

Fluorescence Turn-On Detection of a Protein through the Reduced Aggregation of a Perylene Probe**

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The detection and quantification of proteins is important in basic research as well as in clinical practice. Antibody-based protein detection is the most commonly used method in many areas, such as medical diagnostics, biochemical studies, and environmental analyses, owing to its high sensitivity and specificity. However, the immunoassay method has certain drawbacks: the antibody identification/isolation process relies on animal and cell cultures, and complex conjugation chemistry is required for antibody immobilization and the attachment of signal-amplification elements. As a result, the process is time-consuming and expensive.^[1]

Aptamers are DNA or RNA oligonucleotides obtained through an in vitro screening process known as SELEX (systematic evolution of ligands by exponential enrichment). Just like antibodies, aptamers can bind a variety of targets with high selectivity and sensitivity.^[2] Many aptamer-based sensing methods have been developed.^[2c–g] The biggest advantage of aptamer-based methods is that oligonucleotides can be synthesized chemically with ease and extreme accuracy at quite a low cost nowadays. Furthermore, aptamers can be labeled readily and can recognize more targets, such as small inorganic ions. They are thermally stable, reusable, and show good stability during long-term storage.

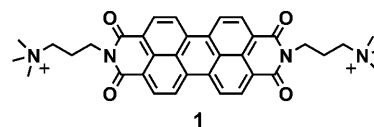
Many analytical tools have been employed to construct aptamer-based sensors for protein detection.^[3] However, most of these approaches involve tedious labeling, modification, or immobilization techniques that are technically demanding, time consuming, and cost-intensive, and may also affect the affinity of the aptamer. Therefore, the development of label-free aptamer-based methods for sensitive protein detection is a promising strategy.

Perylene tetracarboxylic acid diimide (PTCDI) derivatives have been used extensively as pigments as a result of their high thermal stability and excellent chemical inertness. They have been shown to be the best fluorophores for single-molecule spectroscopy owing to their high fluorescence

quantum yield and photostability.^[4a] However, most PTCDI derivatives are not water-soluble, and those that are soluble show a strong tendency to aggregate extensively in aqueous solution. This behavior results in fluorescence quenching.^[4] As a result, PTCDI derivatives have seldom been used as fluorescent labels for biosensing applications.

Herein, we report a label-free fluorescence turn-on approach for the sensitive and selective detection of proteins on the basis of a nucleic acid aptamer and a water-soluble perylene probe. In comparison with previous methods, our approach has the advantage that it is simple, fast, and inexpensive. More importantly, the highly specific interactions between the nucleic acid aptamer and the protein provide high selectivity, and the strongly fluorescent perylene probe offers high sensitivity. Our strategy could be viewed as a variation of the well-documented fluorimetric displacement assay.^[5]

The cationic PTCDI derivative **1** was synthesized as the fluorescent probe. Compound **1** contains two positive charges



and thus shows considerable water solubility (> 30 mM). Because compound **1** contains a planar aromatic structure, it has a tendency to aggregate through aromatic π - π stacking interactions like other PTCDI derivatives. However, since it also contains two positive charges, repulsive interactions between the electrostatic charges decrease its tendency to aggregate. As a result, in aqueous solution at ambient temperature, compound **1** exists in equilibrium between the aggregated form and the free monomeric form.

Lysozyme was employed as the model protein for our study. Its primary sequence has 129 amino acids, and it has a high isoelectric point (pI) value of 11.0. At pH 7, lysozyme is positively charged. Lysozyme exists universally in body tissues and secretions. Its abnormal concentration in serum and urine is related to many diseases, such as leukemia, renal diseases, and meningitis.^[6] Therefore, lysozyme-selective sensing is of considerable importance. An anti-lysozyme aptamer (5'-ATC AGG GCT AAA GAG TGC AGA GTT ACT TAG-3') was employed to quantify lysozyme. The aptamer shows high affinity for lysozyme with a dissociation constant (K_d) of 31 nM.^[7]

The overall detection strategy is shown in Figure 1. 1) In an aqueous solution, compound **1** exists in both the mono-

