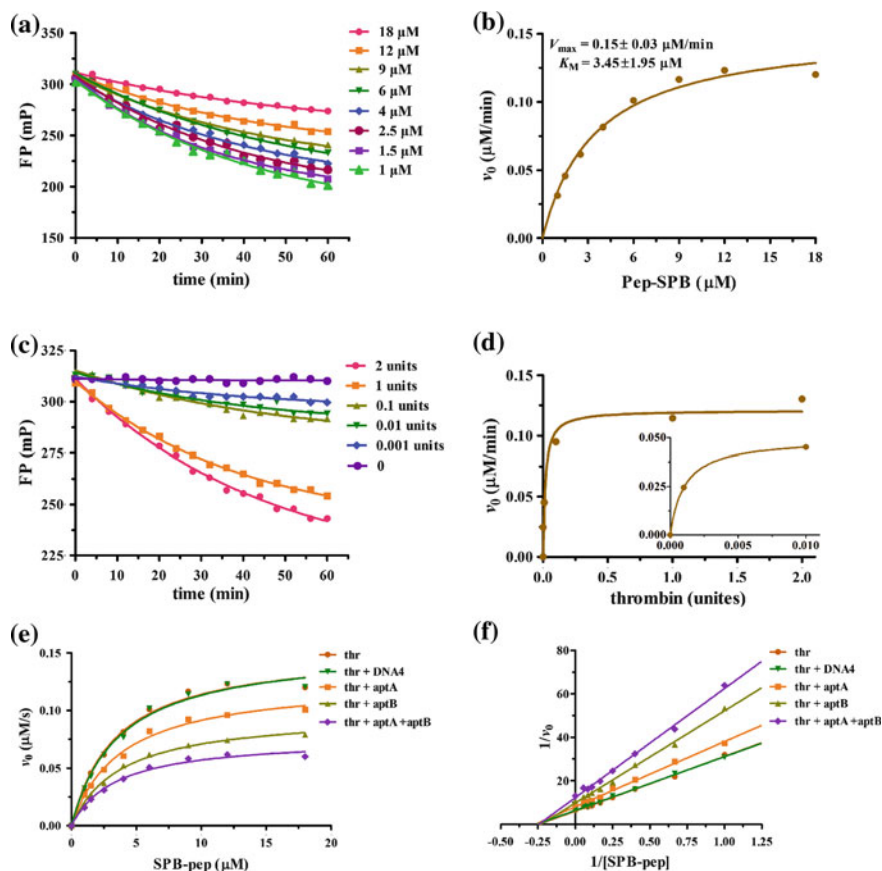


Fig. 2.7 **a** Progress curves at different concentrations of Peptide-SPB composite probes (1–18 μM , corresponding to peptide concentrations). Thrombin of 2.5 units was used. **b** Plot of the initial velocities for an increasing concentration of Peptide-SPB composite probes at a constant amount of 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. **e** 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. **f** Lineweaver–Burk (L-B) double reciprocal plot of the same data for determination of K_i values. Reproduced from Ref. [95], with the permission of Wiley John and Sons

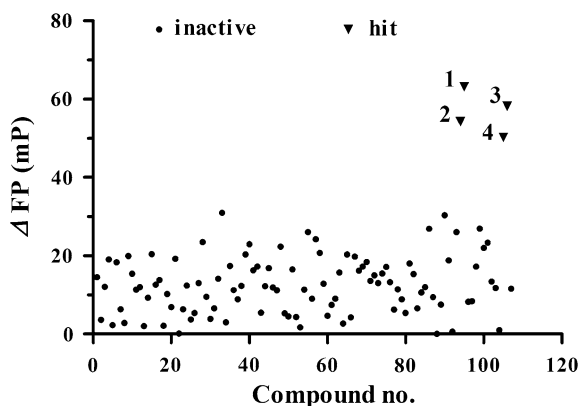
$0.15 \pm 0.03 \mu\text{M}/\text{min}$, and the k_{cat} was 0.30 min^{-1} , which were comparable to the literature values of $0.2 \pm 0.01 \mu\text{M}/\text{min}$ and 0.20 min^{-1} [94]. The K_M value was $3.45 \pm 1.95 \mu\text{M}$, which was 7.5-fold greater than the reported value of $0.46 \pm 0.12 \mu\text{M}$ [94]. 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 FPA is also suitable for quantitative determination of the amount of thrombin present in solution. Fig. 2.7c 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 ($1.85 \sim 5.55$ nM) based on three times the standard deviation (3σ). At a thrombin concentration of 1.0×10^{-3} units ($1.85 \sim 5.55$ nM), the rate of the hydrolysis of peptide was $0.0245 \mu\text{M}/\text{min}$ (Fig. 2.7d). This detection limit was better than the recently reported gold nanoparticle methods of 20 nM [5] and 5 nM [92], as well as electrochemical sensors of 1 nM [103] and 0.5 nM [104], 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 FPA. One is 15-mer ssDNA (aptA: 5'GGTTGGTGTGGTTGG3'), which forms an intramolecular quadruplex structure and primarily binds to thrombin at the fibrinogen-recognition exosite [105, 106]. The other one is 29-mer ssDNA (aptB: 5'AGTCCGTGGTAGGG CAGGTTGGGGTGACT3') binding at the heparin-binding exosite with a higher affinity [107]. Fig. 2.7e shows representative plots of velocity derived from monitoring changes in the FP 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 ± 15 and $20 \pm 14 \mu\text{M}$, respectively (Fig. 2.7f). 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 good agreement with the reported result [107].

Following the successful demonstration using the SPB-based FPA 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 co-solvent. 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 well tolerated by thrombin and trypsinase (data not shown). In the screening experiment, we selected the negative-control reactions without added compounds and positive-control reactions with a 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

Fig. 2.8 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. Reproduced from Ref. [95], with the permission of Wiley John and Sons



α -iodoacetamide (51.8%), sodium fluoride (60.1%), aptA (55.9%), and aptB (65.2%) all showed obvious inhibitory effects on the thrombin, while none of the organic compounds exhibited a significant inhibitory effect on thrombin (Fig. 2.8). 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 developed a simple and sensitive protease assay that utilizes the self-assembled Peptide-SPB probe to enhance the FP 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 FPA demonstrates superior sensitivity. The LOD 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 PE 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.

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