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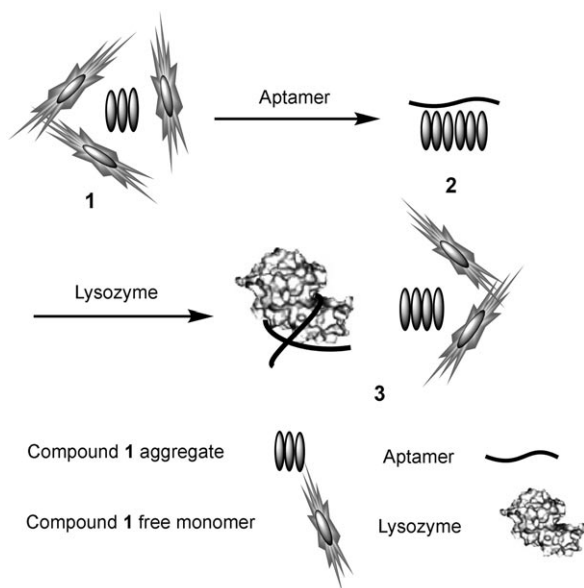


Figure 1. Strategy for selective lysozyme sensing. 1) Coexistence of the monomer and aggregates of compound **1** in equilibrium; 2) binding of the nucleic acid aptamer to compound **1** aggregates; 3) lysozyme binding to the nucleic acid aptamer: compound **1** monomer is released, and turn-on fluorescence is detected.

meric and the aggregated forms. Because of the existence of the free dye monomer, strong fluorescence is detected. 2) Since nucleic acid contains multiple negatively charged phosphate functional groups, it is a polyanion. When the anti-lysozyme aptamer is added to the aqueous solution of compound **1**, strong electrostatic interactions between the dye monomer/aggregates and the polyanionic nucleic acid result in rapid binding of the dye to the nucleic acid, and because the positive charge on the dye is largely neutralized by the nucleic acid, repulsive electrostatic interactions among the dye molecules are greatly diminished. As a result, an enhanced degree of dye aggregation and a significant decrease in the fluorescence intensity are observed.^[8,9] 3) Upon the addition of lysozyme to the solution, specific binding of lysozyme to the nucleic acid aptamer weakens the binding between the aptamer and the dye aggregates. As a result, dye-monomer molecules are released, and a turn-on fluorescence signal is detected.

The aggregation of compound **1** was demonstrated by temperature-dependent UV/Vis and emission spectroscopic studies (Figure 2). When the solution temperature was increased from 10 to 95 °C, a significant increase in the intensity of the 0→0 transition absorption was observed; this result indicates the presence of the aggregated forms of compound **1** at lower temperatures (see the Supporting Information). Changes in the temperature-dependent emission spectrum showed a similar trend. Since the aggregated compound **1** is not fluorescent, the gradual increase in the emission intensity observed with the increase in the solution temperature suggests a gradual conversion of compound **1** aggregates into the free dye monomer. When all aggregated forms of compound **1** have been converted into the free monomer, maximum fluorescence intensity is reached (see

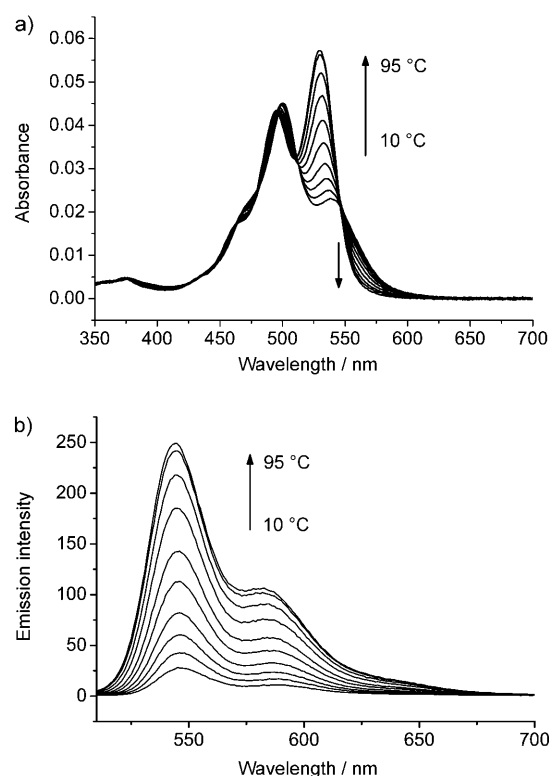


Figure 2. a) UV/Vis absorption spectra and b) emission spectra of compound **1** (1 μM) in MOPS buffer (5 mM, pH 7.0) at different temperatures. MOPS = 3-(*N*-morpholino)propanesulfonic acid.

Figure S1 in the Supporting Information). We also studied the aggregation of compound **1** in the nanomolar concentration range. A nonlinear curve was found for concentration-dependent fluorescence intensity (see Figure S2 in the Supporting Information). The results clearly suggest that in the nanomolar concentration range, compound **1** also exists in equilibrium between the monomeric and aggregated forms.

Figure 3 shows emission spectra of compound **1** in the absence and presence of the anti-lysozyme aptamer. Compound **1** displayed strong fluorescence at ambient temperature owing to the existence of the free dye monomer. Upon

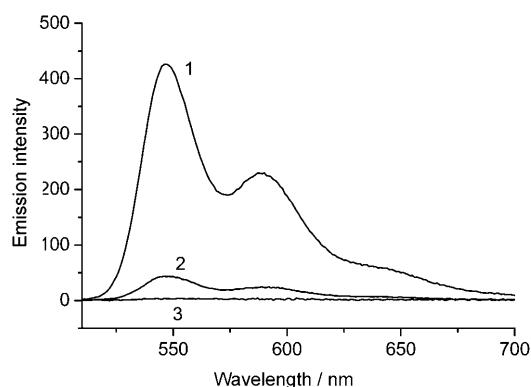


Figure 3. Emission spectra of compound **1** (5 nM) in the absence (line 1) and presence of the DNA aptamer (line 2: 0.25 nM, line 3: 0.5 nM).

the addition of the aptamer, the emission intensity decreased dramatically. A significant decrease in the emission intensity was observed when the DNA aptamer (0.25 nM) was added. When the concentration of the added DNA aptamer was increased to 0.5 nM, complete quenching of the fluorescence of the dye monomer was observed, a result which suggests the complete aggregation of compound **1**. To study the possible quenching effect of the nucleic acid bases, we tested two synthetic polyanions, namely, poly(vinyl sulfonate) and poly(4-styrene sulfonate). Significant quenching of the fluorescence of compound **1** was observed (see Figure S3 in the Supporting Information). The anti-lysozyme aptamer was also predigested with nuclease; after digestion, no induced aggregation of compound **1** would be expected. There was little change in the fluorescence of compound **1** after nuclease digestion (see Figure S4 in the Supporting Information). These results suggest that induced aggregation plays a major role in the induced quenching of compound **1** fluorescence.

When lysozyme was added to the mixture of the DNA aptamer and compound **1**, reappearance of the fluorescence due to the compound **1** monomer was observed. The fluorescence intensity became stronger as the lysozyme concentration was increased and eventually reached a saturation point after which any further increase in the lysozyme concentration caused little increase in the emission intensity (Figure 4).

Since the anti-lysozyme DNA aptamer can bind lysozyme specifically, the results show that binding to lysozyme weakens the ability of the compound **1** aggregates to bind to the DNA aptamer. Previous studies have shown that PTCDI aggregates are linear chain structures.^[4a] The results suggest that whereas the DNA aptamer adopted a random conformation before binding to lysozyme, its binding to the compound **1** aggregates was unrestricted. However, binding to lysozyme resulted in considerable changes in the conformation of the aptamer, and these changes decreased the ability of the aptamer to bind to compound **1** aggregates. As a result, molecules of the compound **1** monomer dissociated, and enhanced emission of the dye monomer was observed.

Our results show that the fluorescence intensity is in direct proportion to the concentration of lysozyme in the range of 0–10.5 nM. The mixture reached equilibrium fairly rapidly: a simple “add and mix” process was sufficient. The fluorescence signal obtained was also fairly stable with no noticeable decrease in intensity during continuous monitoring for 60 min.

We estimate the lysozyme-detection limit of our approach to be 70 pM ($\approx 1 \text{ ng mL}^{-1}$). Without the use of signal amplification, the sensitivity of our method rivals that of antibody-based ELISA.^[10] The method is to our knowledge one of the most sensitive developed to date for lysozyme-selective sensing^[11] and is much more sensitive than a number of other label-free lysozyme-selective sensing methods developed in recent years (Table 1).

We also investigated the selectivity of our method (Figure 5). We tested six proteins that differ dramatically in terms of their size and pI value (see Table S1 in the Supporting Information). Despite these differences, our assay system showed high lysozyme selectivity against these proteins. This

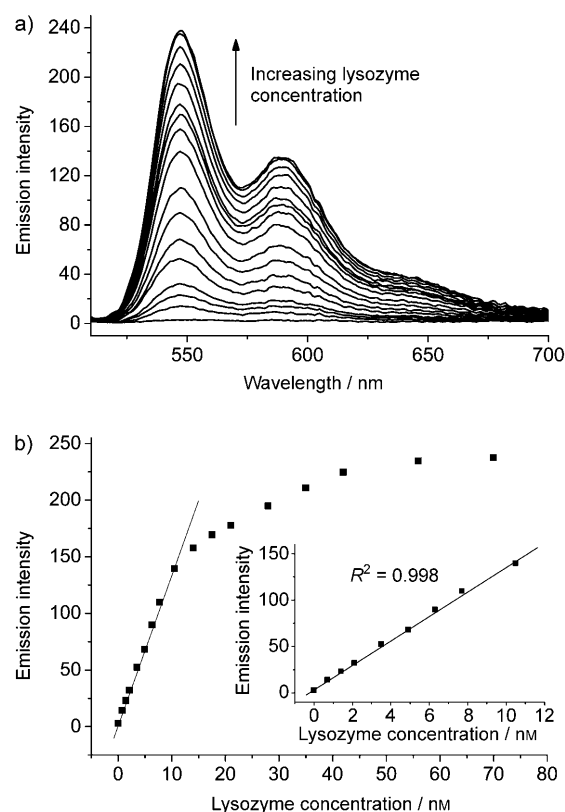


Figure 4. a) Changes in the emission spectrum upon the addition of lysozyme in different concentrations to the mixture of compound **1** (5 nM) and the anti-lysozyme aptamer (0.5 nM). b) Plot of the fluorescence intensity at 545 nm against the lysozyme concentration; inset: expanded linear region of the curve.

Table 1: Comparison of different label-free methods for specific lysozyme detection.

Method	Transducer	Detection limit [nM]
perylene-probe aggregation (this study)	fluorescent	0.07
gold-nanoparticle aggregation	colorimetric	50 ^[11c]
acidified-sulfate-induced aggregation ^[a,b]	surface-enhanced Raman scattering	350 ^[11d]
DNA-base electrooxidation	electrochemical	18 ^[11e]
[Ru(NH ₃) ₆] ³⁺ -probe voltammetry ^[b,c]	electrochemical	35 ^[11a]
[Fe(CN) ₆] ³⁻ -probe impedance ^[b,d]	electrochemical	14 ^[11f]

[a] Detection limit: 5 $\mu\text{g mL}^{-1}$. [b] The concentration was converted into a nanomolar value for straightforward comparison. [c] Detection limit: 0.5 $\mu\text{g mL}^{-1}$. [d] Detection limit: 0.2 $\mu\text{g mL}^{-1}$.

selectivity apparently originates from the high selectivity of the DNA anti-lysozyme aptamer. The possible interference of a randomly selected oligonucleotide in the sample solution was also tested. We found that its interference could be removed easily by pretreatment of the sample by nuclease digestion (see Figure S5 in the Supporting Information).

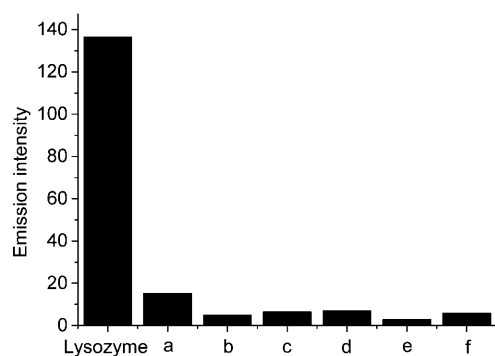


Figure 5. Selectivity studies. The proteins studied were a) hemoglobin, b) thrombin, c) bovine serum albumin, d) collagenase, e) cytochrome c, and f) trypsin. The experimental conditions were the same as those described for Figure 4, with a protein concentration of 10 nM.

However, we realize that longer polynucleotides may be more difficult to digest, and sample pretreatment could potentially introduce an intrinsic and systematic error. Our method could also be used in complex mixtures: we determined the lysozyme concentration in human saliva samples. The obtained values of 6.9–10.5 μM are within the normal range of previously reported values.^[12] The addition of lysozyme (3.5 nM) to the diluted sample mixtures led to recovery values of 3.3–3.5 nM with a relative standard deviation of 2.89%.

In conclusion, we have developed an ultrasensitive biosensor based on an aptamer and the aggregation of compound **1** for the selective detection of lysozyme. In aqueous solution, compound **1** displays strong monomer fluorescence, which was effectively eliminated by the addition of the DNA aptamer. In the presence of the aptamer-binding protein, fluorescence recovery was observed, and the recovered fluorescence intensity was directly proportional to the concentration of the protein added. Our method could be viewed as a special case of the ligand-displacement assay. It shows high sensitivity as well as high selectivity, and because it is a label-free method, the assay is fairly simple and inexpensive. Our aptasensor provides a new approach to sensitive and selective protein quantification.

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